Doctoral Dissertation

Medicinal and Pharmaceutical Properties of Momilactones A and B

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SUMMARY

Background

Momilactones belong to diterpenes group which are typically characterized by a lactone ring linked with the pimarane skeleton. According to the first definition of "momilactone" described by Kato and colleagues in 1973, every lactone structure derived from the rice husk ("籾" - "momi") can be named as a momilactone. Among identified momilactones, momilactones A (MA) and B (MB) are the most common but only detected in rice (*Oryza sativia*) and the moss *Hypnum plumaeforme*. Previous studies soon manifested the potent allelopathic function of MA and MB against plant's natural enemies such as weeds and blast fungus. Later studies confirmed these compounds are authoritative allelochemicals against several pathogens of crops as well. Recently, MA and MB were found more implicative with salinity and drought tolerance of rice more than allelopathy. They are also exhibited antioxidant, cytotoxic, antitumor and anticancer activities. Though MA and MB are promising bioactive constituents in rice, the isolation and purification of the metabolites are complicated and laborious. At present, there are very few laboratories in the world that can successfully isolate and purify MA and MB. As a result, no commercial MA and MB from chemical companies in Japan or abroad can be purchased; thus, knowledge on medicinal and pharmaceutical properties of these compounds has been limited.

Main objectives

Therefore, this research was conducted to at first examine medicinal and pharmaceutical properties of pure momilactones A and B isolated from rice husks focusing on anti-diabetes, anti-obesity, antioxidant and anti-skin-aging; and, to determine the potential sources of momilactones A and B by quantifying their contents among rice plant parts (bran and endosperm) and among common rice varieties.

Structure of dissertation

Chapter 1: Introduction

Chapter 2: Isolation and purification of momilactones A and B and momilactone-like compounds from rice husk

Chapter 3: Momilactones A and B are α-amylase and α-glucosidase inhibitors

Chapter 4: Contributions of momilactones A and B to diabetes and obesity inhibitory potentials of rice bran: Evidence from in vitro assays

Chapter 5: Antioxidant and anti-skin-aging properties of momilactones A and B

Chapter 6: General discussion and conclusion

Materials and methods

Materials: Rice husk of *Oryza sativa* (var. Koshihikari) was collected in Higashi-Hiroshima. Chemicals and reagents at high grade were purchased from prestigious providers including Junsei Chemical Co., Ltd., Fujifilm Wako Pure Chemical Corporation, Japan, Fisher Scientific company, and Sigma-Aldrich, USA.

Methods:

- Pure compounds were isolated and purified by repeated normal phase column chromatography over silica gel;

- Momilactones were identified and confirmed by reliable advance-spectroscopic techniques consisting of high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), ^{1}H and ^{13}C nuclear magnetic resonance (NMR);

- Biological activities were assayed in vitro by using antioxidant and inhibitory experiments on targeted enzymes relevant to diabetes, obesity and skin-aging comprising α-amylase, α-glucosidase, pancreatic α-amylase, trypsin, elastase, and tyrosinase.

Main results

In chapter 2, four bioactive compounds including momilactone E (ME), 7-ketostigmasterol (7KS), momilactone A (MA), and momilactone B (MB) were isolated by column chromatography. The inhibitory activities of MA, MB, ME, and 7KS were examined on lettuce (*Lactuca sativa*), barnyard grass (*Echinochloa crus-galli*), and tall goldenrod (*Solidago altissima*) in bioassays. The allelopathic activities of ME and 7KS were compared with those of potent phytoalexin momilactones A (MA) and B (MB), and the standard *p*-hydroxybenzoic acid (*p*HA). Results showed that both MA and MB exhibited stronger inhibitory activity than ME and 7KS. MB exerted greater inhibitions than MA but the mixture of MA and MB (1:1, *v*/*v*) possessed a similar level of inhibition to MB. On the other hand, although ME and 7KS presented non-significant inhibition, their mixture of ME-7KS (1:1, *v*/*v*) displayed a remarkable inhibition on the growth of *S. altissima*. Findings of this chapter revealed that MA, MB, and the mixture ME-7KS had the potential to control the invasive plant *S. altissima* and the noxious paddy weed *E. crus-galli* in vitro, but their mode of actions should be further investigated.

In chapter 3, the results of enzymatic in vitro assays showed that both MA and MB exerted potent inhibition on α-amylase and α-glucosidase activities. The inhibitory effect of MB on these two key enzymes was greater than that of MA. Both MA and MB exerted greater α-glucosidase suppression as compared to that of the commercial diabetic inhibitor acarbose. Quantities of MA and MB in rice grain were 2.07 and 1.06 μg/dry weight (DW), respectively. This study was the first to confirm the presence of MA and MB in refined rice grain and reported the α-amylase and α-glucosidase inhibitory activity of the two compounds.

Chapter 4 was the first to detect the presence of the two compounds MA and MB in rice bran using the LC-ESI-MS technique. By in vitro assays, both MA and MB exhibited potent inhibitory activities on pancreatic α-amylase and α-glucosidase which were

significantly higher than γ-oryzanol, a well-known diabetes inhibitor in rice bran. Remarkably, MA and MB indicated an effective inhibition on trypsin with the IC_{50} values of 921.55 and 884.03 μg/mL, respectively. By HPLC, quantities of MA (6.65 μg/g dry weight) and MB (6.24 μg/g dry weight) in rice bran were determined. Findings of this study revealed the α-amylase, α-glucosidase and trypsin inhibitors MA and MB contributed an active role to the diabetes and obesity inhibitory potentials of rice bran.

Chapter 5 reported the potential antioxidant and anti-skin-aging activities of MA and MB. Results from antioxidant assays presented a synergistic antioxidant activity of the MA and MB (MAB, 1:1, v/v) by ABTS and reducing power assays. Remarkably, in ABTS assay, IC_{50} value of MAB (0.319 mg/mL) was 4 folds and 9 folds greater than that of individual MB (1.28 mg/mL) or MA (2.84 mg/mL), respectively. Enzymatic assays on pancreatic elastase and tyrosinase indicated that MA and MB were potential cosmeceuticals in protecting the skin from wrinkles and freckles. Besides, the validated method for quantification of MA and MB in rice grains showed that brown rice grains contained more MA and MB than white rice grains due to the MA and MB involvement in bran. Among refined grains, Koshihikari possessed the highest amount of these active compounds. The usage of a specific technique in preparation of samples and an advanced UPLC-ESI-MS method helped improve the sensitivity of the quantification of MA and MB in various rice grains.

Conclusion

This study showed the first-time inhibitory effects of momilactones A (MA) and B (MB) on the key enzymes related to human diseases including diabetes, obesity, and skin aging. The results can be applied widely in medicine and contribute to the development of the prospective therapeutics. Significantly, for the first time, the present research successfully detected and quantified MA and MB in bran and white rice grains. Since most

of the studies agree with the fact that white rice intake can increase diabetes risk, the existence as diabetic and obesity inhibitors of MA and MB in white rice grain may alter the antecedent perspective that white rice consumption induced risks of type 2 diabetes and obesity. The HPLC/UPLC-ESI-MS methods and the protocol of sample preparation were validated, which helped determine and quantify MA and MB at higher reliability and sensitivity. However, further studies should be implemented to assert applicable doses of MA and MB before conducting medicinal production, pre-clinical and clinical trials on the two compounds. Finally, given that quantities of MA and MB are largely varied among rice cultivars, the breeding of new rice cultivars with high amounts of MA and MB may be useful and economical to help control several chronic diseases in human.

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

SUPPLEMENTARY DATA

CHAPTER 1: GENERAL INTRODUCTION

1.1. Rice and its by-products

Rice (*Oryza sativa*) is one of the most important crops, which provides the staple calorie for more than half of the world population. Rice grain has a long use history as the main food source of human owing to its high nutritional value. Recently, rice by-products have had increasing interests from food processing industry and pharmaceutical fields (Esa, Ling, & Peng, 2013; Sohail, Rakha, Butt, Iqbal, & Rashid, 2016). While rice endosperm contains an abundant amount of polysaccharides, rice by-products including rice bran, husk, germ and straw include some beneficial nutrients, such as dietary fiber and proteins, vitamins, oryzanol and phenolics (Esa et al., 2013; Rahaie, Gharibzahedi, Razavi, & Jafari, 2014; Tan & Norhaizan, 2017).

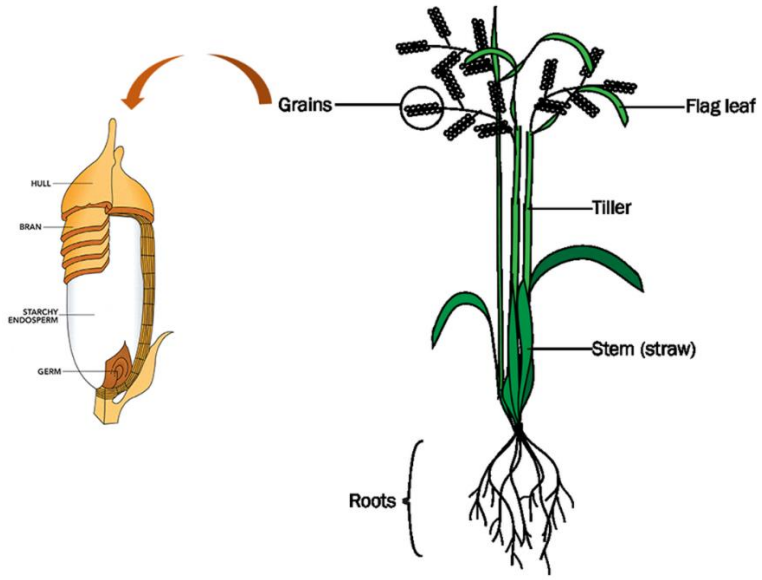


Figure 1. Rice plant parts anatomy (Marquez, 2013; USA Rice, 2019).

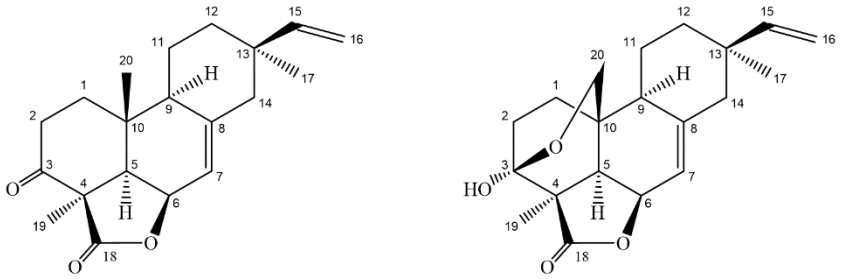
Theoretically, the paddy-field produces approximately 70% white rice, 20% husk and 10% bran (Esa et al., 2013). If there is no proper utilization, a large amount of rice by-products will be possibly wasted. Although there are several approaches targeting to the utilization of rice by-products such as animal feeds, biofuels, fertilizers, and nutritional

media for mushroom cultivation. However, to take maximum advantage of these sources, pharmaceutical and medicinal aspects should be more considered. Moreover, there has been no report on toxicities of natural compounds from either rice grain or its by-products affecting human health. Therefore, the investigation and discovery of new ingredients or bioactive compounds that possess medicinal and pharmaceutical properties from rice and its by-products are potential, actual, and essential.

1.2. Momilactones

1.2.1. Discovery and definition of momilactones in rice Oryza sativa

The term "momilactone" was first introduced by Kato et al. (1973), which was a combination of a chemical component, lactone and a Japanese word, "籾" - "momi", the rice husk. Accordingly, every lactone from rice husk can be considered as a member of the momilactone group. In the study of Kato et al. (1973), two momilactones named momilactone A (MA) and momilactone B (MB) were described (Figure 1), in which, 150 mg of MA and 100 mg of MB were purified from 200 kg of Koshihikari rice husks. Later, Tsunakawa et al. (1976) found momilactone C (MC) with a minor amount of only 3 mg from 300 kg of dried rice husks. In 2015, Cho and partners reported two new momilactones D (MD) and E (ME) from rice roots, however, the compound named ME was mistakenly defined because its structure did not obtain any lactone moiety. Zhao et al. (2018) had a statistic of total 47 momilactone-like compounds reported from 1973 to 2017. However, only seven compounds were derived from rice (*O. sativa*), of which, four molecules were officially characterized as momilactones consisting of momilactones A, B, C, D. All specified MA-D have a similar point in their structure where a γ-butyrolactone linked with the pimarane skeleton at carbon-4,5,6.



Momilactone A Momilactone B

Figure 2. Structures of momilactone A and momilactone B.

1.2.2. Origin and biosynthesis of momilactones A and B

Among known momilactones, momilactone A (MA) or (1*R*,2*R*,5*R*,9*R*,12*R*,16*R*)-5-ethenyl-1,5,12-trimethyl-10-oxatetracyclo[7.6.1.$0^{2,7}$.$0^{12,16}$]hexadec-7-ene-11,13-dione and momilactone B (MB) or (1*S*,2*R*,5*R*,12*R*,18*R*)-5-ethenyl-13-hydroxy-5,12-dimethyl-10,14-dioxapentacyclo[11.2.2.$1^{1,9}$.$0^{2,7}$.$0^{12,18}$]octadec-7-en-11-one, have been the most thoroughly scrutinized. To date, the two metabolites have been only detected in rice (*O. sativa*) and the moss *Hypnum plumaeforme* (Nozaki et al., 2007; Kato-Noguchi & Kobayashi, 2009). Structurally, MA and MB belong to the diterpene lactone group, which have a 19,6β-lactone structure (Figure 2).

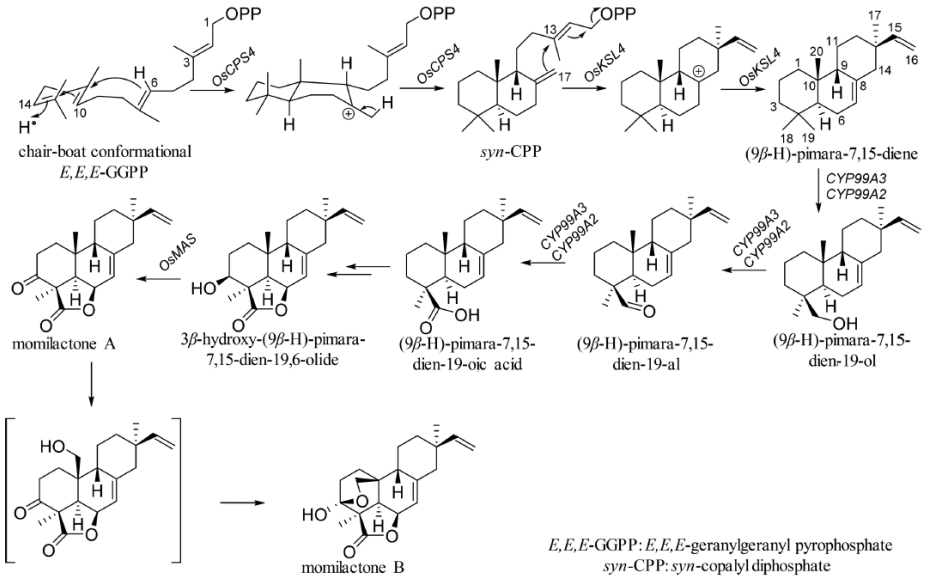


Figure 3. Biogenetic pathway for MA and MB (Zhao et al., 2018).

In rice, the biosynthesis of MA and MB was elucidated by genetic and protein kinase evidence (Otomo et al., 2004; Xu et al., 2012; Schmelz et al., 2014; Miyamoto et al., 2016; Zhao et al., 2018). Figure 3 summarizes the biogenetic pathway of MA and MB. In brief, the process initializes from a catalyzation of the precursor (*E,E,E*)-geranylgeranyl diphosphate (*E,E,E*-GGDP) via the methylerythritol phosphate pathway in chloroplasts (plastids). The first product *syn*-copalyl diphosphate (*syn*-CPP) generated by CPP synthase OsCPS4 enzyme is further cyclized to form (9*β*-H)-pimara-7,15-diene (*syn*-pimaradiene) by kaurene synthase-like OsKSL4. Sequentially, the involvement of cytochrome P450 enzymes (CYPs) comprising CYP99A3, CYP99A2, CYP701A8 (Kitaoka, Wu, Xu, & Peters, 2015) and CYP76M8 (Wang et al., 2012) leads to formations of (9*β*-H)-pimara-7,15-dien-19-oic acid and (9*β*-H)-pimara-7,15-dien-19,6*β*-olide, respectively. Finally, MA is synthesized by OsMAS, momilactone A synthase (Atawong, Hasegawa, & Kodama, 2002) meanwhile MB is "proposed to from MA via C_{20}-hydroxylation and hemiketal ring closure" (Zhao et al., 2018). Though the final step in MB synthesis has remained little vague, the genes encoding cyclase (OsCPS4 and OsKSL4), oxidase or P450 members (CYP99A3, CYP99A2, CYP701A8, CYP76M8) and dehydrogenase (OsMAS) are mapped close together in the chromosome 4 of rice (Figure 3). The evidence manifested the important role of P450 family in finalization of biosynthesis of diterpenoids like MA and MB.

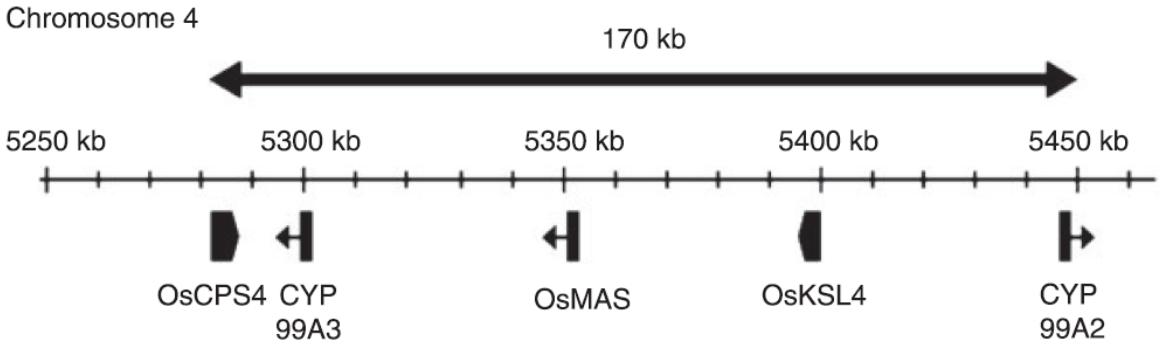


Figure 4. Biosynthetic gene clusters of MA and MB in rice (Shimura et al., 2007; Xu et al., 2012).

1.3. Biological activities of momilactones

1.3.1. Allelopathic activity

Previous studies soon signified the potent allelopathic function of MA and MB against plant's natural enemies such as weeds and blast fungus. Table 1 indicates the potent inhibitory activity of MA and MB on the growth of toxic weeds (barnyard grass, *Echinochloa crus-galli* and Monochoria, *Monochoria vaginalis*) and the blast fugus *Magnaporthe oryzae*. Accordingly, the potency of MB was basically stronger than that of MA. The mechanism that MA and MB inhibit the plant growth was validated by gene expressions and transcriptions of tested plants as barnyard grass (Fang et al., 2015) and *Arabidopsis* (Kato-Noguchi, Ota, Kujime, & Ogawa, 2013). Among the evidence, some important proteins associated with cell development and defense response during plant growth stages were attributed to be disturbed by treating with MA and MB.

Besides, the effects of MA and MB on several rice seedlings were tested, of which, these metabolites presented positive activity or no visible damage on rice growth (Kato Noguchi & Ota, 2013).

Table 1. IC_{50} values of MA and MB displaying inhibitory effect on some weeds and blast fungus

Indicator species	**Inhibitory activities**	**IC_{50} value (μM)**		**References**
		MA	**MB**	
Arabidopsis thaliana	Seeds germination inhibition	742	48	(Kato-Noguchi et al., 2013)
Cyperus difformis	Seeds germination inhibition; growth inhibition	33; 35	30; 31	(Chung, Hahn, & Ahmad, 2005)
Leptochloa chinenesis		899; 1210	6; 30	
Amaranthus retrof lexus		1276; 1393	31; 32	

Indicator species	Inhibitory activities	IC_{50} value (µM)		References
		MA	MB	
Arabidopsis thaliana	Root inhibition; shoot inhibition	204; 86	10; 12	(Kato Noguchi & Ota, 2013)
Medicago sativa		379; 315	68; 82	
Lactuca sativa		472; 395	54; 80	
Lepidum sativum		476; 337	35; 41	
Phleum pretense		77; 157	6; 8	
Echinochloa crus-galli		91; 145	7; 6	
Echinochloa colonum		67; 238	7; 12	
Digitaria sanguinalis		99; 275	10; 12	
Lolium multiflorum		92; 138	7; 7	
Monochoria vaginalis	Germination; root; shoot inhibitions	[(1)]44; 0.5; 24	[(1)]27; 1; 17	(Fukuta et al., 2007)
Magnaporthe oryzae	Spore germination inhibition; germ tube growth inhibition	48; 74	9; 3	(Cartwright, Langcake, Pryce, Leworthy, & Ride, 1977; Zhao et al., 2018)
Botrytis cinerea	Mycelia growth inhibition	[(1)]78	[(1)]1.2	(Fukuta et al., 2007)
Fusarium solani		[(1)]198	[(1)]124	
Colletrotrichum gloeosporioides		[(1)]95	[(1)]53	
Fusarium oxysporum		[(1)]129	[(1)]137	

[(1)] a unit is expressed in µg.

1.3.2. Antibacterial and antioxidant activities

Fukuta et al. (2007) examined the antibacterial activity of MA and MB on four strains that commonly cause digestive problems in human such as diarrhea and gastroenteritis. The

results from in vitro assays showed that the growths of *Pseudomonus ovalis*, *Bacillus cereus*, and *Bacillus pumilus* were significantly inhibited by MB rather than by MA. Meanwhile, the inhibitory activity of the two bioactive substances was at the same level against *Escherichia coli*. In the same study, authors also reported the antioxidant activity of MA (EC_{50} = 783.9 µg) and MB (EC_{50} = 790.7 µg) by DPPH assay (Fukuta et al., 2007).

1.3.3. Cytotoxic, antitumor, and anticancer activities

By using the MTT assay, Chung et al. (2005) found that MA and MB obtained a remarkable cytotoxic activity on the murine P388 leukemia cells. The concentrations required for suppressing 50% growth of the P388 cancer cell were 0.85 and 0.07 µg/mL for MA and MB, respectively. On the other research, MB was convinced to have antitumor efficacy on several blood cancer cells in human (Lee et al., 2008). Particularly, MB at concentration less than 1.98 µg/mL could inhibit the growths of HL-60 (human myeloblastic leukemia cells), Jurkart (human leukemic T cells), RBL-2H3 (a basophilic leukemia cell line), and p815 (mouse mastocytoma cells). The mechanism was substantiated by the apoptosis pathway through caspase and mitochondria. Additionally, MB presented a strong inhibitory effect on the human colon cancer cells HT-29 and SW620 (IC_{50} was 0.33 µg/mL approximately) (Kim, Park, Park, & Lee, 2007). By an assay on the U937 cell model, Park et al. (2014) confirmed that the anticancer property of MB was resulted by an apoptosis and a suspension in G1 cell cycle via the induction of p21 expression, an inhibition of Cdk/cyclin-associated kinase activities, and a reduction of phosphorylation of pRB. Moreover, MB possessed a potent inhibition on human breast cancer cell line (T47D) with the IC_{50} value of about 17 µg/mL (Joung et al., 2008). By STAT5b model and a caspase-3 dependent pathway, the authors proved that MB inhibited the expression of proteins related to the oxygen deficiency in the human breast cancer cell, consequently, increased the death rate (apoptosis) of cancer cells (Figure 5).

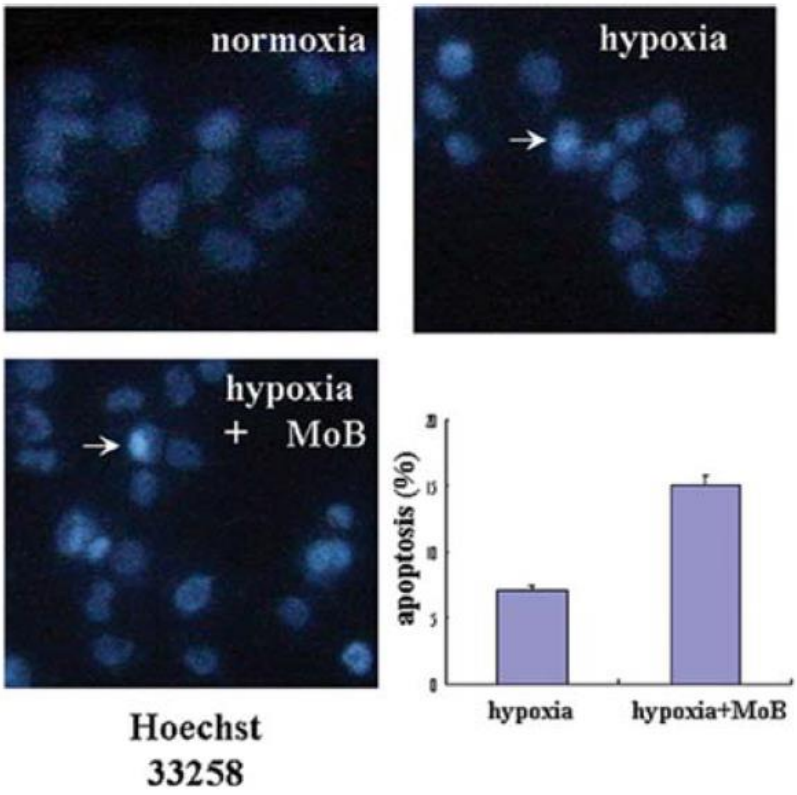


Figure 5. T47D cells were treated with normoxia, hypoxia, or hypoxia plus momilactone B (50 μM) before Hoechst staining and analysis of nuclei (Joung et al., 2008).

Apoptotic cells exhibited blue, peripherally clumped or fragmented chromatin, as indicated by the arrows. MoB, momilactone B. Data are representative of three independent experiments. The error bars represent the standard deviation from three experiments.

1.3.4. Other biological functions

Since 1973, MA and MB have been considered as potent phytoalexins which can protect rice from many risks of natural enemies. However, in recent years, these metabolites have been reported to be more implicative with physiological functions as salinity and drought tolerances of rice more than the allelopathic role (Xuan, Minh, Anh, & Khanh, 2016; Quan & Xuan, 2018). The similar statement by Jensen et al. (2001) was also suggested that the momilactone biosynthetic gene cluster was not associated to allelopathy.

On the other hand, MB was recorded as a ketosis inhibitor by regulating the angiopoietin-like-3 (ANGPTL3) and lipoprotein lipase (LPL) pathways and suppressing the expression

of 3-hydroxy-3-methylglutaryl-CoA synthase-2 (HMGCS2) (Kang et al., 2017). The described mechanism of such effect played an important role in the treatment of the ketosis, an abnormality of the human body that can cause a seriously harmful effect on the liver and kidney if without a suitable control.

1.4. Objectives of this study

Though biological activities of MA and MB are potential, the isolation and purification of MA and MB are very complicated. Presently, very few laboratories in the world can successfully isolate and purify MA and MB. As a result, a scanty commercial source of MA and MB can be purchased, therefore, knowledge on medicinal and pharmaceutical properties of these compounds has been limited. Thus, the main objective of this study was, firstly, to study unknown medicinal and pharmaceutical properties of MA and MB isolated from rice husks focusing on anti-diabetes, anti-obesity, antioxidant and anti-skin-aging; and secondly, to determine the potential sources of momilactones A and B by quantifying their contents among rice plant parts (bran and endosperm) and among common rice varieties by using advanced spectroscopic techniques.

CHAPTER 2: ISOLATION AND PURIFICATION OF MOMILACTONES A AND B AND MOMILACTONE-LIKE COMPOUNDS FROM RICE HUSK

2.1. Summary

Rice husk has been exploited as a potential source of allelochemicals. In this study, four bioactive compounds including momilactone E (ME), 7-ketostigmasterol (7KS), momilactone A (MA), and momilactone B (MB) were isolated by column chromatography (CC) to yield 2.7, 0.3, 11.7, and 8.3 mg/kg rice husk, respectively. The structures of the isolated compounds were identified and confirmed by spectroscopic techniques consisting of ^{1}H and ^{13}C nuclear magnetic resonance (NMR), electrospray ionization mass (ESI), high-resolution mass spectrometry (HR-MS) and infrared spectroscopy (IS). An advanced quantitative method for MA and MB was achieved to increase the detectable yields of MA and MB in rice husk to 51.96 and 42.33 µg/mL, respectively. The inhibitory activities of MA, MB, ME, and 7KS were examined on lettuce (*Lactuca sativa*), barnyard grass (*Echinochloa crus-galli*), and tall goldenrod (*Solidago altissima*) in bioassays. The allelopathic activities of ME and 7KS were compared with those of potent phytoalexin momilactones A (MA) and B (MB), and the standard *p*-hydroxybenzoic acid (*p*HA). Results showed that both MA and MB exhibited stronger inhibitory activity than ME and 7KS. MB exerted greater inhibitions than MA but the mixture of MA and MB (1:1, *v*/*v*) possessed a similar level of inhibition to MB. On the other hand, although ME and 7KS presented non-significant inhibition, their mixture of ME-7KS (1:1, *v*/*v*) displayed a remarkable inhibition on the growth of *S. altissima*. Findings of this study revealed that MA, MB, and the mixture ME-7KS had the potential to control the invasive plant *S. altissima* and the noxious paddy weed *E. crus-galli* in vitro, but their mode of actions should be further investigated.

2.2. Introduction

Allelopathy is defined as a natural phenomenon when a plant produces and liberates phytochemicals into the environment (Rice, 1985). Subsequently, the substances may inhibit or stimulate the growth of ambient species (Xuan, Elzaawely, Deba, Fukuta, & Tawata, 2006). In agriculture, the application of allelopathy in weed management is not a novelty. The use of either plant products or derivatives of bioactive compounds from plants has increasingly become common, owing to their eco-friendly sustainable agricultural characteristics (Khanh, Xuan, & Chung, 2007). Mimosine, a potent allelochemical derived from *Leucaena leucocephala* and *Mimosa* spp., is a typical pattern of the utilization of a natural product which reduced weed emergence but increased rice yield (Xuan, Tawata, & Khanh, 2013). As mimosine is effective for weed inhibition and is easily degradable, the synthesis of mimosine derivatives to develop novel pesticides is promising (Xuan et al., 2013). Among agricultural by-products which can reduce weed emergence, rice husk has been reported to possess promising allelochemicals (Xuan, Tsuzuki, Tawata, & Khanh, 2004). In addition, the application of rice husk and rice bran were effective in paddy weed management (Chung et al., 2005). The incorporation of rice husk in paddy field also increased approximately 20% of rice yield (Xuan et al., 2004; Esa, Ling, & Peng, 2013). Moreover, the allelopathic capability of rice husk extract was extensively examined in a laboratory, greenhouse and paddy field (Khang et al., 2016). There was a number of allelochemicals isolated from rice husk, including phenolics, fatty acids, and momilactones (Kato et al., 1973; Khanh et al., 2007; Berendji, Asghari, & Matin, 2008).

Momilactones A (MA) and B (MB) were first time-isolated and identified from rice husk by Kato et al. (1973). Among biological activities, MA and MB have been known as potent phytoalexins and allelochemicals (Khanh et al., 2007; Minh et al., 2018a; Minh et al., 2018b), but recent researches show that they are also diabetes inhibitors (Quan et al., 2019a;

Quan et al., 2019b). Subsequently, momilactone C (MC) (Tsunakawa et al., 1976) and momilactone D (MD) (Cho et al., 2015) were identified in rice husk and root, respectively. However, only MC exhibited an effective inhibition on germination of *L. sativa* (Tsunakawa et al., 1976) whilst MD displayed a weak anti-inflammatory activity compared to MA by using nitric oxide production models (Cho et al., 2015). Notably, in the research of Cho et al. (2015), authors isolated a pimarane diterpenoid and named it momilactone E (ME). However, it was mistakenly defined because the constituent did not obtain any lactone moiety in its chemical structure. On the other hand, orizaterpenoid retrieved from rice hulls was reported to have a cytotoxic activity on P388 murine leukemia cells (Chung et al., 2005). Structurally, orizaterpenoid consists of a lactone ring linked with the diterpenoid skeleton; therefore, such a compound can be considered as a momilactone. And, in this study, I suppose to name orizaterpenoid as momilactone E (ME). Meanwhile, 7-ketostigmasterol (7KS) was first time-isolated from rice husk (Ahmad, Xuan, Minh, Siddiqui, & Quan, 2019). The compound showed protective effects on human intestinal (Caco-2) cells (Alemany, Laparra, Barberá, & Alegría, 2012), immune system (Laparra, Alfonso-García, Alegría, Barberá, & Cilla, 2015), and antiviral (herpesvirus 1) activity (Marinho et al., 2017). Nevertheless, to date, no study on the allelopathic property of ME and 7KS was carried out.

The selection of test plants in the assessment of allelopathic activity of natural compounds is a crucial tool to evaluate the actual role and applicability of these compounds. Lettuce (*Lactuca sativa*) is widely used as a typical indicator plant, while barnyard grass (*Echinochloa crus-galli*), a major natural enemy of rice and crops in agricultural fields, is applied as an indicator weed in the allelopathic bioassays (Xuan et al., 2004; Khanh, Cong, Chung, Xuan, & Tawata, 2009). In addition, among invasive plants which currently cause trouble for environment, ecology, and crop production in Japan, tall goldenrod (*Solidago altissima*) is one of the most problematic. This plant species distributes throughout the

country and affects many native species (Fujii, 2017). In order to preliminarily clarify the allelopathic role of MA, MB, ME, and 7KS, this research was conducted to examine the inhibitory effects of these compounds on the growth of noxious *E. crus-galli* and invasive *S. altissima*. The inhibition levels from different doses as well as the mixtures of the four compounds on the indicator plants were evaluated.

2.3. Materials and methods

2.3.1. Materials

Rice husks of *Oryza sativa* (var. Koshihikari) were collected from rice mills in Higashi-Hiroshima, Japan in September 2016. Lettuce (*Lactuca sativa*) and barnyard grass (*Echinochloa crus-galli*) seeds were purchased from the Japan Agriculture, Hiroshima, Japan; and P2 and Associates Inc., Fukuoka, Japan, respectively. Meanwhile, tall goldenrod (*Solidago altissima*) seeds were collected in fields at 34.39°N, 132.74°E, Higashi-Hiroshima, Japan in November 2017. The dormancy of *E. crus-galli* and *S. altissima* was broken by keeping them in a freezer for 2 weeks at −20 °C to achieve the maximum germination rate. Pure standard compounds were obtained from previous studies of our laboratory (Ahmad et al., 2019).

2.3.2. Extraction and isolation of active compounds from rice husk

The extraction and isolation of the four pure compounds MA, MB, MD, and 7KS from rice husk were described previously (Ahmad et al., 2019). In brief, 30 kg of dried husk was extracted by methanol for 2 weeks at room temperature. The crude methanolic extract (485 g) was then separated by liquid–liquid extraction method using hexane, ethyl acetate (EtOAc) and water solvents. The resulting EtOAc extract (350 g) was subjected to repeated column chromatography over 60 Å pore size–silica gels (60–100 mesh in a 5 × 60 cm column followed by 200–400 mesh in a 2 × 50 cm column). Four pure compounds were yielded including momilactone A (MA, 350 mg), momilactone B (MB, 200 mg), orizaterpenoid (80

mg) that was defined as momilactone E (ME), and 7-ketostigmasterol (7KS, 10 mg), see Figure 1. The structures of the four isolated compounds were identified and confirmed by spectroscopic techniques consisting of ^{1}H and ^{13}C nuclear magnetic resonance (NMR, Bruker DRX-500 spectrophotometer, Bruker India Scientific Pvt. Ltd., New Delhi, India), electrospray ionization mass (ESI, LTQ Orbitrap XL mass spectrometer, Thermo Fisher Scientific, CA, USA), high-resolution mass spectrometry (HR-MS, 6545Q-TOF LC/MS, Agilent technology, Santa Clara, CA, USA), and infrared spectroscopy (IS, Shimadzu 8201 PC, Shimadzu Corporation, Kyoto, Japan). The spectroscopic methods followed the previous studies (Minh et al., 2018a; Minh et al., 2018b; Ahmad et al., 2019) and data were compared with those in the literature (Ahmad et al., 2019).

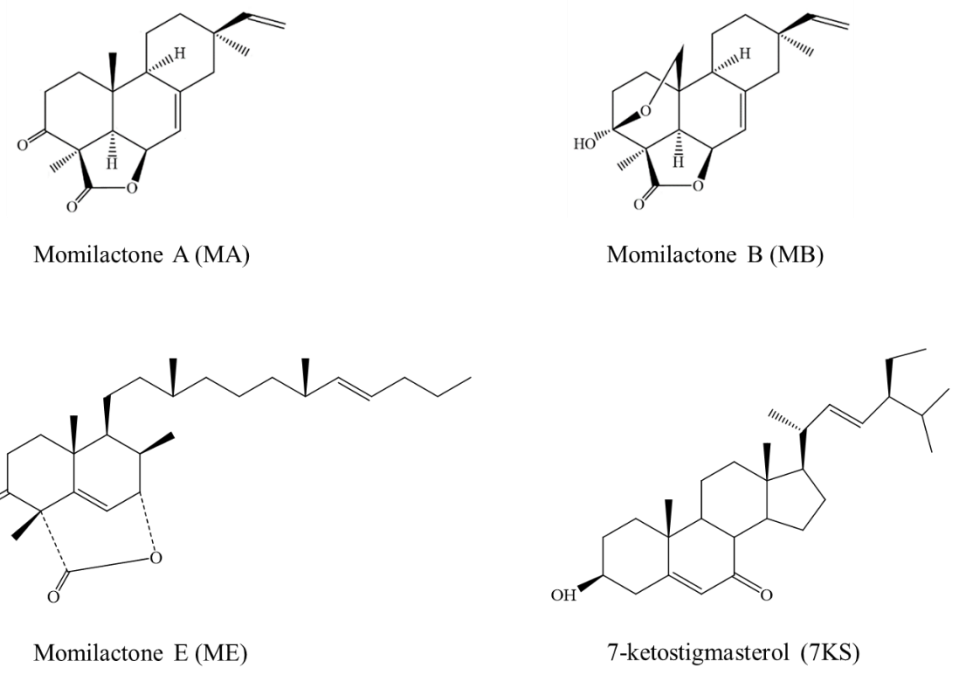


Figure 6. Chemical structures of MA, MB, ME, and 7KS.

2.3.3. Allelopathic bioassays

Seeds of *L. sativa*, *E. crus-galli* and *S. altissima* were soaked in 0.5% aqueous sodium hypochlorite for 20 min and washed thoroughly with distilled water. *S. altissima* seeds were then dried by an oven at 35 °C for 4 days and then kept in a fridge at −20 °C for 2 weeks. Before experiments, selected healthy seeds were incubated in 300 mL of distilled water

(30 °C) for 2 days. Germination and growth of the three species were conducted using Nunc™ 12-well plates (Thermo Fisher Scientific, Jiangsu, China) in a growth chamber (Biotron NC system, Nippon Medical & Chemical Instrument, Co. Ltd., Osaka, Japan) with a photoperiod of 14h day/10h night at 30 °C for 8 days (Figure 7).

The four isolated compounds MA, MB, ME, and 7KS were dissolved in methanol at different dilutions. An aliquot of 0.2 mL of tested solution was penetrated to a filter paper disc (20 mm diameter) followed by evaporating in an oven at 40 °C for 30 min to subtract the effect of methanol on bioassay. Subsequently, a treated disc was placed into the corresponding well of the 12-well plate for the bioassay (Figure 2). Pure methanol was used as a negative control, while *p*-hydroxybenzoic acid (*p*HA) was a positive control. Each experiment was repeated three times. The germination rate of all species was determined, and root and shoot lengths of *L. sativa* and *E. crus-galli* were recorded. The inhibitory and stimulatory percentages were calculated over the negative control. The IC_{50} value represented a concentration that provided 50% inhibition and was calculated by a method of Fukuta et al. (2007). Baseds on the preliminary screening result and a limitation of isolated amount, the pure 7KS was used in *L. sativa* assay only.

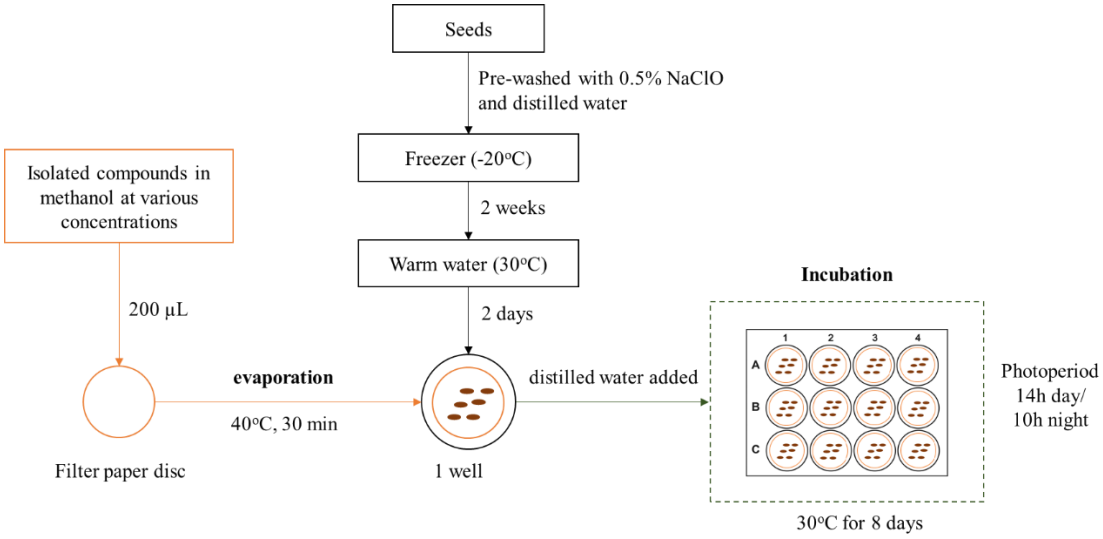


Figure 7. General experimental design of the in-vitro allelopathic assay.

For the allelopathic assay on *L. sativa*, a total of eight seeds were laid on the treated disc in each well. Afterwards, an amount of 0.4 mL of distilled water was added, and the plate was covered by wrapping paper prior to incubating in a growth chamber. In the case of *E. crus-galli* and *S. altissima*, each well of the Nunc™ 12-well plate was at first lined by a layer of 0.5% agar gel (1 mL), and then a treated paper disc was put on the surface followed by pipetting 0.2 mL of distilled water. A total of 8 seeds of *E. crus-galli* and 20 seeds of *S. altissima* were used in each trial.

2.3.4. Quantification of momilactones A and B by HPLC

In this study, the quantitative methods for MA and MB by HPLC were advanced as compared with a previous study described by Quan et al. (2019a). Rice husk (100 g) was extracted with 200 mL of methanol for 1 week. The obtained methanolic extract was mixed with hexane in a separatory funnel for 2 h. Afterward, the methanolic phase was filtered and reconstituted by a vacuum evaporator (Rotavapor® R-300, Nihon Buchi K.K., Tokyo, Japan). The extract of rice husk was homogenized in 50% aqueous methanol and loaded into a Sep-Pak® Plus C18 cartridge (Waters Cooperation, Milford, MA, USA). The cartridge was washed with 2 mL of 50% methanol and then eluted with 10 mL of 100% methanol. Subsequently, the methanol solutions were combined and adjusted into the concentration of 10 mg/mL which was used for HPLC analysis. Five microliters of the extract were injected into the HPLC system comprising PU-4180 RHPLC pump, LC-Net II/ADC controller, and UV-4075 UV/Vis detector (Jasco, Tokyo, Japan). The stationary phase was Waters Spherisorb ODS2 column (10 μm, 150 mm × 4.6 mm i.d.) acquired from Waters Cooperation, Milford, MA, USA. The mobile phase consisted of 0.1% trifluoroacetic acid in 70% aqueous acetonitrile. The operation time was set for 25 min with a flow rate of 0.5 mL/min. The wavelength of 210 nm was used for detecting appearances of MA and MB. The

quantification of MA and MB in rice husk extract was calculated according to the retention times and peak areas of the standards MA and MB with the sample.

The sensitivity of the HPLC system was expressed as limits of detection (LOD) and limits of quantitation (LOQ) by linear regression analyses of peak areas against concentrations of isolated momilactones A and B.

2.3.5. Statistical analysis

All bioassays were carried out thrice in a completely randomized design ($n = 3$). Data were analyzed by the Minitab 16.0 software (Minitab Inc., State College, PA, USA). Results were displayed as means ± standard errors (SE). The IC_{50} value was the amount required to inhibit 50% germination or growth of the test plants. Significant differences among assays were evaluated by one-way ANOVA using Tukey's test at $p < 0.05$.

2.4. Results

The inhibitory effects of the four isolated compounds on germinations of *L. sativa*, *E. crus-galli* and *S. altissima* are shown in Table 2. It was indicated that the suppressive influences of MA, MB, ME, and 7KS on germination of tested plants were differed. Of which, MB and the mixture of MA and MB (MAB) at a ratio 1:1 (*v*/*v*) exerted much stronger inhibition compared to ME and 7KS. Particularly, MAB showed significant inhibitions on the germination of *L. sativa* (IC_{50} = 327.20 µg/mL), *E. crus-galli* (IC_{50} = 28.26 µg/mL), and *S. altissima* (IC_{50} = 23.97 µg/mL). The IC_{50} values of MA (on *L. sativa*), ME, *p*-hydroxybenzoic acid (*p*HA), and the mixture of ME-7KS (on *L. sativa* and *E. crus-galli*), as well as 7KS (on all assays) were not calculated for *L. sativa* assay when the germination rate was 100 % at all treated concentrations.

As shown in Table 2, ME showed a similar inhibition to the positive control *p*HA while other compounds and mixtures exhibited greater suppression on germination of *S. altissima*. The IC_{50} values of MA, MB, and MAB were 119.80, 20.36, and 23.97 µg/mL, respectively.

Besides, the mixture ME-7KS (IC_{50} = 358.30 μg/mL) also expressed notable suppressions on the *S. altissima* germination. The mixture MAB presented a similar level of inhibition on *S. altissima* compared with MB. IC_{50} values of MB and MAB were approximately 5- and 22-fold greater than those of MA and ME-7KS, respectively, which indicated that MB may play a crucial role in the allelopathic activity.

Table 2. IC_{50} value (μg/mL) for inhibitory effect of isolated compounds on germination rate of tested plants

Sample	*L. sativa*	*E. crus-galli*	*S. altissima*
MA	nd	229.67 ± 1.20 b	119.80 ± 11.50 b
MB	178.46 ± 0.03 b	31.88 ± 0.18 a	20.36 ± 0.91 a
MAB	327.20 ± 27.50 a	28.26 ± 3.05 a	23.97 ± 1.89 a
ME	nd	nd	1162.50 ± 14.10 d
7KS	nd	nd	nd
ME-7KS	nd	nd	358.30 ± 8.39 c
*p*HA	nd	nd	1074.20 ± 66.60 d

Means ± standard error. Means within a column followed by similar letters are not significantly different by Turkey's test ($p < 0.05$); nd = not determined; MA, momilactone A; MB, momilactone B; MAB, mixture of momilactones A and B (1:1, *v*/*v*); ME, momilactone E; 7KS, 7-keto-stigmasterol; ME-7KS, mixture of momilactone E and 7-keto-stigmasterol (1:1 *v*/*v*); *p*HA, *p*-hydroxybenzoic acid.

The inhibitory effects of MA, MB and their mixture MAB on growths of *L. sativa* and *E. crus-galli* are shown in Table 3. Of which, MB and the mixture MAB showed similar levels of suppression and it was much greater than either MA or the control *p*HA. MA, MB and MAB inhibited root and shoot elongations of *E. crus-galli* more strongly than those of *L. sativa*. In contrast, the positive control *p*HA showed greater effects on *L. sativa* than *E. crus-galli* (Table 2).

Table 3. IC_{50} value (µg/mL) for inhibitory effect of MA and B on growth of *L. sativa* and *E. crus-galli*

Sample	*L. sativa*		*E. crus-galli*	
	RL	**SL**	**RL**	**SL**
MA	348.00 ± 11.30 b	388.20 ± 10.70 b	147.60 ± 0.37 b	123.87 ± 1.78 b
MB	6.49 ± 0.04 a	58.64 ± 0.99 a	4.00 ± 0.03 a	4.46 ± 0.05 a
MAB	5.63 ± 0.05 a	63.77 ± 0.64 a	5.13 ± 0.05 a	4.47 ± 0.05 a
*p*HA	820.57 ± 9.8 c	1453.30 ± 48.10 c	936.66 ± 4.49 c	1464.6 ± 40.30 c

Data presented a means ± standard error. Means within a column followed by similar letters are not significantly different by Turkey's test ($p < 0.05$); RL, root length; SL, shoot length; MA, momilactone A; MB, momilactone B; MAB, mixture of momilactones A and B (1:1, *v*/*v*); *p*HA, *p*-hydroxybenzoic acid.

In Table 4, the inhibitory activity of MA, MB, ME, 7KS and their mixtures were compared at two concentrations 400 and 1000 µg/mL. In general, the inhibitory levels of MA, MB, as well as the mixture MAB were greater than ME, 7KS and the mixture ME-7KS. At 400 µg/mL, the germination and growth of *E. crus-galli* and *S. altissima* were almost suppressed, although *L. sativa* was inhibited at a lower level (Table 4). In contrast, even at 1000 µg/mL, shoot length of *L. sativa* was strongly promoted by ME, 7KS, and ME-7KS, whilst no effect on germination of both *L. sativa* and *E. crus-galli* was observed. Although the inhibitory effects of ME, 7KS and the mixture ME-7KS on germination of *S. altissima* were significantly stronger than on that of *L. sativa* and *E. crus-galli*, MA, MB and the mixture MAB recorded much greater inhibition on germination and growth of all tested plants (Table 4).

The quantitative methods for MA and MB by HPLC showed a significantly greater quantity of the two compounds in rice husk as compared with the previous study (Quan et al., 2019a) (Table 5). Of which, the use of column type and flow rate were modified to

enhance the detective level of both MA and MB to quantify much greater amounts of MA and MB (Table 5).

Table 4. Comparative allelopathic effects (%) of isolated compounds on growth of tested plants

Sample	Concentration (μg/mL)	*L. sativa*			*E. crus-galli*		*S. altissima*	
		GR	**RL**	**SL**	**GR**	**RL**	**SL**	**GR**
Methanol		(8/8)[a]	(1.95)[b]	(0.76)[c]	(8/8)	(4.53)	(3.03)	(13/20)
		0.0	0.0	0.0	0.0	0.0	0.0	0.0
MA	400	0.0	52.38	50.67	78.57	95.96	96.70	86.75
MB		100.00	94.71	84.59	100.00	100.00	100.00	100.00
MAB		58.33	91.82	82.82	100.00	100.00	100.00	100.00
ME	1000	0.0	+92.68	6.61	0.0	+16.48	+8.01	39.53
7KS		0.0	+60.93	13.65	nd	nd	nd	nd
ME-7KS		0.0	+93.00	5.71	0.0	13.33	29.52	94.66
*p*HA		0.0	63.14	37.01	0.0	58.46	36.12	44.66

Data shows inhibition percentages over the control; +, stimulation percentages over the control; MA, momilactone A; MB, momilactone B; MAB, mixture of momilactones A and B (1:1, *v*/*v*); ME, momilactone E; 7KS, 7-keto-stigmasterol; ME-7KS, mixture of momilactone E and 7-keto-stigmasterol (1:1, *v*/*v*); *p*HA, *p*-hydroxybenzoic acid; GR, growth rate; RL, root length; SL, shoot length; nd, not determined. Numbers in parentheses are baselines of negative control; [a], number of germinated seeds; [b,c], root and shoot lengths (cm).

In this study, the amounts of MA and MB in rice husk were calculated as 51.96 μg/mL and 42.33 μg/mL, respectively, which were 3.2- and 4.6-fold higher than those of MA and MB quantified by the previous method (Quan et al., 2019a). The principal factor was attributed to the use of a Sep-Pak C18 cartridge in preparation of husk extract. By this application, some other components were firstly eliminated by 50% aqueous methanol, then the purer MA and MB were obtained from the 100% methanol solution.

Table 5. Advanced development of the quantitative methods by HPLC for MA and MB

Parameters	This Study	Previous Study (Quan et al., 2019a)
Sep-Pak C18 cartridge	Used	Not use
HPLC column	10 µm, 150 mm × 4.6 mm i.d.	10 µm, 250 mm × 4.6 mm i.d.
Flow rate	0.5 mL/min	0.4 mL/min
LOD	0.05 ng/mL (MA), 0.48 ng/mL (MB)	0.43 ng/mL (MA), 0.18 ng/mL (MB)
LOQ	0.14 ng/mL (MA), 1.46 ng/mL (MB)	1.31 ng/mL (MA), 0.54 ng/mL (MB)
Quantity of MA	51.96 µg/Ml	16.44 µg/mL
Quantity of MB	42.33 µg/Ml	9.24 µg/mL

HPLC, high performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MA, momilactone A; MB, momilactone B.

The other factor might be HPLC column length and flow rate. The use of a shorter analytical column (150 mm) and a slight increase in flow rate (0.5 mL/min) might influence the sensitivity of the HPLC system by enhancing LOD (0.05 ng/mL) and LOQ (0.14 ng/mL) of MA. The result provided an efficient option for selecting the most appropriate method of sample preparation and quantification of MA and MB.

2.5. Discussion

MA and MB have been admitted as plant growth-inhibitors and phytoalexins in rice plant organs and rice husk for more than 40 years, thus both MA and MB are believed to play a crucial role in protecting rice from multiple natural enemies including weeds and pathogens (Kato et al., 1977; Cartwright et al., 1981; Chung et al., 2005; Fukuta et al., 2007). This study confirmed and specially emphasized the potent biological activity of MB in the growth restraint of some indicator species. To the best of our knowledge, this is the first time

the growth inhibition of the mixture MAB has been investigated. MB exerted a stronger inhibition on plant growth than MA, and a mixture of MA and MB (1:1, *v*/*v*) did not show stronger effects than the solo treatment by MB (Table 2). The result can be construed by the exclusive structure of MB. A bridge -O- between C-3 and C-20 attached with a hydroxyl group in MB structure may bring about potent herbicidal activity compared with MA (Fukuta et al., 2007; Quan et al., 2019a). This finding may provide a new approach in the green production sof herbicides which is environmentally friendly and cost saving. The use of mixture MA and MB did not show stronger inhibition than the individual MB did, thus the search for single constituents with strong bioactive effects to develop natural herbicides should be targeted. The application of a solo compound is apparently easier and lower cost than a mixture of many constituents. Additionally, the improved method for sample purification and HPLC analysis from this study increased the quantifying effectiveness of momilactones A and B by 3–4 times (Table 5), which helped determine more correctly the real contents of these active compounds in rice.

For the other consideration, ME and 7KS were the first investigated for the allelopathic activity. While ME portrayed an exceptional stimulation on root growth of *L. sativa* and a minor enhancement on root and shoot elongations of *E. crus-galli*, it showed a considerable inhibition on germination of *S. altissima*. Given the synergistic effect of a mixture ME and 7KS (1:1, v/v) on the suppression of root and shoot lengths of *E. crus-galli* and germination of *S. altissima*, 7KS might possess a promising inhibitory effect on the growth of these indicator plants (Table 4). In fact, each plant has a specific way to respond with both abiotic and biotic stresses from the habitat. It depends on various factors including dose and intensity of factors (Xuan, Tawata, Khanh, & Chung, 2005) as well as the defense mechanism of a plant itself (War et al., 2012). Presently, a large number of allelochemicals has been elucidated belonging to phenolics, flavonoids, terpenoids, alkaloids, and their

derivatives (Khanh, Xuan, Chung, & Tawata, 2008). The earlier study also manifested the potential role of sterols in the allelopathic activity of plants (Fischer & Quijano, 1985). However, information of natural products being allelopathic on different plants has not been provided sufficiently. This study was the first to demonstrate the difference in allelopathic effects of a terpenoid (ME, orizaterpenoid) and a sterol (7KS, 7-ketostigmasterol) isolated from rice husk on the growth of various indicator plants including *L. sativa*, *E. crus-galli*, and *S. altissima*.

Fujii and partners (1991) reported that L-3,4-Dihydroxyphenylalanine (L-DOPA) derived from velvetbean (*Mucuna pruriens* (L.) DC. var. *utilis*) possessed a potent inhibition on radicle growth of *L. sativa* (IC_{50} = 50 μg/mL) and *S. altissima* (IC_{50} = 115 μg/mL). In comparison with MA and MB, L-DOPA presents a stronger inhibitory effect on the growth of radicle while MA and MB exhibits more powerful suppressive capacity on germination of indicator plants. In term of contribution of individual compounds to the total allelopathic activity of original plants, L-DOPA is more relatively implicative than MA and MB because of its high amount in velvetbean (1% in leaves and 6 to 9% in seeds) (Fujii, Shibuya, & Yasuda, 1991), whereas, the content of MA and MB in rice is minor (11.7 and 8.3 part per billions, respectively). Ecologically, therefore, MA and MB may not merely be allelochemicals but phytoalexins. Other models such as electrolyte leakage, lipid peroxidation, proline content, and gene expression should be further carried out in order to verify these biological activities of momilactone compounds.

The most significant finding of this study was the inhibitory effect of active compounds from rice husk on the germination of tall goldenrod (*Solidago altissima*), a harmful invasive plant. Several studies were implemented to evaluate the germination of *S. altissima* seeds, however, most of them used sand or soil as the media (Walck, Baskin, & Baskin, 1997; Meyer & Schmid, 1999). For the current study, the germination of *S. altissima*

was successfully assayed under the laboratory condition by using agar gel (0.5%) as a growth media. The method was designed in order to ensure the shortest time and the highest efficiency of plant germination. In nature, *S. altissima* grows very fast and wildly distributes in miscellaneous habitats (Sakata et al., 2013) such as abandoned fields, edges of rice-fields, and pavements. It strongly competes with other plants in the habitat due to the tenacious rhizome system which can regenerate to form a new individual (Heath, Kessler, Woebbe, Cipollini, & Stireman, 2014). Additionally, *S. altissima* seed has a powerful dispersal ability thanks to its peculiar structure (wind spread) and a vast number of seeds. In Japan, *S. altissima* does not only affect the landscape, agricultural fields, and social infrastructures but also influences the health of humans and animals. There is an unofficial report that seeds of *S. altissima* when spreading in the air, may cause respiratory allergies in human and pets. The allelopathic results in this study indicated that the germination of *S. altissima* was the most susceptible by momilactones and was potentially inhibited by the mixture of ME-7KS. In addition, the active compounds besides MA, MB, ME, and 7KS isolated from rice husk and their derivatives should be further examined on harmful weeds and invasive species to improve the potential use of rice husk for weed management. The synthesis of derivative constituents derived from MA, MB, ME, and 7KS might have potential to develop novel and environmentally safer herbicides.

2.6. Conclusions

Findings of this study showed that MA, MB, ME, and 7KS were plant growth-inhibitors in rice husk, although the levels of inhibition of these compounds varied. Among the four compounds, MB was the most inhibitory, followed by MA. Both ME and 7KS exhibited lower suppressive effects than MA and MB. MA, MB, and the mixture MAB had potential to inhibit the growth of both barnyard grass (*E. crus-galli*) and tall goldenrod (*S. altissima*), whilst only the mixture of ME-7KS showed strong inhibition towards the

emergence of *S. altissima*. This study also achieved an advanced quantitative method to detect MA and MB in rice husk by 51.96 and 42.33 μg/mL, respectively, or 3.16- and 4.58-fold higher as compared with our previous research. MA, MB, and the mixture ME-7KS showed promise in controlling the harmful paddy weed *E. crus-galli* and the invasive *S. altissima* in vitro, but their modes of action should be investigated further.

CHAPTER 3: MOMILACTONES A AND B ARE α-AMYLASE AND α-GLUCOSIDASE INHIBITORS

3.1. Summary

Momilactones A (MA) and B (MB) are the active phytoalexins and allelochemicals in rice. In this study, MA and MB were purified from rice husk of *Oryza sativa* cv. Koshihikari by column chromatography, and purification was confirmed by high-performance liquid chromatography, thin-layer chromatography, gas chromatography-mass spectrometry, liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS), and ^{1}H and ^{13}C nuclear magnetic resonance analyses. By in vitro assays, both MA and MB exerted potent inhibition on α-amylase and α-glucosidase activities. The inhibitory effect of MB on these two key enzymes was greater than that of MA. Both MA and MB exerted greater α-glucosidase suppression as compared to that of the commercial diabetic inhibitor acarbose. Quantities of MA and MB in rice grain were 2.07 ± 0.01 and 1.06 ± 0.01 μg/dry weight (DW), respectively. This study was the first to confirm the presence of MA and MB in refined rice grain and reported the α-amylase and α-glucosidase inhibitory activity of the two compounds. The improved protocol of LC-ESI-MS in this research was simple and effective to detect and isolate MA and MB in rice organs.

3.2. Introduction

Diabetes has become a worldwide health problem in developed and developing countries. It is reported that about 425 million people are suffering from diabetes which accounts for 12% of global health expenditure (International Diabetes Federation, 2017). Among diabetic types, type 2 is the most common occurrence in adults due to a complex metabolic abnormality including insulin resistance, hyperglycemia, and deficient insulin secretion. The pathogenesis of type 2 diabetes may launch from many factors such as genetic

predisposition, environment, and pancreatic beta-cell dysfunction (Leahy, 2005). One of the most beneficial therapeutics was proposed as maintaining blood glucose at normal levels after a meal (Abesundara, Matsui, & Matsumoto, 2004). This approach may gradually help avoid chronic hyperglycemia, reduce insulin resistance and consequently stabilize the insulin production of pancreatic beta-cell. In humans, the digestion of carbohydrates is carried out by multiple hydrolytic enzymes. Of which, α-amylase and α-glucosidase are crucial in the hydrolysis of polysaccharides to obtain glucose more suitable for absorption (Ercan & El, 2016). Hyperglycemia in patients with type 2 diabetes was attributed to starch breakdown by pancreatic α-amylase and glucose uptake by intestinal α-glucosidase (Apostolidis, Kwon, & Shetty, 2007). In fact, there are many available antidiabetic drugs such as biguanides, sulfonylureas, meglitinides, thiazolidinediones, α-glucosidase inhibitors, incretin mimetics, dipeptidyl peptidase-IV inhibitors and insulin; however, the use of these pharmaceutical drugs may cause undesired and severe side effects (Arulselvan et al., 2014). Therefore, the search for and discovery of new α-amylase and α-glucosidase inhibitors from natural sources are valuable approaches to more securely decelerating the glucose production in the human intestine.

Momilactones A (MA) and B (MB) belonging to diterpenes group (Figure 1), have been detected only in rice and the moss *Hypnum plumaeforme* (Kato-Noguchi, 2011; Minh et al., 2018a). The allelopathic function of MA and MB against plant's natural enemies such as weeds and blast fungus has widely been reported (Cartwright et al., 1981; Obara, Hasegawa, & Kodama, 2002; Chung, Jung, & Kim, 2006; Fukuta et al., 2007; Toyomasu et al., 2008; Kato-Noguchi, Hasegawa, Ino, Ota, & Kujime, 2010). Recently, MA and MB were found more implicative with salinity and drought tolerance of rice more than allelopathy (Xuan et al., 2016; Quan & Xuan, 2018). MA and MB also exhibited antioxidant (Fukuta et al., 2007), cytotoxic (Chung et al., 2005), antitumor (Kim et al., 2007) and anticancer

activities (Joung et al., 2008; Park et al., 2014). Of which, MB was in lower quantity in rice husk and other plant parts but exerted greater biological activities than MA (Kang et al., 2017; Minh et al., 2018a). In addition, Kang et al. (2017) showed that MB was effective in controlling ketosis associated with low blood sugar levels, however, trials in hyperglycemic conditions have not been performed. Hitherto, the search of natural compounds with potent anti-diabetes properties has been expanded, but none of the compounds with diterpene lactone structure possessing antidiabetic property was reported, except eremanthin (a sesquiterpene lactone) and andrographolide (a diterpenoid lactone). Among these, eremanthin exhibited hypoglycemic and hypolipidemic activities (Eliza, Daisy, Ignacimuthu, & Duraipandiyan, 2009), while andrographolide was potent for diabetic control (Yu, Hung, Chen, & Cheng, 2003; Xu, Liu, Dai, Wu, & Liu, 2007; Subramanian, Asmawi, & Sadikun, 2008; Brahmachari, 2017). There were several other reports on antidiabetic activity of diterpenes and their synthetic derivatives (Reddy et al., 2009; Ayinampudi, Domala, Merugu, Bathula, & Janaswamy, 2012; Ghosh & Rangan, 2014; Jelenković, Jovanović, Palić, Mitić, & Radulović, 2014; Li, Ding, Wan, & Li, 2016).

Moreover, no reports on toxicities of natural compounds from either rice grain or its by-products affecting human health have been published. Several compounds involved in the diabetic inhibition were found in rice bran and color rice (Cheng et al., 2010; Boue, Daigle, Chen, Cao, & Heiman, 2016; Uraipong & Zhao, 2016; Chiou, Lai, Liao, Sung, & Lin, 2017). Though MA and MB are promising bioactive constituents in rice, the isolation and purification of MA and MB are complicated and laborious. At present, there are very few laboratories in the world that can successfully isolate and purify MA and MB. As a result, no commercial MA and MB from chemical companies in Japan or abroad can be purchased; thus, research on biological activities of the two compounds has been limited. My group recently developed a new protocol for extracting conditions and solvents to

provide optimal yields of MA and MB by column chromatography combined with different extracting solvents and temperature (Minh et al., 2018b; Ahmad et al., 2019). In this study, I investigated the inhibition of MA and MB on α-amylase and α-glucosidase activities and reported the presence of the two compounds in white rice grain using LC-ESI-MS technique.

3.3. Materials and methods

3.3.1. Collection and extraction of rice husk

Rice husk of Koshihikari variety (Japonica subtype) was collected from rice mills allocated near Hiroshima University, Higashi-Hiroshima Campus, Japan in July 2017. The identification of variety was authenticated by a voucher specimen (KOS-MOMI 17HJ) which was deposited at Plant Physiological Laboratory, IDEC, Hiroshima University, Japan. Extraction and isolation methods were referred from previous studies (Minh et al., 2018a; Ahmad et al., 2019) with slight modifications. Briefly, rice husks (7 kg) were dried at 50 °C for 6 days by an oven and steeped in 50 L methanol 100% for 2 weeks at room temperature to yield 92 g crude extract. After mixing with an adequate volume of distilled water, the extract was partitioned consecutively with hexane and ethyl acetate (EtOAc) at high grades (>99.5%).

3.3.2. Isolation of momilactones A and B from EtOAc extract

The dry EtOAc extract (57 g) was chromatographed on silica gel (60–100 mesh) column (5 × 60 cm) to yield 35 fractions with the following eluants: 5 fractions in hexane (500 mL/elution), 20 fractions in hexane:EtOAc (9:1) (200 mL/elution), 10 fractions in hexane:EtOAc (8:2) (200 mL/elution). Fractions from hexane:EtOAc (8:2) were combined, evaporated and further purified by column chromatography over silica gel (200–400 mesh) with chloroform. Similarity among fractions was tested by TLC prior to combination (Ahmad et al., 2019).

3.3.3. Identification and confirmation of momilactones A and B by HPLC, TLC, GC-MS, and ^{1}H-NMR and ^{13}C-NMR analyses

The presence and purity of MA and MB by HPLC and TLC analyses were compared with the standards MA and MB attained from a previous study (Ahmad et al., 2019). For HPLC analysis, the isolated MA and MB were separately dissolved in methanol and filtered by a 0.45 μm pore size polytetrafluoroethylene filter (FILTSTAR Syringe Filter, Starlab Scientific Co., Ltd, China). The final concentration of tested samples was adjusted to 20 μg/mL, the standard MA and MB were mixed at ratio 1:1 (v/v). 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, Milford, 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 momilactone A and B.

TLC analysis was carried out on the TLC silica gel 60 plates (Merck KGaA, Darmstadt, Germany) with a layer thickness of 175–225 μm. Solution of the standards MA and MB (1:1, v/v) was used as a reference. Specifically, MA and MB were spotted on a TLC plate and run with the solvent system of chloroform:methanol (9.5:0.5, v/v) for 2.4 min. The plate was then dipped into a chamber comprising of 1% vanillin-sulfuric acid in pure ethanol and dried in an oven at 100 °C for 2 min. Subsequently, the separation of MA and MB was observed visually and the retention factor (R_f) value of each MA and MB was calculated as:

R_f = distance of MA or MB spot traveled/distance of solvent system moved (1)

where the molecular mass of the isolated MA and MB was confirmed by spectrum data from GC-MS analysis. The GC-MS system (JMS-T100 GCV, JEOL Ltd., Tokyo, Japan) equipped with an auto sampler coupled with a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-5MS column (Agilent Technologies, J & W Scientific Products, Folsom, CA, USA). A concentration of 1000 μg/mL of either isolated momilactones was used. Helium was used as a carrier gas at split ratio 5:1. The GC oven conditions were as follows: the initial temperature was 50 °C without hold time, boosted temperature up to 300 °C at 10 °C/min, and held for 20 min. The injection port and detector temperature were set at 300 °C and 320 °C, respectively. The mass range scanned from 29 to 800 amu. The control of the GC-MS system and the confirmation of analytes were conducted using JEOL's GC–MS Mass Center System Version 2.65a.

Both ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra were achieved on a Brucker DRX-500 model spectrometer (Bruker India Scientific Pvt. Ltd., New Delhi, India) operated at 500 and 125 MHz, respectively. The NMR spectra were received in deuterated $CHCl_3$ 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 spectrometry (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 (4000–400 cm^{-1}) (Shimadzu Cooporation, Kyoto, Japan).

3.3.4. α-Amylase inhibition assay

The inhibitory effect of MA and MB on α-amylase was assessed by starch-iodine method (Ercan & El, 2016) with a modified model as follows: in each well of a microplate (U-shape, Greiner Bio-one, NC, USA), 20 µL of each either MA or MB were pre-incubated with 20 µL of 1 U/mL α-amylase solution (from *Aspergillus oryzae*, Sigma-Aldrich, St. Louis, MO, USA) at 37 °C for 10 min. The reaction was initiated by pipetting 30 µL of starch (0.5% in deionized water) (soluble ACS reagent, Sigma-Aldrich, St. Louis, MO, USA). After 6 min incubation at 37 °C, an aliquot of 20 µL of hydrochloric acid (1 M) were added to stop reaction, followed by 120 µL of 0.25 mM aqueous iodine solution. The absorbance at 565 nm was read by a microplate reader (Multiskan^{TM} Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). The inhibitory activity of MA and MB on α-amylase was performed as the inhibition percentage that was calculated by the following formula:

$$\% \text{ inhibition} = (A - C)/(B - C) \times 100 \quad (2)$$

where A is the absorbance of reaction with presence of MA or MB, B is the absorbance of reaction without enzyme, C is the absorbance of reaction with absence of the momilactones. A commercial diabetes inhibitor acarbose was used as a positive reference. Dilutions of test samples and dissolutions of enzyme used 20 mM sodium phosphate buffer (pH 6.9 comprising of 6 mM sodium chloride). α-Amylase solution and soluble starch solution were prepared and used on the day of experiment. The IC_{50} value was calculated to exhibit 50% inhibitory capacity of reaction at a certain concentration. Therefore, lower value of IC_{50} indicates stronger activity.

3.3.5. α-Glucosidase inhibition assay

The anti-α-glucosidase activity of MA and MB was evaluated using a method as described previously (Johnson, Lucius, Meyer, & Gonzalez De Mejia, 2011) with some alterations. In brief, an amount of 20 µL methanolic stock solution of MA and MB were pre-

mixed with an equal volume of 0.1 M potassium phosphate buffer (pH 7) and 40 µL of α-glucosidase (from *Saccharomyces cerevisiae*, Sigma-Aldrich, St. Louis, MO, USA) enzyme solution (0.5 U/mL in 0.1 M potassium phosphate buffer, pH 7). After 6 min incubation at 25 °C, a 20 µL aliquot of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (pNPG) substrate (in 0.1 M potassium phosphate buffer, pH 7) was added to each reaction and the mixture was incubated for another 8 min. Eventually, the reaction was terminated by adding 100 µL of 0.2 M Na_2CO_3, and absorbance was recorded at 405 nm. The inhibition percentage was calculated by the following equation:

$$\% \text{ inhibition} = (1 - A_{sample}/A_{control}) \times 100 \quad (3)$$

where A_{sample} is absorbance of reaction with either MA or MB or positive controls (acarbose or quercetin) and $A_{control}$ is absorbance of reaction with 20% methanol as a negative control. IC_{50} value was obtained by the same way as above.

3.3.6. Quantification and confirmation of MA and MB in rice plant parts by HPLC and LC-ESI-MS

Rice grain, husk, leaf and root of Koshihikari rice variety were collected in July–October 2018, Higashi Hiroshima, Japan. Samples were pre-soaked into NaOCl 0.5% for 15 min, then cleaned several times by distilled water. After blotting, they were dried by an oven at 50 °C for 6 days. Dried samples were separately extracted by MeOH for 5 days. Subsequently, each crude methanolic extract was obtained and mixed with an equal volume of hexane in a separatory funnel. After 2 h, the lower phase of every extraction was collected, filtered, and concentrated to yield a concentration of 100 mg/mL. HPLC analysis was conducted in a similar method as described above. The identification and quantification of MA and MB in rice plant parts were compared and calculated according to the retention times and peaks of the standards MA and MB with the samples. LC-ESI-MS analysis was implemented by using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific,

CA, USA) connected with an electrospray ionization (ESI) source. LC condition was set in the same way with HPLC analysis. ESI condition comprised: ion spray voltage was 4.5 kV, sheath gas flow rate was 60 and aux gas flow rate was 20. MS analysis was run by a positive fourier transform mass spectrometer (FTMS) at a resolution of 60000 with a scan range of m/z 100–1000. Ten μL of samples (50 mg/mL) and standard momilactones (0.5 mg/mL) were injected to the above system by an autosampler. The presence of momilactones in samples was confirmed by comparing their extracted ion chromatograms (EIC) and mass spectra with those of standard momilactones.

3.3.7. Statistical analysis

Data of this study were elaborated on the Minitab 16.0 software (Minitab Inc., State College, PA, USA). All analyses were in a complete randomization with three replications, and results are displayed as mean ± standard error (SE). Significant differences were determined by one-way and two-way ANOVA using Tukey's test at $p < 0.05$.

3.4. Results

3.4.1. Isolation and confirmation of momilactones A and B

3.4.1.1. HPLC

By an open column chromatography with chloroform as mobile phase, two compounds were purified including MA (52 mg) and MB (44 mg). The presence of MA and MB was confirmed by HPLC at 210 nm spectra (Figure 8). The peaks were affirmed by measuring a mixture of standards and the isolated MA and MB at ratio 1:1 (data not presented). According to Figure 8i, the isolated MA and MB appeared at 17.03 ± 0.02 min and 14.06 ± 0.01 min, respectively. The separation order was in accordance with standard MA (17.03 ± 0.03 min) and MB (14.06 ± 0.02 min). The retention times were also coincident with those reported in previous research (Cartwright et al., 1977; Lee et al., 1999; Kang et al., 2017; Minh et al., 2018a). Detection limits of MA and MB were 0.43 and 0.18 ng/mL,

respectively. Meanwhile, limits of quantitation were calculated as 1.31 ng/mL for MA and 0.54 ng/mL for MB.

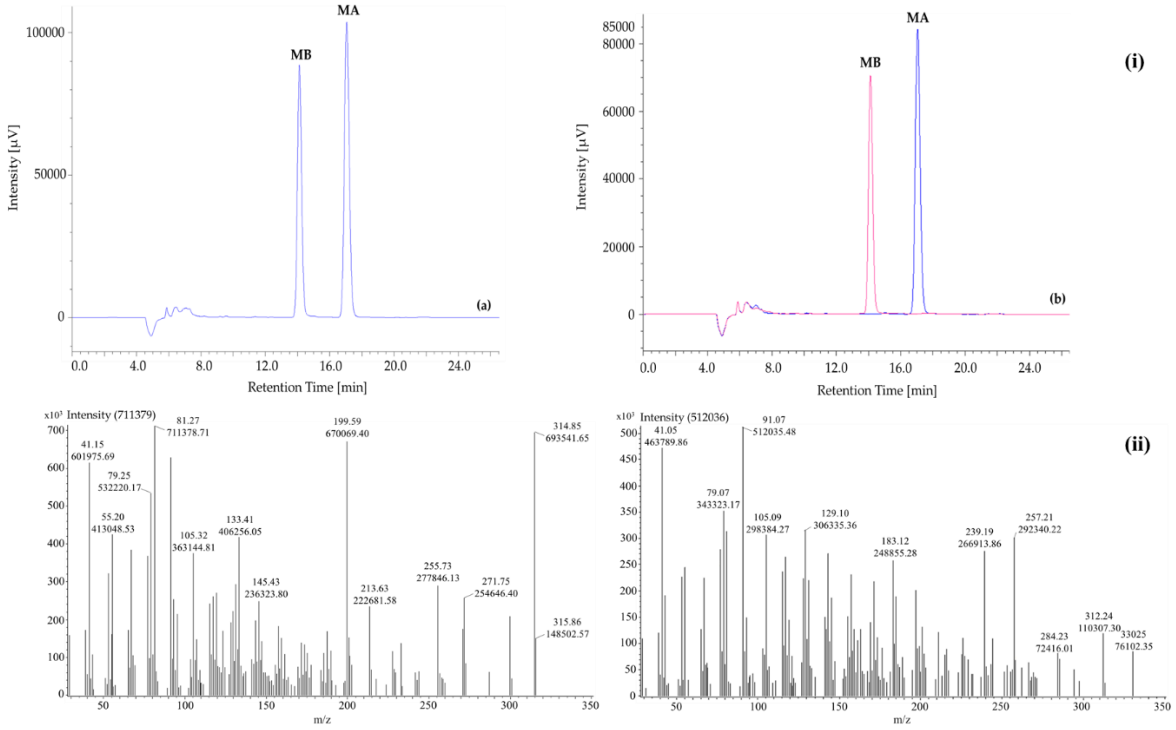


Figure 8. (**i**) HPLC chromatograms of momilactones A and B: (**a**) mixture of standard momilactones A (MA) and B (MB), (**b**) isolated momilactones A and B (overlaid chromatogram); and (**ii**) Mass spectra of the purified (**a**) momilactone A and (**b**) momilactone B.

3.4.1.2. GC-MS

The mass spectral data of the isolated MA and MB were shown in Figure 8(ii). The earlier detected peak (retention time = 23.50 min) showed a molecular ion at 315.86 m/z while this value of later one (retention time = 23.70 min) was 330.25 m/z. There were 161 and 165 fragments of MA and MB were obtained, respectively. The fragmentation patterns (161 and 165 for MA and MB, respectively) and intensity data of the two compounds were presented in the Supplementary Materials, Tables S1 and S2.

3.4.1.3. ^{1}H-NMR and ^{13}C-NMR

MA*:* Colourless crystalline compound; R_f 0.71 ($CHCl_3$: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, J = 17.0, 11.0, H-15), 4.97, 4.93 (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 (Cartwright et al., 1981; Kim et al., 2007; Minh et al., 2018b).

MB: Colourless crystalline compound; R_f 0.63 ($CHCl_3$: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 $C_{20}H_{27}O_4$, 331.1909). (Compare NMR data with previous literature (Cartwright et al., 1981; Kim et al., 2007; Minh et al., 2018b).

3.4.2. In vitro inhibition of α-amylase and α-glucosidase

To the best of our knowledge, no study has before examined the inhibitory activities of MA and MB on the two key enzymes α-amylase and α-glucosidase which are relevant to diabetes. The inhibition of MA and MB was positively correlated with their concentrations (r^2 = 0.94 and 0.80 for α-amylase; r^2 = 0.83 and 0.95 for α-glucosidase, respectively) (Supplementary Materials, Figures S1 and S2). The IC_{50} values were presented in Table 6. Accordingly, both MA and MB exerted inhibitory activity against α-amylase (IC_{50} = 266.68 ± 2.74 and 146.85 ± 1.95 µg/mL, respectively) and α-glucosidase (IC_{50} = 991.95 ± 0.96 and 612.03 ± 0.39 µg/mL, respectively). These effects were also in line with those of the well-known commercial diabetes inhibitors (acarbose: IC_{50} = 117.08 ± 1.47 µg/mL for α-amylase and 2549.00 ± 5.15 µg/mL for α-glucosidase; quercetin: IC_{50} = 105.68 ± 0.09 µg/mL for α-glucosidase). Both MA and MB and acarbose exhibited stronger inhibition on α-amylase than on α-glucosidase activities. By comparing the IC_{50} values, it was clear that MA and MB have levels of antidiabetic capacity similar to acarbose. More specifically, MA and MB even exhibited much stronger suppressive properties than acarbose on α-glucosidase inhibitory assay, for which MA was 2.5-fold and MB was 4.2-fold greater than acarbose.

Table 6. α-Amylase and α-glucosidase inhibitory activities of momilactones A and B

	α-Amylase inhibitory assay (µg/mL) (IC_{50})	**α-Glucosidase inhibitory assay (µg/mL) (IC_{50})**
MA	266.68 ± 1.58 [c]	991.95 ± 0.96 [c]
MB	146.85 ± 1.12 [b]	612.03 ± 0.39 [b]
Acarbose	117.08 ± 0.85 [a]	2549.00 ± 5.15 [d]
Quercetin	-	105.68 ± 0.09 [a]

Data presented means ± standard errors. Means within a column followed by different superscript letters ([a, b, c, d]) are significantly different at $p < 0.05$ level. -, not measured; MA, momilactone A; MB, momilactone B.

3.4.3. Contents of MA and MB in rice plant parts

Results from a two-way ANOVA analysis revealed that there was a significant difference between MA and MB contents. Furthermore, the momilactone contents significantly varied among rice plant organs. Additionally, interaction value represented the mismatch between MA and MB contents in various rice parts. In particular, MA was detected in a higher amount than MB in grain, husk, and root, but in contrast, the content was lower in leaf ($p < 0.05$). The highest MA quantity was in husk (16.44 ± 0.09 µg/g DW), whereas the maximum MB content was found in leaf (12.73 ± 0.36 µg/g DW) as compared with other plant parts (Table 7). Because both MA and MB were antidiabetic chemicals, rice grain and different plant parts of rice might be useful to exploit for antidiabetic treatment. This finding was in line with previous reports that in rice organs, the amount of MA was higher than MB (Lee et al., 1999; Xuan et al., 2016; Kang et al., 2017; Minh et al., 2018a).

Table 7. Momilactone contents in rice grain, husk, leaf, and root (µg/g DW*)*

Rice Organs	Momilactone A	Momilactone B
Grain	2.07 ± 0.01 ^{d}	1.06 ± 0.01 ^{d}
Husk	16.44 ± 0.09 ^{a}	9.24 ± 0.04 ^{b}
Leaf	4.28 ± 0.03 ^{c}	12.73 ± 0.36 ^{a}
Root	8.06 ± 0.13 ^{b}	5.69 ± 0.19 ^{c}

Data presented means ± standard errors. Means within a column followed by different superscript letters ($^{a, b, c, d}$) are significantly different; and * indicates a significant difference at $p < 0.05$.

By this study, the LC-ESI-MS method was the first to confirm the presence of MA and MB in rice grain. The use of a positive FTMS mode, mass range scanned from *m/z* = 315.193−315.198 and *m/z* = 331.188−331.192, the EIC of all samples (Figure 9) provided two major peaks which were finally confirmed as MA (RT~19.89, $C_{20}H_{27}O_3$, 315.196) and MB (RT~16.03, $C_{20}H_{27}O_4$, 331.190). Figure 4 illustrated the LC-ESI-MS results which confirmed the presence of the standards MA and MB in rice grain sample by analyzing the

similarities of retention time integrated with fragmentation patterns. Mass spectra data of grain sample showed that patterns at 315.19574 and 331.19073 m/z entirely matched with standard patterns at 315.19580 for MA and 331.19073 for MB (Figures 9 and 10).

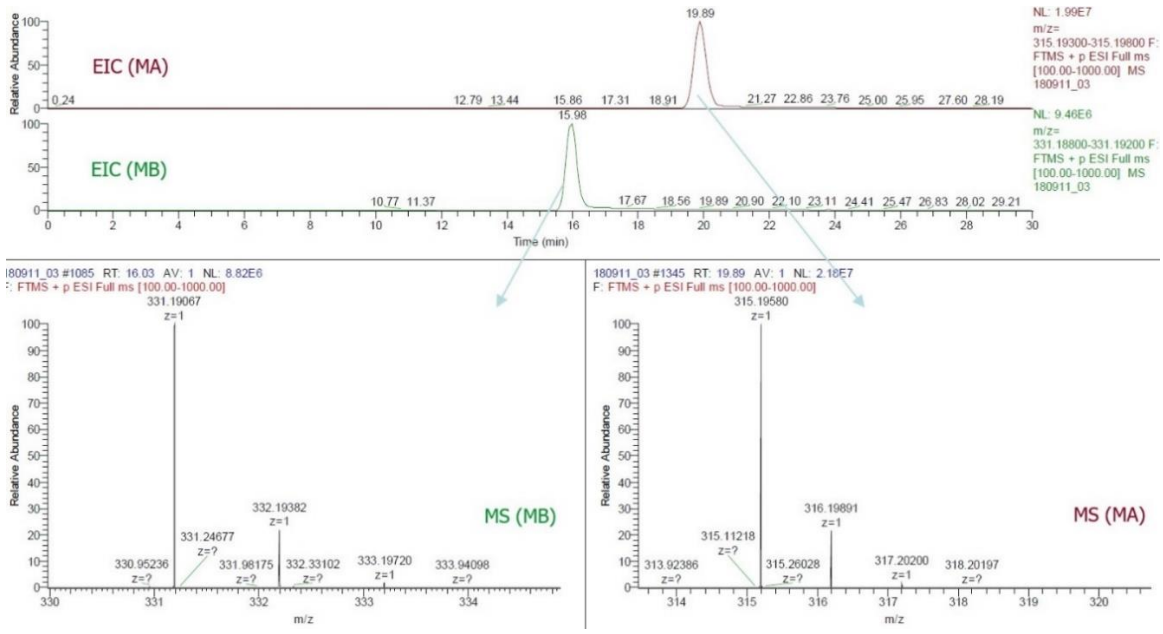


Figure 9. Extracted ion chromatograms and mass spectra of momilactones A and B.

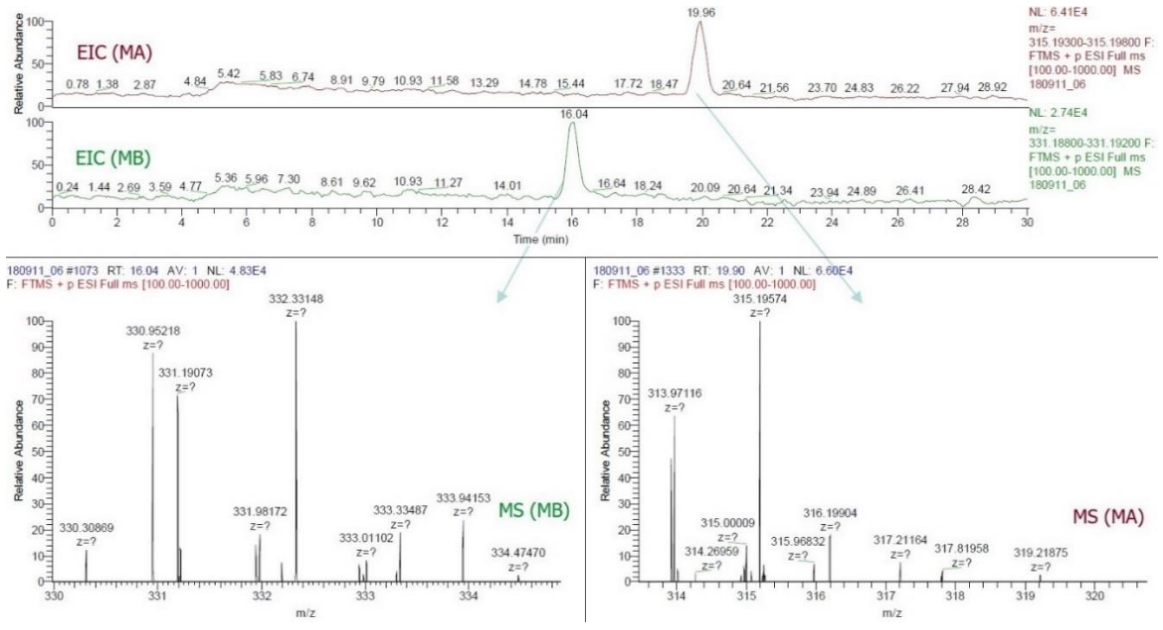


Figure 10. Extracted ion chromatograms and mass spectra of momilactones A and B detected in rice grain.

3.5. Discussion

In this study and for the first time, I reveal that MA and MB (diterpene lactones) are found in white rice grain and exhibited potential inhibitory effects on α-amylase and α-glucosidase, two key enzymes relevant to type 2 diabetes (Apostolidis et al., 2007). MA and MB were principally found in rice husk (Lee et al., 1999; Kato-Noguchi et al., 2010; Kang et al., 2017; Minh et al., 2018a), leaf (Xuan et al., 2016), root and root exudates (Kato-Noguchi et al., 2010; Kato-Noguchi, 2011). The content and presence of MA and MB varied among rice cultivars and growing stages (Lee et al., 1999; Xuan et al., 2016). The present research highlighted the promising antidiabetic activity of MA and MB, detected and quantified the two compounds in rice grain. Individually, MB displayed a stronger inhibitory activity than MA on both α-amylase (1.8-fold) and α-glucosidase (1.6-fold) (Table 6). The presence of a lactone ring in chemical structure has been reported to play a major role for α-glucosidase suppression (Yin, Zhang, Feng, Zhang, & Kang, 2014). On the other hand, the presence of a hydroxyl group in the diterpenoid part might explain the difference in bioactivity between MA and MB. Particularly, the hydroxyl group at C-3 in the structure of MB could increase the antidiabetic competence as compared to MA (Figure 2). The examination of functional groups in bioactive compounds is important in order to develop novel synthesized derivatives, which possess a stronger activity and are more economical than their parent compounds. For instance, DDK and DK are the two kavalactones in *Alpinia zerumbet*, a plant that has been demonstrated to play a potential role in longevity of Japanese living in one of the southernmost islands of the Ryukyus, Japan (Teschke & Xuan, 2018). Accordingly, many derivatives of DDK and DK have been synthesized and show promising medicinal and pharmaceutical properties (Xuan & Teschke, 2015; Xuan, Khanh, Khang, Quan, & Elzaawely, 2016; Van, Xuan, Minh, & Quan, 2018). Further elaboration on functional chemical groups in the diterpene lactone structures of MA and MB should be

conducted to be compared in terms of their potential inhibition on diabetes both in vitro models and in human randomized clinical trials. In addition, other potent antidiabetic compounds in rice grain might be further identified by using the advanced techniques from the LC-ESI-MS protocol developed by this study.

There were sporadic studies to examine the diabetes inhibitory potential of rice husk and straw, but they sorely investigated activity of the complete rice extracts. Yang, Kang, Nam, & Friedman (2012) demonstrated the effect of liquid rice hull smoke extract against diabetes using an in vivo model, but the chemical composition of the extract was not mentioned. Yehia & Saleh (2012) only noted the inhibitory effect of rice straw extract on amylase activity of some fungi without successfully identifying bioactive substances as well as antidiabetic competency. Heretofore, the digestion of white or refined rice grain has been thought to be positive on diabetes occurrence, as rice grain is rich in carbohydrates (Sun et al., 2011; Hu, Pan, Malik, & Sun, 2012). Therefore, diabetic patients are often advised to ingest as low as possible white rice grain in their meals or to use other alternative grains such as brown and color rice. Several compounds derived from brown or colored rice grains containing γ-oryzanol and polyphenols such as phenolic acids and anthocyanins were reported to be potent in type 2 diabetes reduction (Yawadio, Tanimori, & Morita, 2007; Yao, Sang, Zhou, & Ren, 2010; Zhang et al., 2010; Kozuka et al., 2013; Shao & Bao, 2015). Instead by this study, I hypothesized that the ingestion of rice might be beneficial against diabetes because of the presence of MA and MB which showed the higher inhibitory level on α-glucosidase activity than the commercial diabetes inhibitor acarbose by in vitro assays (Table 6). The contents of 2.07 μg MA and 1.06 μg MB per g dry weight in rice grain (Table 7) were equivalent to 128.8–138.5 and 479.2–577.3 g rice grain that was estimated to provide effective MA and MB on 50% inhibition of α-amylase and α-glucosidase activity, respectively.

Several natural products have been reported to be α-amylase and α-glucosidase inhibitors (Kim et al., 2007; Tran et al., 2014; Yin et al., 2014; Jhong, Riyaphan, Lin, Chia, & Weng, 2015). However, there was no uniformity in the inhibition results compared with the standard acarbose, which might be due to different sources of tested enzymes. Hence, further assays on human and mammalian enzymes are apparently needed to examine the benefits of MA and MB against diabetes risk. In addition, side effects of MA and MB should be carefully searched for, although no negative report on any natural rice compound is available. Finally, an in vivo model is absolutely needed to confirm the positive effects of MA and MB on insulin content, cardiovascular as well as intestine problems, and absorption processes. Furthermore, the search for other α-amylase and α-glucosidase inhibitors including derivative constituents of MA and MB in rice grain should be further conducted.

Thus far, MA and MB have been known as phytoalexins and allelochemicals in rice leaf, root, husk, and root exudate. This present research was the first one to detect the presence of MA and MB as new α-amylase and α-glucosidase inhibitors in rice grain by using the improved protocol of LC-ESI-MS (Figures 9 and 10). It provided detailed mass spectra on detecting of MA and MB in both GC-MS (Figure 8) and LC-ESI-MS (Figures 9 and 10). It will thus help achieve more accessible and accurate detection of MA and MB, as well as their potent derivatives in rice grain and rice organs. Although MA and MB showed persuasive α-amylase and α-glucosidase inhibitory activities, more in vivo and clinical trials should confirm the efficacy of MA and MB against diabetes. This observation can estimate the effective amounts of MA and MB in rice grain, thus aiding the breeding of rice cultivars rich with MA and MB.

3.6. Conclusion

By in vitro assays and for the first time, the present study discovered that MA and MB detected in and isolated from rice were effective inhibitors of α-amylase and α-

glucosidase, which may be explored as novel and potent candidates for antidiabetic therapy. However, further studies should be implemented to assert applicable doses of MA and MB before conducting medicinal production, pre-clinical and clinical trials on the two compounds. The identification and quantification of MA and MB in refined rice grain by the HPLC-ESI-MS technique were the principal findings of this study.

CHAPTER 4: CONTRIBUTION OF MOMILACTONES A AND B TO DIABETES AND OBESITY INHIBITORY POTENTIALS OF RICE BRAN: EVIDENCE FROM IN VITRO ASSAYS

4.1. Summary

This study was the first to detect the presence of the two compounds momilactone A (MA) and momilactone B (MB) in rice bran using liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS). By in vitro assays, both MA and MB exhibited potent inhibitory activities on pancreatic α-amylase and α-glucosidase which were significantly higher than γ-oryzanol, a well-known diabetes inhibitor. Remarkably, MA and MB indicated an effective inhibition on trypsin with the IC_{50} values of 921.55 and 884.03 μg/mL, respectively. By high-performance liquid chromatography (HPLC), quantities of MA (6.65 μg/g dry weight) and MB (6.24 μg/g dry weight) in rice bran were determined. Findings of this study revealed the α-amylase, α-glucosidase and trypsin inhibitors MA and MB contributed an active role to the diabetes and obesity inhibitory potentials of rice bran.

4.2. Introduction

Diabetes mellitus is a chronic disease that appears at all ages and leads to many serious complications including cardiovascular diseases, stroke, kidney failure, blindness, and nerve damage (Steppan et al., 2001). Among diabetic types, type 2 is the most common in adults due to the impaired diet and exercise regimens. The type 2 diabetes may commence from a combination of factors including genetic predisposition, environment, and pancreatic beta-cell dysfunction (Leahy, 2005). In these patients, their body cannot efficiently use insulin - a hormone that regulates glucose uptake into the cells. Without a proper control, this process will progressively lead the body to chronic insulin resistance, hyperglycemia, fatigue, weight loss, and other severe consequences. In fact, therapeutic interventions were

proposed tended to control those pathogenic factors. However, the most beneficial method is keeping blood glucose at normal levels after a meal (Abesundara et al., 2004). In human, the initial ingested foods are diverse, and they are complicatedly digested by multiple hydrolytic enzymes. Among these, α-amylase and α-glucosidase play a vital role in the hydrolysis of polysaccharides to liberate more suitable glucose for absorption (Ercan & El, 2016). The earlier study indicated that the hyperglycemia in type 2 diabetes patients was attributed to starch and glucose metabolism by pancreatic α-amylase and intestinal α-glucosidase (Apostolidis et al., 2007). On the other consideration, trypsin, an important proteolytic enzyme, takes part in many metabolic processes of human. Previous studies proved that trypsin inhibition might attenuate protein digestibility (Quesada, Bartolomé, & Estrella, 1996) and indirectly reduce blood glucose level by enhancing insulin absorption (Morishita, Morishita, Takayama, Machida, & Nagai, 1992; Park, Kwon, Lim, Park, & Kim, 2007). Recent researches also demonstrated that trypsin inhibitors could restrain food intake by promoting satiety impression and therefore, prevented several metabolic diseases as diabetes and obesity (Komarnytsky, Cook, & Raskin, 2011; Nakajima, Hira, Tsubata, Takagaki, & Hara, 2011; Carvalho et al., 2016; Serquiz et al., 2016). For those reasons, the discovery of new potent inhibitors from natural origins which can simultaneously inhibit the activities of α-amylase, α-glucosidase and trypsin are a potential approach for the control of risks from type 2 diabetes.

Momilactones A (MA) and B (MB) (Figure 2) have been known as authoritative allelochemicals against several pathogens of crops (Cartwright et al., 1977; Obara, et al., 2002; Chung, Jung, & Kim, 2006; Fukuta et al., 2007; Toyomasu et al., 2008; Kato-Noguchi et al., 2010; Kato-Noguchi, 2011). In addition, MA and MB might have a certain role to support tolerance of rice to salinity and drought stresses (Xuan et al., 2016; Quan & Xuan, 2018). MA and MB also exhibited several beneficially bioactivities including antioxidant

(Fukuta et al., 2007), cytotoxic (Chung et al., 2005), antitumor (Kim et al., 2007) and anticancer activities (Joung et al., 2008; Park et al., 2014). Of which, MB is commonly determined in lower quantity in rice plant parts but exerted greater biological activities than MA. Nevertheless, the isolation and purification of MA and MB, chiefly from rice husk, are not straightforward. Currently, very few laboratories in the world can successfully isolate and purify MA and MB. As a result, the commercial price of pure standards MA and MB is not affordable, and therefore, studies on biological activities of the two compounds has been limited. Hitherto, the antidiabetic property of MA and MB has not been fully studied.

On the other hand, rice is the staple food for more than half of the world population. Besides using white rice as a main food source owing to its well-known nutritional values, rice by-products have had an increasing interest from food processing industry and pharmaceutical fields (Esa et al., 2013; Sohail, Rakha, Butt, Iqbal, & Rashid, 2016). Theoretically, paddy-field produces approximately 70% white rice, 20% husk and 10% bran (Esa et al., 2013). The diabetes inhibitory potential of crude extracts from rice by-products was sorely investigated (Yang, Kang, Nam, & Friedman, 2012; Yehia & Saleh, 2012). Among rice by-products, rice bran versus diabetes was the most thoroughly studied. In which, tocotrienol, γ-oryzanol, fiber and functional peptides including amino acid sequences and protein hydrolysates were prerequisite compounds that could combat diabetes (Tashiro, Hashino, Shiozaki, Ibuki, & Maki, 1987; Ghatak & Panchal, 2012; Esa et al., 2013; Boonloh et al., 2015; Uraipong & Zhao, 2016; Liu, Strappe, Shang, & Zhou, 2017;). However, the antidiabetic role of individual compounds in rice bran has been sporadically studied. Recently, although MA and MB have been reported to be α-amylase and α-glucosidase inhibitors and appeared in different rice plant parts including leaf, husk, root as well as refined rice grain (Quan et al., 2019a), the presence of MA and MB and their contribution to the antidiabetic property in rice bran has not been revealed yet. Thus, in this study, I at first

documented and compared the diabetic inhibitory potential of MA and MB in rice bran and γ-oryzanol (an outstanding diabetes inhibitor in rice bran) through in vitro assays of pancreatic α-amylase and trypsin inhibitions. The protocol to integrate HPLC and LC-ESI-MS to identify and quantify MA and MB in rice bran was also described.

4.3. Materials and methods

4.3.1. Reagents

The extraction and isolation solvents were purchased from Junsei Chemical Co., Ltd., Tokyo, Japan. Acetonitrile used for LC analyses was obtained from Fisher Scientific company, Hampton, NH, USA. Iodine solution, acarbose, and γ-oryzanol were procured from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Silica gel, α-amylase from porcine pancreas (type VI-B), α-glucosidase from *Saccharomyces cerevisiae*, trypsin from porcine pancreas, soluble starch, *p*-nitrophenyl-α-D-glucopyranoside (pNPG), Nα-benzoyl-L-arginine 4-nitroanilide hydrochloride (BAPNA) and all buffer components were acquired from Sigma-Aldrich, St. Louis, MO, USA.

4.3.2. Collection and preparation of samples

Rice husk and rice bran of Koshihikari variety (Japonica subtype) were collected from rice mills allocated near Hiroshima University, Higashi-Hiroshima Campus, Japan in July 2017. After drying by sunlight for one week, samples were deliberately sifted and winnowed to remove impurities. The samples were then dried in an oven at 50 ^{o}C for 3 days and kept in a container for further extractions.

4.3.3. Extraction and isolation process of momilactones A and B from rice husk

Extraction and isolation procedures were described previously (Quan et al., 2019a). Briefly, from 7 kg of rice husks, 52 mg of MA and 44 mg MB were isolated by repeated column chromatography (Figure 11). The identification and confirmation of MA and MB were conducted by high performance liquid chromatography (HPLC), thin-layer

chromatography (TLC), and gas chromatography-mass spectrometry (GC-MS) techniques. The structures of MA and MB were re-confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analyses and compared with those in the previous research (Minh et al., 2018b). The purified MA and MB were used for biological activities and quantification of these compounds in rice bran.

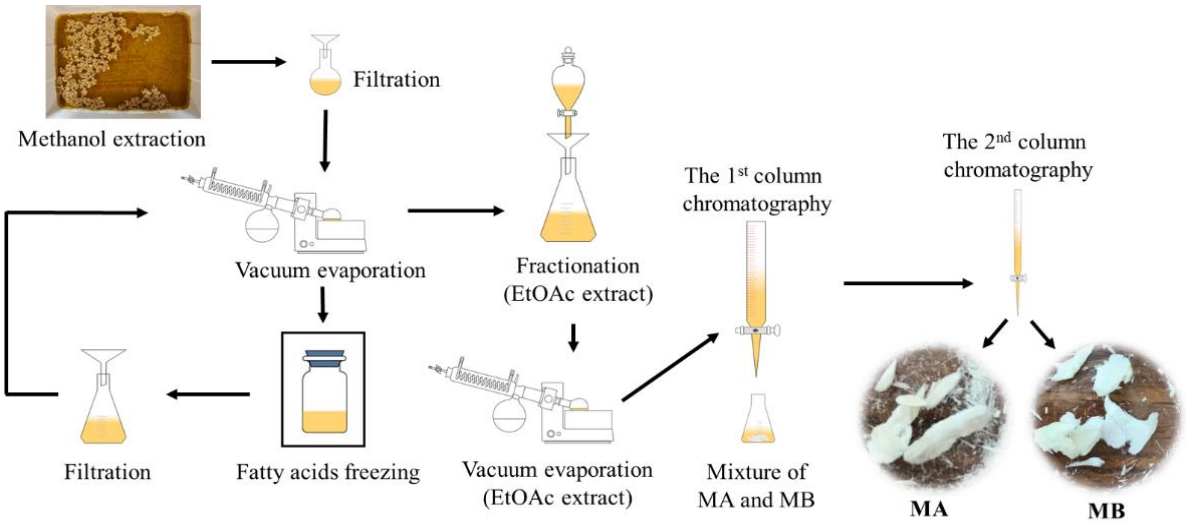


Figure 11. The extraction and isolation process of momilactones A and B.

4.3.4. Quantification and confirmation of MA and MB in rice bran by HPLC and LC-ESI-MS

Rice bran (100 g) was extracted by 400 mL of MeOH for 1 week. After filtering, crude methanolic extract was obtained and mixed with an equal volume of hexane in a separatory funnel. After 2 hours, the hexane layer with fatty compounds was removed, the methanol layer was filtered and kept in a fridge (4 ^{o}C) for 1 day. The methanol-soluble part was separated from crystallized sugars by another filtration and evaporated to yield a defatted bran extract (DBE) with the stock concentration of 100 mg/mL (Figure 12). The HPLC analysis was done in a similar method as described previously (Quan et al., 2019a). The contents of MA and MB in rice bran were compared and calculated in consonance with the retention times (RT) and peak areas of the standards MA and MB with the samples.

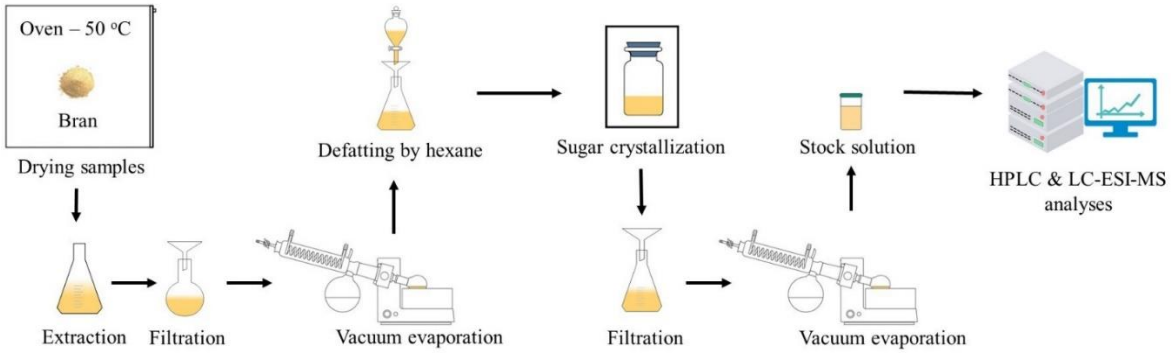


Figure 12. Preparation of rice bran for HPLC and LC-ESI-MS analyses.

The DBE sample was analyzed by LC-ESI-MS technique. The analytical system included an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, CA, USA) equipped with a source of electrospray ionization (ESI). The column Waters Spherisorb ODS2 (10µm, 250 mm x 4.6 mm i.d.) and mobile phase comprising of 0.1% trifluoroacetic acid in 70% acetonitrile were used in LC phase. The operation time was 30 min with a flow rate of 0.4 mL/min under the room temperature. ESI condition was set up as follows: ion spray voltage (4.5 kV), sheath gas flow rate (60 arb) and aux gas flow rate (20 arb). MS analysis was run by a positive Fourier transform mass spectrometer (FTMS) at a resolution of 60000 with a scan range of *m/z* 100-1000. An amount of 10 µL of DBE sample (50 mg/mL) and standard momilactones (0.5 mg/mL) were separately injected to the system by an autosampler. The presence of MA and MB in DBE was confirmed by comparing their extracted ion chromatograms (EIC) and mass spectra with those of standard momilactones.

4.3.5. α-Amylase and α-glucosidase inhibition assays

A modified model of the starch-iodine method described by Al-Dabbas, Kitahara, Suganuma, Hashimoto, & Tadera (2006) was used to assess the porcine pancreatic α-amylase (PPA) inhibition of momilactones A and B, DFE, and γ-oryzanol. Concisely, in each well of a microplate (U-shape, Greiner Bio-one, NC, USA), 20 µL of sample were pre-incubated with 20 µL of PPA solution (2 mg/mL in 20 mM phosphate buffer containing 6

mM sodium chloride, pH 6.9) at 37 °C for 10 min. The reaction was activated by pipetting 30 µL of soluble starch (0.5%). After 6 min of incubation, an aliquot of 20 µL of hydrochloric acid (1 M) were added to stop the reaction, followed by 100 µL of 0.25 mM iodine solution. The absorbance at 565 nm was read by a microplate reader (Multiskan^{TM} Microplate Spectrophotometer, Thermo Fisher Scientific, Osaka, Japan). The inhibition percentage of samples on PPA was calculated by the following formula:

$$\% \text{ inhibition} = (S - W)/(C - W) \times 100, \quad \text{(i)}$$

where S is the absorbance of reaction with presence of samples, C is the absorbance of reaction without enzyme, W is the absorbance of reaction without samples. Acarbose was used as a positive reference. The IC_{50} value was determined to exhibit 50% inhibitory capacity of reaction at a certain concentration.

The α-glucosidase inhibitory assay was conducted, adhered to the earlier described protocol (Quan et al., 2019a). In brief, 40 µL of samples including momilactones A and B, DBE, and γ-oryzanol which were two-times diluted by 0.1 M potassium phosphate buffer (pH 7), were incubated with 40 µL of α-glucosidase enzyme solution (0.5 U/mL in the buffer) at 25 °C. After 6 min, 20 µL of 5 mM pNPG substrate were added to inaugurate the reaction. The mixture was incubated for another 8 min and eventually terminated by adding 100 µL of 0.1 M Na_2CO_3. The absorbance was recorded at 405 nm by a microplate reader. The inhibition percentage was calculated by the following equation:

$$\% \text{ inhibition} = (B - A)/B \times 100, \quad \text{(ii)}$$

where A is absorbance of reaction with either MA or MB or positive controls (acarbose or quercetin) and B is absorbance of reaction with 50% methanol in buffer. IC_{50} value was obtained by the same way as above.

4.3.6. Trypsin inhibition assay

The trypsin inhibition was assayed using an adapted protocol of method reported previously (Balisteiro, Rombaldi, & Genovese, 2013). Initially, the stock solution of samples was diluted two times by 50 mM Tris buffer (containing 20 mM $CaCl_2$, pH 8.2). In each test sample, 50 µL of trypsin and 100 µL of BAPNA substrate were added, followed by incubation at 37 °C for 12 min. Finally, the reaction was stopped by pipetting 25 µL of acetic acid 30% into the mixture. Then absorbance was measured at 410 nm by a microplate reader. The inhibition percentage was calculated by the formula (ii) and IC_{50} value was achieved by the same way as mentioned above. Positive controls were caffeic acid and tannic acid. Final concentrations of enzyme and BAPNA substrate in the reaction were 40 and 160 µg/mL, respectively.

4.3.7. Statistical analysis

Data were elaborated on the Minitab 16.0 software (Minitab Inc., State College, PA, USA). All analyses were in a complete randomization with three replications, and results were displayed as mean ± standard error (SE). Significant differences were determined by one-way ANOVA using Tukey's test at $p < 0.05$.

4.4. Results

4.4.1. Isolation and confirmation of momilactones A and B

By using the repeated column chromatography, MA and MB were successfully isolated from rice husk extract. The confirmation of MA and MB by HPLC, TLC, GC-MS, and NMR was reported previously (Minh et al., 2018; Ahmad et al., 2019; Quan et al., 2019a). The results can be seen in supplementary data (Figure S3- S5).

4.4.2. Inhibition of α-amylase, α-glucosidase and trypsin

In the previous study, I reported the inhibitory effects of MA and MB on α-amylase and α-glucosidase from bacteria (Quan et al., 2019a). In the present research, I confirmed

such activity on porcine pancreatic α-amylase (PPA) together with a comparison of inhibitory activities of momilactones, defatted bran extract, and a well-known diabetic inhibitor γ-oryzanol. Additionally, to the extent of our knowledge, this is the first study that investigates inhibitory activities of MA and MB on trypsin, the enzyme linked to both diabetes and obesity.

Table 8. IC_{50} values of pancreatic α-amylase and α-glucosidase inhibitory activities of momilactones A and B, defatted bran extract, and γ-oryzanol

Sample	IC_{50} value of α-amylase inhibition (μg/mL)	IC_{50} value of α-glucosidase inhibition (μg/mL)
MA	132.56 ± 0.51 b	991.95 ± 0.96 b (Quan et al, 2019b)
MB	129.02 ± 0.09 b	612.03 ± 0.39 a (Quan et al, 2019b)
DBE	779.03 ± 3.87 c	16653.00 ± 59.00 e
GO	-	1754.20 ± 6.38 c
Acarbose	80.26 ± 0.24 a	2549.00 ± 5.15 d (Quan et al, 2019b)

Data present means ± standard errors. Means within a column followed by different letters are significantly different at $p<0.05$ level. -: not calculated. MA: momilactone A; MB: momilactone B; DBE: defatted bran extract; GO: γ-oryzanol.

As shown in Table 8, both MA and MB obtained inhibitory activities against PPA (IC_{50} = 132.56 and 129.02 μg/mL, respectively) and α-glucosidase (IC_{50} = 991.95 and 612.03 μg/mL, respectively). These effects were also relatively compared with the well-known commercial diabetic inhibitor (acarbose: IC_{50} = 80.26 μg/mL for PPA and 2549.00 μg/mL for α-glucosidase). By comparing the IC_{50} values, it might conclude that MA and MB are asymptotic with the antidiabetic level of acarbose. On the other hand, DBE performed an IC_{50} value of 779.03 μg/mL for PPA inhibition while γ-oryzanol showed a negligible activity which was recorded as 18.65% inhibition at a concentration of 4 mg/mL. However, in α-glucosidase assay, γ-oryzanol (IC_{50} = 17.54 mg/mL) evinced a higher inhibitory activity than

DBE (16.65 mg/mL). To sum up, MA and MB remarkably exhibited stronger suppressive effects than γ-oryzanol and DBE on both PPA and α-glucosidase assays.

The inhibitory effect of MA and MB on trypsin activity was delineated by IC_{50} value (μg/mL) in Figure 13. In particular, trypsin inhibition of MA (921.55 μg/mL) was significantly lower than that of MB (884.03 μg/mL). Both MA and MB comparably exhibited the inhibitory level as two phenolic inhibitors tannic acid (75.66 μg/mL) and caffeic acid (7.31 mg/mL). Conspicuously, trypsin inhibitory activity of MA was 7.9-folds and of MB was 8.2-folds stronger than caffeic acid. Meanwhile, DBE showed a trivial inhibition of 11.94% at a concentration of 20 mg/mL. γ-oryzanol did not offer an inhibition on trypsin activity.

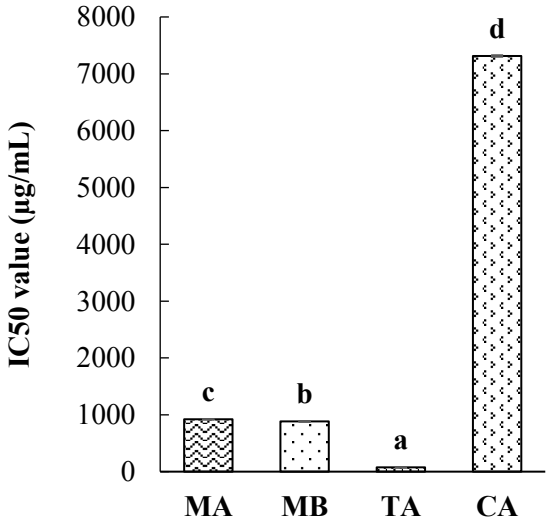


Figure 13. Trypsin inhibitory effect of momilactones A and B.

MA: momilactone A; **MB**: momilactone B; **TA**: tannic acid; **CA**: caffeic acid; Different letters indicate significant differences ($p<0.05$).

4.4.3. Contents of MA and MB in rice bran

Results of HPLC and LC-ESI-MS showed that MA and MB contents in rice bran can be reliably identified and quantified. By HPLC, MA and MB in bran were determined by comparing the similarity of retention time and peak areas between sample and standards (Fig S6). As shown in Table 9, quantity of MA and MB in rice bran was similar to each other

(6.65 and 6.24 µg/g dry weight, respectively). This is the first study so far to detect and quantify MA and MB contents in rice bran.

Table 9. Peak information of momilactones A and B detected in rice bran by HPLC

Sample	Peak estimation	Retention Time	Area
DBE50 + Std (3:1)	MB	14.53	2322546
	MA	17.73	3138205
DBE50	MB	14.53	291048
	MA	17.74	164757

DBE50 was defatted bran extract at 50 mg/mL; DBE50 + Std0.5 (3:1) was defatted bran extract (50 mg/mL) mixed with pure MA and MB (0.5 mg/mL) at ratio 3:1 (v/v).

LC-ESI-MS method was introduced to be able to confirm the presence of MA and MB in rice grain (Quan et al., 2019a). In this research, by integration of a specific extraction and LC-ESI-MS methods, I certified the existence of these bioactive compounds in rice bran extract (Figure 12). The use of a positive FTMS mode and certain mass range scans resulted in an extracted ion chromatogram (EIC) of the sample which illustrated two major peaks. The retention time and fragmentation patterns from peaks of DBE sample were confirmed as MA (RT~19.87, 315.196) and MB (RT~15.98, 331.190) which were totally coincided with those of standards MA and MB (Figure 9). Figure 14 illustrates LC-ESI-MS results, which first confirms the presence of MA and MB in rice bran.

4.5. Discussion

Nowadays, the use of available antidiabetic drugs may bring undesirable and severe side effects (Arulselvan et al., 2014). The quest of new antidiabetic compounds is indispensable to overcome diabetic problems worldwide. Nonetheless, the isolation and identification of active compounds with either no or minimal adverse effects are greater challenges to biomedical and scientific researches. To date, compounds with diterpene lactone structure possessing antidiabetic property have been released intermittently. An exhaustive search of

related literature brought only one result which was andrographolide, a diterpenoid lactone isolated from *Andrographis paniculata*, was potent for diabetic control (Yu et al., 2003; Subramanian et al., 2008; Brahmachari, 2017). Hence, MA and MB regarded as a new chemical class performing antidiabetic ability in this study should be attached more special importance.

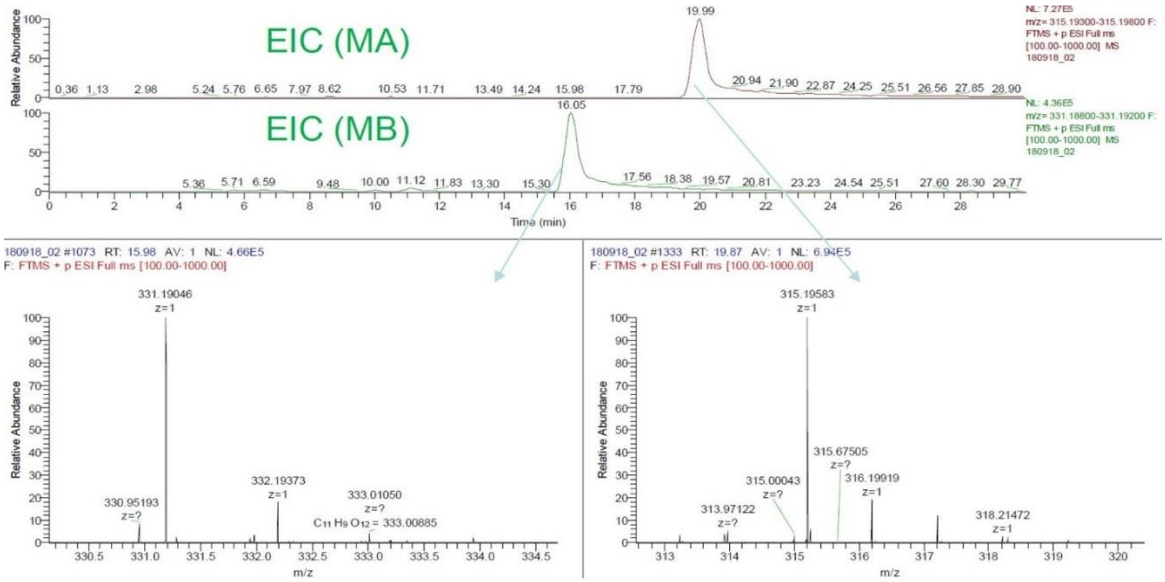


Figure 14. Extracted ion chromatograms and mass spectra of momilactones A and B detected in rice bran.

MA and MB have been principally detected in rice husk (Ahmad et al., 2019; Chung et al., 2006; Kato-Noguchi et al., 2010; Minh et al., 2018a, 2018b), leaf (Lee et al., 1999; Xuan, et al., 2016), root and root exudates (Kato-Noguchi et al., 2010; Kato-Noguchi, 2011). The content of MA and MB varied among rice cultivars and growing stages (Lee et al., 1999; Xuan, et al., 2016). This research highlighted the antidiabetic activity of pure MA and MB, detected and quantified the two compounds in rice bran. Among enzymatic assays, MA and MB presented the most remarkable inhibition on porcine pancreatic α-amylase activity (IC_{50} = 132.56 and 129.02 µg/mL for MA and MB, respectively) which were closely in line with the activity of the standard inhibitor acarbose (IC_{50} = 80.26 µg/mL). These inhibitory activities might stem from the presence of lactone ring in the structure of MA and MB, which

was reported to play a potent role in anti-α-glucosidase activity (Yin, Zhang, Feng, Zhang, & Kang, 2014). Additionally, diterpenoid component was convinced to be involved in trypsin deduction (Xing et al., 2003; Kang et al., 2006; Kim et al., 2006). Moreover, the appearance of hydroxyl group at C-3 in the diterpenoid part of MB might increase the antidiabetic competence as compared to MA (Quan et al., 2019a). The influence of number and position of the hydroxyl groups of natural compounds on α-glucosidase and trypsin activities was substantiated by earlier studies (Rohn, Rawel, & Kroll, 2002; Reddy et al., 2009; Yin et al., 2014). Among plant-derived compounds, the phenolic group was most intensively elaborated due to its abundance and availability. Tan et al. (2013) reported the α-glucosidase inhibitory activity of kaempferol and chlorogenic acid isolated from *Gynura medica* leaf with the IC_{50} value of higher than 2 mg/mL. In research on trypsin inhibition of phenolics from extracts of pears, lentils and cocoa beans by Quesada et al. (1996), gallic acid and catechin were potential inhibitors with IC_{50} values of 4.8 and higher than 10 mg/mL, respectively. By comparing with results of the present study, I documented that MA and MB were noteworthy diabetes inhibitors in term of α-amylase, α-glucosidase, and trypsin inhibitions.

Although previous studies introduced several techniques to isolate and purify MA and MB (Cartwright et al., 1981; Chung et al., 2005; Chung et al., 2006; Minh et al., 2018a, 2018b), none of them proposed a detailed process that can be extensively applicable for isolation these diabetic inhibitors as our study. Furthermore, I successfully developed a simple method that helped precisely detect MA and MB in rice bran for the first time. Results from the advanced technique LC-ESI-MS were reliable (Figure 14), nevertheless, the key of achievements might emanate from the sample processing (Figure 12). Particularly, after withdrawing fatty and low polarity components by hexane, I proceeded with a sugar abolishment based on the crystallization of sugars at low temperature. Basically,

momilactones are minor constituents in rice and the productivity of MA and MB isolation may be accelerated by various factors as UV-irradiation (Cartwright et al., 1981; Kodama, Suzuki, Miyakawa, & Akatsuka, 1988), temperature and extracting solvents (Minh et al., 2018b). The rejection of compounds with high molecular weight or lower polarity may enhance the sensitivity in detecting MA and MB, which has not been mentioned in the earlier researches. Though contents of MA and MB quantified in rice bran were 6.65 and 6.24 μg/g dry weight, respectively, their individual activity on the suppression of hydrolytic enzymes linked to diabetes was considerable. Therefore, the contribution of MA and MB to the anti-diabetic capacities of rice bran should be further endorsed by in vivo models as well as clinical trials.

In addition, γ-oryzanol, a commercially-important bioactive phytochemical of rice bran, is a mixture of ferulic acid esters of triterpene alcohols and sterols, which possesses a wide spectrum of health-beneficial effects, including anticarginogenic, anti-inflammatory, antihyperlipidemic, antidiabetic, and neuroprotective (Lemus, Angelis, Halabalaki, & Skaltsounis, 2014). Most of the evidence about antidiabetic effect of γ-oryzanol was from in vivo assays, but no in vitro study on inhibitions of the key enzymes linked to diabetes was investigated. This current study for the first time resolved this concern. Results from in vitro assays pointed out that the inhibitory effect of MA and MB on α-amylase, α-glucosidase and trypsin were more significantly potent than that of γ-oryzanol and defatted bran extract. As a result, along with quantification results, MA and MB can be considered as new members of diabetic inhibitors and might contribute an active role to the diabetes inhibition of rice bran. Trends in the use of rice bran as a source of anti-diabetes, therefore, have been more fortified by this study.

4.6. Conclusion

By in vitro assays, for the first time, the present study manifested the decisive function of MA and MB in the inhibition of key enzymes related to diabetes. The first identification and quantification of MA and MB in rice bran using advanced techniques may launch a new direction for further isolations of these diabetic inhibitors in larger scales. Findings of this research highlighted that MA and MB contributed an important role in anti-diabetes property of rice bran, although in vivo trials on MA and MB should be further explored. Given that quantities of MA and MB are largely varied among rice cultivars, the breeding of new rice cultivars with high amounts of MA and MB may be useful and economical to help control diabetes.

CHAPTER 5: ANTIOXIDANT AND ANTI-SKIN-AGING PROPERTIES OF MOMILACTONES A AND B

5.1. Summary

This chapter reported the potential antioxidant and anti-skin-aging activities of MA and MB. Results from antioxidant assays presented a synergistic activity in the mixture of MA and MB (MAB, 1:1, v/v) by 2,2'-azino-bis (ABTS) and reducing power assays. Remarkably, in ABTS assay, IC_{50} value of MAB (0.319 mg/mL) was 4 folds and 9 folds greater than that of individual MB (1.28 mg/mL) and MA (2.84 mg/mL), respectively. In vitro enzymatic assays on pancreatic elastase and tyrosinase indicated that MA and MB were promising cosmeceuticals in protecting the skin from wrinkles and freckles. Interestingly, at a concentration of 2 mg/mL, MA exerted higher inhibitory effects on both enzymatic activities (30.86 and 37.59 % for elastase and tyrosinase inhibition, respectively) than MB (18.50 and 12.60 %) and MAB (32.03 and 19.71 %). The results of enzymatic inhibitions were in line with that of β-carotene bleaching assay, in which, MA and MAB also presented a higher lipid peroxidation inhibition than MB. Besides, the validated method for quantification of MA and MB in rice grains showed that brown rice grains contained more MA and MB than white rice grains due to the MA and MB involvement in bran. Among refined grains, Koshihikari rice possessed the highest amount of MA (0.46 μg/mL) and MB (0.41 μg/mL). The usages of ultra performance liquid chromatography - electrospray ionization - mass spectrometry (UPLC-ESI-MS) and specific techniques in preparation of samples helped improve the sensitivity of the quantification of MA and MB in various rice grains.

5.2. Introduction

In human, the skin is the most important organ which plays a crucial role as the first protective barrier. The skin protects our body from not only physical factors, such as collisions, clashes, UV radiation, etc. but also from chemical agents, for instance, toxics, oxidants, or oxidative stresses. These factors may impact on many aspects of the skin function, especially on skin cells which are able to be dropped in an undesired condition of apoptosis (Chompoo, Upadhyay, Fukuta, & Tawata, 2012). Among skin injuries caused by UV radiation, the elasticity reduction or wrinkles and melanogenesis increases or freckles are the most detectable because they directly expose via visual phenotypes (Thring, Hili, & Naughton, 2009; Kim, Ishihara, & Lee, 2012; Tu & Tawata, 2015). The stability of skin is maintained by elastin and collagen fibers while melanin is an important pigment that helps protect skin from UV radiation. Basically, wrinkles and freckles are associated with the surge of dermal enzymatic activities containing elastase and tyrosinase, respectively. While elastase acts as the elastin's breaker, tyrosinase enhances the occurrence of melanin, consequently, results in the formation of freckles. Besides, practical investigations reported that skin disorders and complications would sooner or later be developed in all diabetic and obesity patients (Linda, 2002; Demirseren et al., 2014; Duff, Demidova, Blackburn, & Shubrook, 2015). These physiological disturbances are mainly attributed to the excess accumulation of reactive oxygen species (ROS), "which can interact with proteins, lipids, and DNA, and alter cellular functions, thus causing aging-related disorders or melanogenesis" (Tu & Tawata, 2015). Therefore, the identification of elastase and tyrosinase inhibitors is the most effective approach in the therapeutics of skin diseases.

Rice (*Oryza sativa*) is a global crop which has a long history of safe usage as an indispensable food for human. The use of rice products in cosmeceuticals is not a novelty, of which, a diverse number of bioactive compounds has been identified as ferulic, γ-oryzanol,

and members of flavonoids, flavones, etc. (Miyazawa, Oshima, Koshio, Itsuzaki, & Anzai, 2003; Chang, 2009; Zolghadri et al., 2019). In fact, there are many cosmetic firms in the world producing those natural substances and benefiting greatly from selling such products originated from rice. In previous studies, I discovered momilactones A and B as potential diabetic and obesity inhibitors in rice grain as well as in rice bran. Their biological activities were also reviewed in chapter 1 containing allelopathic, antifungal, antibacterial, and anticancer activities. However, the anti-skin-aging activity of MA and MB has not been studied so far. On the other hand, in chapter 2, I found that the use of SEP-PAK C18 cartridge might increase the sensitivity of MA and MB detections by high-performance liquid chromatography (UV-VIS detector). The application of the more advance technique as ultra performance liquid chromatography (UPLC) integrated electrospray ionization-mass spectrometry (ESI-MS) has not been used in quantification of MA and MB in rice grains. Hence, I conducted this research in order to investigate the antioxidant and anti-wrinkle and freckle properties of MA and MB; and to validate the method of quantifying such bioactive metabolites in different common rice grains. The outcomes may provide a good option in selecting the best rice grains which can combat simultaneously several chronic diseases as diabetes type 2, obesity and skin problems.

5.3. Materials and methods

5.3.1. Reagents

The extraction and isolation solvents were purchased from Junsei Chemical Co., Ltd., Tokyo, Japan and Fisher Scientific company, Hampton, NH, USA. Chemicals for antioxidant assays were acquired from Fujifilm Wako Pure Chemical Corporation, Osaka, Japan. Elastase from porcine pancreas Type IV, tyrosinase from mushroom lyophilized powder, N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide (SANA), L-tyrosine, oleanolic acid, vanillin, myricetin and all buffer components were acquired from Sigma-Aldrich, St. Louis,

MO, USA. Pure momilactones A and B were obtained from chapter 3. Tricin was isolated previously from rice husk by our laboratory.

5.3.2. Rice grains and sample preparations

Total nine rice grains were investigated in this study, of which, five Japanese varieties and four Vietnamese varieties were separated into two major groups including refined and brown types. All varieties are the most commercially popular rice in Japan and Vietnam.

The information of rice samples is shown in Table 10, in which, Japanese rice grains were purchased from Japan Agriculture (JA), Hiroshima, Japan while Vietnamese ones were provided by Agricultural Genetics Institute (AGI), Vietnam.

Table 10. Information of rice grains

Code	Name	Type	Origin
Ko	Koshihikari	Refined	Japan
KoCo	Koshihikari (cooked)	Refined	Japan
KT1	Shinnosuke	Refined	Japan
KT2	Seiten no hekireki	Refined	Japan
KT3	Ginga no shizuku	Refined	Japan
KT4	Ho no mai	Brown	Japan
ST24	ST24	Refined	Vietnam
RVT	RVT	Refined	Vietnam
Bin9	a mutant line	Brown	Vietnam
KD18	Khang dan 18	Brown	Vietnam

The extraction of rice grains was done followed methods described in previous chapters 2-4. Briefly, dried and powdered rice grains (200 g) was extracted with 300 mL of methanol for 1 week. After separating from hexane extract, the methanolic phase was filtered and dried by a vacuum evaporator (Rotavapor® R-300, Nihon Buchi K.K., Tokyo, Japan). The normal extracts were then processed by the procedure reported in chapter 4. The purified extract was carried out as follows: a part of normal rice grain extract was evaporated and then blended with 50% aqueous methanol. The mixture was loaded into a Sep-Pak® Plus C18 cartridge (Waters Cooperation, Milford, MA, USA). Subsequently, the cartridge was pre-washed with 2 mL of 50% methanol and then eluted with 10 mL of 100% methanol. Eventually, the methanolic elutions were combined and adjusted into the concentration of 10 mg/mL (named SEP-PAK samples) and stored in a fridge (4^{o} C) for further measurements.

5.3.3. Quantification and confirmation of MA and MB in rice grain by UPLC-ESI-MS

The UPLC-ESI-MS system consisted of an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, CA, USA) equipped with a source of electrospray ionization (ESI). The LC was carried out by injecting 3.0 µL of a sample (at various concentrations) into the Acquity UPLC® BEH C18 (1.7 µm, 50 x 2.1 mm i.d.) column (Waters Cooperation, Milford, MA, USA). The column temperature was maintained at 25 ^{o}C. The chromatography was run in a gradient model with a flow rate of 300 µL/min. The gradient of mobile phase was established as follows: 50% solvent A (0.1% trifluoroacetic acid in water) and 50% solvent B (0.1% trifluoroacetic acid in acetonitrile) over 0-5 min, then increased to 100% B over 5-10 min which was upheld for 0.1 min, finally the column was equilibrated by the initial condition for 5 min. The total operation time was 15.1 min. The ESI condition was set up in a similar index to that described in chapters 3 and 4. The presence of MA and MB in rice grains was confirmed by comparing their extracted ion chromatograms (EIC) and mass spectra of samples with those of standard momilactones. The areas of an identified peaks

those matched with the retention time of standard MA and MB in the EIC were used to calculate the amount of such compounds by a linear model.

5.3.4. Antioxidant activities

The antioxidant capacities of MA, MB and the mixture of MA and MB (MAB, 1:1, v/v) were evaluated by 2,2'-azino-bis radical cation ($ABTS^{\bullet+}$) decolorization, reducing power, and β-carotene bleaching methods described by Quan et al. (2019c).

5.3.5. Elastase and tyrosinase inhibitory activities

Elastase inhibition was assayed basing the method reported by Tu & Tawata (2015) with minor refitted changes. Particularly, 200 µL of the buffer substrate (1 mM SANA 0.1 M Tris-HCl buffer, pH 8.0) was added to 20 µL of a stock sample (in methanol) in a well of the 96-well plate. The solutions were vortexed and incubated for 5 min at 25 °C, and then 15 µL of pancreatic elastase (0.1 U/mL) was added. After mixing, the microplate was incubated at 25 °C for 12 min and subsequently, the absorbance was measured at 410 nm by a microplate reader. Negative controls were performed with methanol, while oleanolic acid was used as a positive control.

The inhibition of tyrosinase was determined by the protocol of Chompoo, Upadhyay, Fukuta, & Tawata (2012) with a minor alteration. Briefly, in each well of a 96-well plate, 20 µL of sample were mixed with 120 µL of phosphate buffer (20 mM, pH 6.8) and 20 µL of tyrosinase (500 U/mL in buffer), respectively. After a five minutes-incubation at 25 ºC, an adequate amount of 50 µL L-tyrosine substrate (2 mM in deionized water) was pipetted to start the reaction. The mixture was mixed and incubated for other 10 min at 25 ºC prior to be measured under 470 nm by a microplate reader. Myricetin, vanillin, and tricin were used as positive references whereas methanol was negative control.

The inhibition percentage of enzymatic assays was calculated as the following formula:

$$\text{Inhibition (\%)} = [1 - (C - D)/(A - B)] \times 100$$

where A is the absorbance of the control with the enzyme, B is the absorbance of the control without the enzyme, C is the absorbance of the sample with the enzyme, and D is the absorbance of the sample without the enzyme. The IC_{50} value was defined as the concentration of a sample that suppressed 50 % of the enzymatic reaction over the negative control.

5.3.5. Statistical analysis

Data were elaborated on the Minitab 16.0 software (Minitab Inc., State College, PA, USA). All assays were thrice implemented, and results were displayed as means ± standard errors (SE). Significant differences among tests were determined by one-way ANOVA using Tukey's test at $p < 0.05$.

Table 11. Antioxidant activities of momilactones A and B in term of ABTS and β-carotene bleaching assays

	IC_{50} value of ABTS assay (mg /mL)	**β-Carotene bleaching assay (% LPI)**
MA	2.838±0.002d	75.234±0.855b
MB	1.283±0.002c	61.690±1.640c
MAB	**0.319±0.002b**	**79.990±1.080b**
BHT	0.080±0.001a	86.667±0.327a

Data presented a means ± standard errors. Means within a same column followed by similar letters are not significantly different by Turkey's test ($p<0.05$). MA, momilactone A; MB, momilactone B; MAB, mixture of MA and MB at 1:1, v/v; BHT, butylated hydroxytoluene.

5.4. Results

5.4.1. Antioxidant activities

Antioxidant properties of MA, MB and MAB are displayed in Table 11 and Figure 15. According to the result of ABTS assay, the individual MA and MB presented a weaker

activity with IC_{50} values of 2.84 and 1.28 mg /mL, respectively compared to the standard antioxidant, butylated hydroxytoluene (BHT). However, the mixture of MA and MB (MAB, 1:1, v/v) exhibited a synergistic activity (IC_{50} = 0.32 mg/mL) which was significantly higher than those of MA and MB individually. Notably, by β-carotene bleaching assay, MA performed a stronger lipid peroxidation inhibition (% LPI = 75.23) than MB did (% LPI = 61.69) at a same concentration of 1 mg/mL. Also, MAB had a significant antioxidant activity (% LPI = 79.99) which was closely comparable with MA and the standard BHT (% LPI = 86.67). Although both MA and MB presented a negligible antioxidant ability in term of reducing power assay, the result showed a similar trend that MB was stronger than MA and the mixture MAB was optically more powerful than either MA or MB.

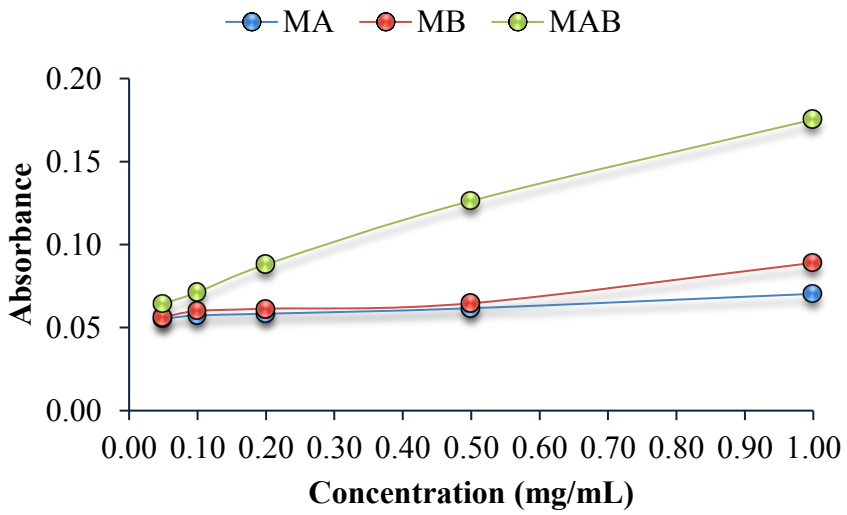


Figure 15. Reducing power activity of momilactones A and B.

5.4.2. Potential anti-wrinkle and anti-freckle activities of momilactones A and B

By in vitro assays, momilactones A and B were remarkably active in the inhibition of two key enzymes elastase and tyrosinase which related to wrinkle and freckle of skin (Table 12). At a concentration of 2 mg/mL, MA inhibited 30.86 and 37.59 % of pancreatic elastase and tyrosinase activities, respectively, meanwhile, MB suppressed only 18.50 and 12.60 % of reactions, respectively. The inhibitory effect of MAB (32.03 %) was in line with

that of MA in case of the elastase assay but its suppression (19.71 %) was similar to that of MB in the tyrosinase test. On the other hand, the standards oleanolic acid caused a 50 % inhibition on elastase at 0.28 mg/mL, whereas myricetin had an IC_{50} value of 0.74 mg/mL for tyrosinase suppression.

Table 12. Inhibitory activities on pancreatic elastase and tyrosinase of momilactones A and B at a concentration of 2 mg/mL

	Inhibition percentage (%)	
	Pancreatic elastase	**Tyrosinase**
MA	30.86 ± 0.27 a	37.59 ± 0.22 a
MB	18.50 ± 0.56 b	12.60 ± 0.43 b
MAB	32.03 ± 0.47 a	19.71 ± 0.42 b
Oleanolic acid (IC_{50})	0.28 ± 1.10 mg/mL	-
Myricetin (IC_{50})	-	0.74 ± 0.01 mg/mL

Data presented a means ± standard errors. Means within a same column followed by similar letters are not significantly different by Turkey's test ($p<0.05$). MA: momilactone A; MB: momilactone B; MAB: mixture of MA and MB at 1:1, v/v; -: not determined.

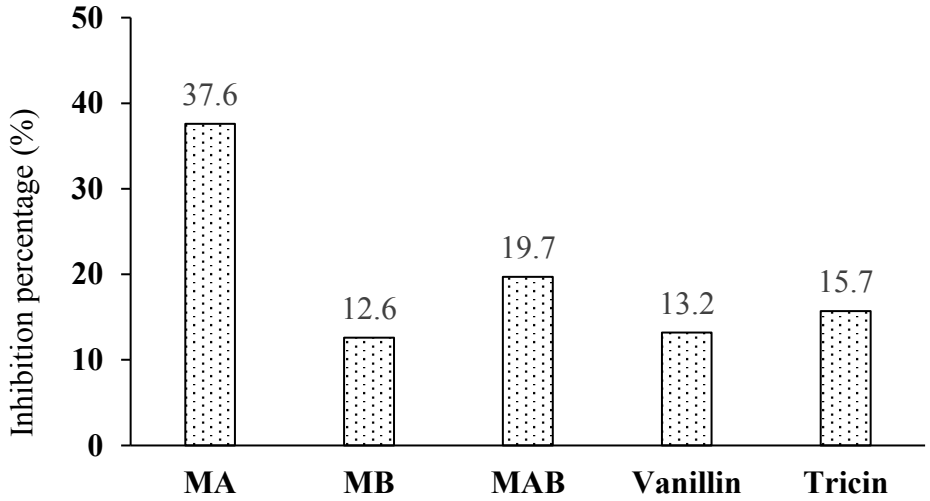


Figure 16. Anti-tyrosinase activities of MA, MB, vanillin and tricin at 2 mg/mL.

Remarkably, the anti-tyrosinase activities of MA and MAB were significantly higher than that of vanillin (13.2 %) and tricin (15.7 %) at a same concentration of 2 mg/mL (Figure 16). Meanwhile, MB's activity was comparable with such two known tyrosinase inhibitors.

5.4.3. Momilactones A and B contents in rice grains

The quantities of MA and MB in various common rice grains are shown in Table 13. Conspicuously, samples prepared by the SEP-PAK cartridge are detected with higher contents than those by the normal filtration.

Table 13. Momilactones A and B contents (μg/g DW) in various rice grains

Code	Type	Normal sample		SEP-PAK sample	
		MA	MB	MA	MB
Ko	Refined	0.22 c	0.19 b	0.46 c	0.41 b
KoCo	Refined	0.03 f	0.03 e	0.09 ef	0.08 de
KT1	Refined	0.02 f	0.03 de	0.05 fg	0.05 ef
KT2	Refined	0.02 f	0.02 ef	0.08 efg	0.07 de
KT3	Refined	0.02 f	0.02 ef	0.13 de	0.15 c
ST24	Refined	0.02 f	0.01 f	0.03 g	0.01 f
RVT	Refined	0.07 e	0.03 e	0.10 ef	0.04 ef
KT4	Brown	0.46 a	0.49 a	1.56 a	1.61 a
Bin 9	Brown	0.13 d	0.09 c	0.18 d	0.11 cd
KD18	Brown	0.40 b	0.05 d	0.58 b	0.07 de

Results are presented in means (n = 3); Ko, Koshihikari; KoCo, cooked Koshihikari, KT1, shinnosuke rice; KT2, seiten no hekireki rice; KT3, ginga no shizuku rice; KT4, ho no mai; KD18, Khang dan 18; SEP-PAK, a sample prepared by SEP-PAK® Plus C18 cartridge. Means within a same column followed by similar letters are not significantly different by Turkey's test ($p<0.05$).

Significantly, the calculated MA and MB amounts in KT3 grain (SEP-PAK sample) were increased by 6 and 7 times for MA (from 0.02 to 0.13 μg/g DW) and MB (from 0.02 to 0.15 μg/g DW), respectively. Except Bin 9, the brown rice KT4 and KD18 contained much higher amounts of MA and MB than other refined rice grains, of which, KT4 rice had the highest contents (1.56 and 1.61 μg/g DW of MA and MB, respectively). Among white rice, Koshihikari contained the greatest amount of momilactones A (0.46 μg/g DW) and B (0.41 μg/g DW) which were even higher than those of the brown rice Bin 9. Generally, MA and MB contents quantified in Japanese rice were greater than those in Vietnamese rice. Nevertheless, the MA content of RVT (0.10 μg/g DW) were higher than that of KT1 (0.05 μg/g DW) and KT2 (0.08 μg/g DW) thought the difference was not statistically significant. Additionally, the extract of cooked Koshihikari rice had a significantly lower momilactones than that of un-cooked Koshihikari.

5.5. Discussion

Momilactones A and B are important compounds in the defense-mechanism pathway of rice (*Oryza sativa*). Recently, my group have revealed the potential antidiabetic and anti-obesity activities of MA and MB via in vitro enzymatic assays. In this chapter, I focused on the novel biological activities including antioxidant and anti-skin-aging properties of these active metabolites. In fact, Fukuta et al. (2007) reported the antioxidant activity of MA and MB, however, the study only showed a weak activity on 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, by which, EC_{50} values for MA and MB were 783.9 and 790.7 μg, respectively. In the present study, by ABTS, reducing power and β-carotene bleaching methods, I successfully evaluated the antioxidant capacity of MA and MB. For the first time, the mixture of MA and MB (MAB, 1:1, v/v) was found to be more significantly potent than the single MA and MB in term of ABTS and reducing power assays. Interestingly, this study indicated the first time MA possessing stronger activities than MB by β-carotene bleaching

and inhibitory elastase and tyrosinase activities. The results suggested that the mechanism of lipid peroxidation inhibition of MA and MB might correlate to that of elastase and tyrosinase inhibitory actions. Prominently, MA and MB performed higher anti-tyrosinase activities than vanillin and tricin which were affirmed as potential tyrosinase inhibitors (Mu, Li, & Hu, 2013; Ashraf, Rafiq, Seo, Babar, & Zaidi, 2015). Moreover, vanillin and tricin were reported as dominant compounds in various parts of rice (Ogo, Ozawa, Ishimaru, Murayama, & Takaiwa, 2013; Khang et al., 2016). On the other hand, rice and rice products were investigated as well-known sources for the treatment of skin's diseases (Manosroi et al., 2012; Kanlayavattanakul, Lourith, & Chaikul, 2016). Among identified cosmeceutical compounds from rice, flavonoids (Miyazawa et al., 2003), ferulic acid, gamma-oryzanol and phytic acid (Marto et al., 2018) were the most common. Therefore, findings of the present study introduced a new member of the anti-aging substance class, namely momilactones A and B. The inhibitory assays on elastase and tyrosinase activities showed that MA and MB can be promising candidates of skin-stretching and skin-whitening materials.

Momilactones A and B have been quantified by various techniques but mostly by HPLC (Xuan et al., 2016; Quan et al., 2019a; 2019b). In chapter 2, I found that the sensitivity of MA and MB quantification by HPLC could be enhanced by applying the SEP-PAK C18 cartridge in preparation of rice husk sample. Therefore, in this chapter, I investigated the difference between the normal filtration and SEP-PAK purification integrated with an advanced UPLC-ESI-MS technique in quantifying MA and MB in various rice grains. The results highlighted that rice grain samples prepared by SEP-PAK C18 cartridge were detected with higher amount of MA and MB than samples prepared by the normal filtration. The UPLC-ESI-MS could disclose MA and MB with a higher sensitivity compared to HPLC-ESI-MS (Quan et al., 2019a).

5.6. Conclusion

This chapter is the first debut of antioxidant and anti-skin-aging abilities of MA and MB and quantification of such bioactive compounds among commercially common Japanese and Vietnamese rice grains. The findings provide a good option in selecting the best rice variety enriched of MA and MB which may help protect our healthy against not only risks from diabetes and obesity but also from their complications, skin's problems.

CHAPTER 6: GENERAL DISCUSSION

6.1. Key findings

This study showed the first-time inhibitory effects of momilactones A (MA) and B (MB) on the key enzymes related to human diseases including diabetes, obesity, and skin aging. The results can be prospectively applied in medicine and pharmaceutical fields and contribute to the development of the future therapeutics.

Secondly, for the first time, this research successfully detected and quantified MA and MB in bran and white rice grain. Since most of the studies agree with the fact that white rice intake can increase diabetes risk, the existence as diabetic and obesity inhibitors of MA and MB in white rice grain may alter the antecedent perspective that white rice consumption induced risks of type 2 diabetes and obesity.

The HPLC/UPLC-ESI-MS methods and the advanced protocols for sample preparations were validated, which helped determine and quantify MA and MB at higher reliability and sensitivity in various rice plant parts and rice varieties.

6.2. Relationships among diabetes, obesity, and skin diseases

As mentioned in previous chapters, the endothelial dysfunction may be caused by many factors which are divided into main types of exo- and endogenous factors. In detail, the exogenous factor includes all biotic and abiotic aspects from the ambient environment that can influence the metabolism of the human body. Meanwhile, endogenous factors may contain genetic problems, metabolic disorders, hormone abnormalities, etc. Basically, in the higher organisms like plant, animal, and human, the activity of hormones plays a prerequisite role in every assimilation and metabolism during the life. Hormone does not only regulate the physiological activities but also maintains the endothelial stability by just minor concentrations through diffusing across cells or transporting along with the bloodstream

(Barrington, 2019). Nowadays, there is a wide spectrum of studies about cardiovascular and metabolic diseases. Most of the causalities among diseases and disorders are adequately explained by various complicated pathways. Among the evidence, the theory revolved around insulin resistance and intrinsic disorders is the most analyzed and elucidated.

In mammals, insulin is an essential hormone secreted by pancreatic β-cells and is responsible for glucose uptake (Schwartsburd, 2017). In human, insulin takes part in most of the important metabolisms and serves as a regulator of both glucose and lipid (Saltiel & Kahn, 2001). The occurrence of insulin resistance is defined by many irregularities at various levels, of which, the activity of intracellular enzymes is regarded as one of the key determinants (Pessin & Saltiel, 2000; Saltiel & Kahn, 2001). Therefore, knowledge on hormone and relevant enzyme activities play a crucial role in development of the proper therapeutics for chronic diseases as diabetes and obesity.

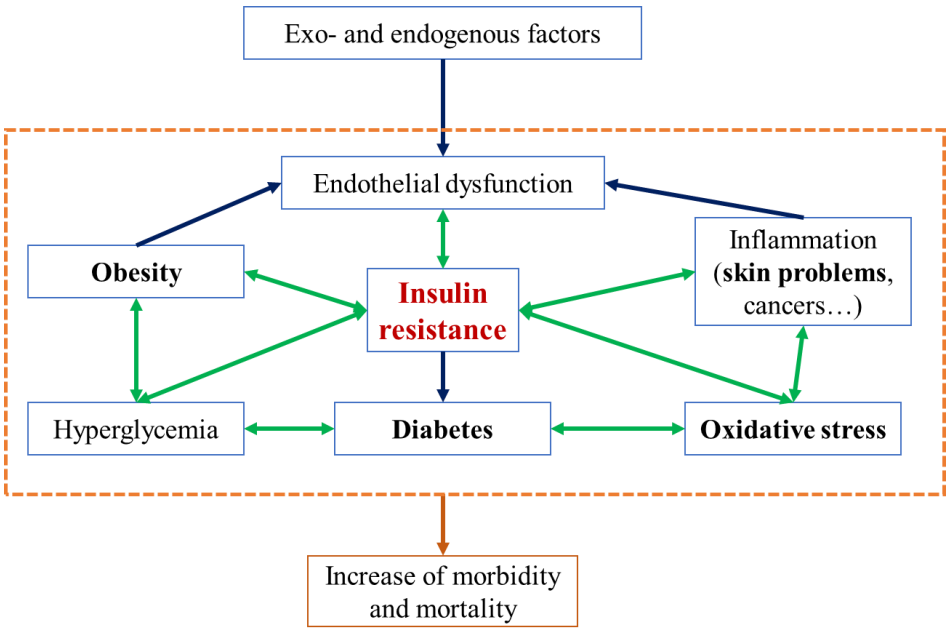


Figure 17. Relations among diabetes, obesity, and skin diseases.

Figure 17 delineates the relation among diabetes mellitus, obesity and oxidative stress which eventually may result in morbidity and mortality. In fact, many studies demonstrated the link between obesity and diabetes, especially type 2 diabetes (Golay &

Ybarra, 2005; Al-Goblan, Al-Alfi, & Khan, 2014). In particular, the literature indicated that body mass index has a strong relationship to diabetes and insulin resistance. Besides, practical investigations reported that skin disorders and complications would sooner or later be developed in all diabetic and obesity patients (Linda, 2002; Demirseren et al., 2014; Duff et al., 2015). The dermatological changes have been reported to be acanthosis nigricans and skin tags (due to insulin resistance); hyperandrogenism; striae due to over extension; stasis pigmentation due to peripheral vascular disease; lymphedema; cutaneous infections; diabetic foot; rubeosis faciei; and diabetic dermopathy in general. Mechanically, these complications are an inevitable consequence of a series of disorders arising from chronic insulin resistance (Figure 17). Whereby, the rise in the accumulation of free oxygen radicals in the body will ultimately lead to a chain of metabolic disturbances consisting of inflammations as ulcers, cancers, skin sores, etc.

Presently, therapeutics pay the most attentions on how to control and minimize diabetes and obesity complications. However, long-term use of synthetic preparations often results in some dangerous side effects. Therefore, the discovery and utilization of alternative medicinal substances originated from natural sources is the best appropriate option in order to both decelerate risks from undesired effects and ensure the safety for patients. By that sense, this study was conducted to reveal the antidiabetic, anti-obesity and anti-skin diseases properties of two bioactive metabolites from rice, momilactones A and B.

6.3. Possible mechanisms of momilactones A and B on diabetes, obesity and skin aging inhibitory activities

In chapters 3 and 4, antidiabetic and anti-obesity activities of MA and MB were reported by inhibition assays on three key enzymes α-amylase, α-glucosidase, and trypsin while anti-skin aging activity was assayed on elastase and tyrosinase. Principally, the normal reaction of an enzyme and its substrate obeys the lock and key hypothesis meaning that an

enzyme reacts only with a suitable substrate to produce the corresponding product and vice versa. However, with the existence of inhibitors, the original reaction can be terminated which is so called the enzymatic inhibition.

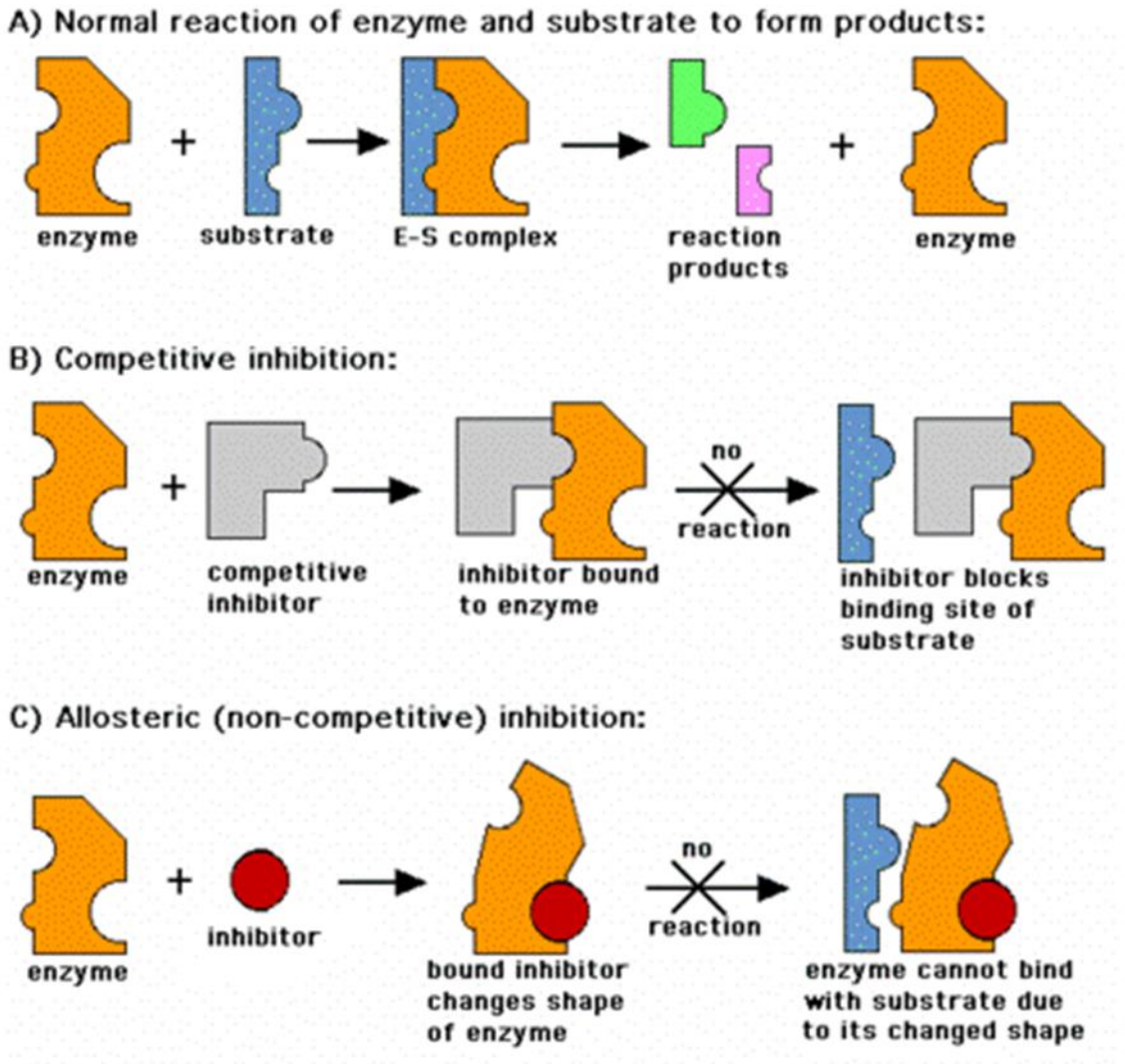


Figure 18. Enzyme reaction and inhibitions.

(*Source*: http://blog.canacad.ac.jp/bio/BiologyIBHL1/files/195649.gif)

Based on the targeted direct of inhibitors on the active site of enzymes, the inhibitory effects can be divided into two main types containing competitive or active site-directed inhibition and non-competitive or non-active site-directed inhibition (Figure 18). To investigate a substance or a novel natural product is either competitive or non-competitive inhibitor, the enzyme kinetic assay should be carried out (Figure 19).

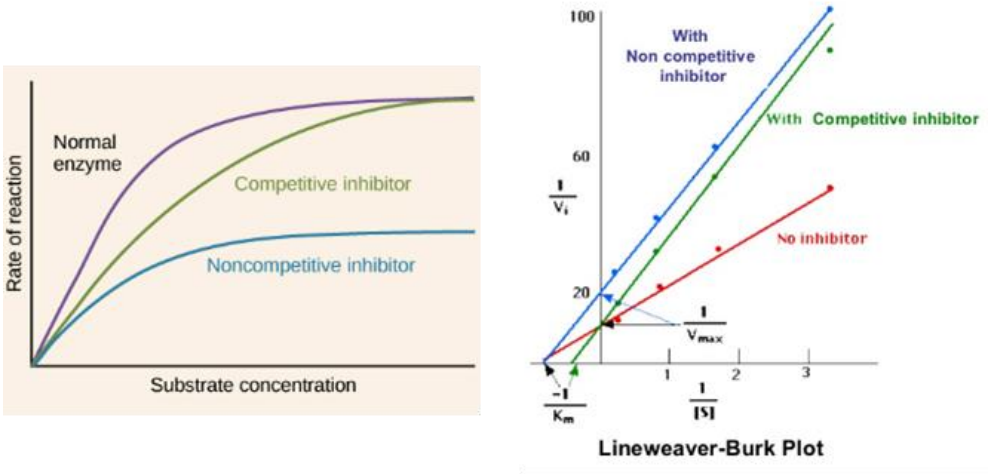


Figure 19. Enzymatic kinetic model.

(*Source*: https://cnx.org/contents/GFy_h8cu@9.85:MnC6GuJi@7/Enzymes; https://www.biology-pages.info/E/EnzymeKinetics.html)

By this method, the reaction rate can be examined by different factors such as varying concentrations of substrate, subsequently, the inhibitory mechanism influencing the catalytic level of reaction is able to be revealed and clarified (Engelking, 2015). Therefore, the mechanism of inhibitory effects of MA and MB on key enzymes related to diabetes, obesity and skin aging may be elucidated by enzyme kinetic assays.

Nevertheless, the other possible aspects may impact on the mechanism of MA and MB inhibiting enzyme activities, for example, whether there is a link between momilactones and substrates, or the effects of pH on solubility and stability of momilactones, or functional groups of momilactones, or types of enzyme which may cause enzymatic inhibitions. For example, previous studies indicated the effect of functional groups of inhibitors as well as their impacts on sulfhydryl (SH) enzymes and non-SH enzymes (Keizo, 1979; Barros et al., 2015). Accordingly, Barros et al. reported some unsaturated lactones that could inhibit *Aedes aegypti* larval growth via non-SH enzyme, trypsin. On the other hand, Barron & Singer (1943) soon investigated the inhibitory effect of glutathione on enzyme systems containing active SH groups. Therefore, the relation between functional groups of MA and MB and

different SH and non-SH enzymes related to human diseases should be further scrutinized. In summary, the discovery of major factors and co-factors related to the inhibition of enzyme activity may help shorten the time to study appropriate remedies and therapeutics.

As reviewed in chapter 1, the inhibition mechanism of MA and MB could be scrutinized via the expression levels of genes encoding targeted enzymes and relevant kinase activities. In case of diabetes type 2, there is a strong evidence that the pathogenesis is genetically correlated to the multifactorial transmission (Owen & McCarthy, 2007). However, genetic risk of type 2 diabetes contains few genes of major effect (Lindgren & McCarthy, 2008), therefore, the relevant study has been difficult and ambiguous so far. In a review paper, Rich, Norris and Rotter discussed these conventional problems and suggested new approaches which could cover the human genome at high resolution and characterize individual candidate genes (2008). To date, at least 18 genes have been discovered to be probably associated with risk of type 2 diabetes, however, the key pathway or expression mechanism towards pathophysiology has not been satisfactorily explained (Rich et al., 2008; Gaulton et al., 2008). Lastly, these genetic achievements implied much pragmatic information for further researches on the discovery of the most effective drug for anti-targeted diseases at the molecular level. In fact, various targeted gene expression including glucokinase, glucose-6-phosphatase, monocyte chemoattractant protein-1, peroxisome proliferator-activated receptor-gamma (alpha), tumor necrosis factor, phosphoenol pyruvate carboxykinase, aldose reductase were currently used to examine and evaluate the inhibitory effect of new diabetic inhibitors (Armoni et al., 2003; Nakamura et al., 2006; Chaurasia, Kharya, Sharma, & Roy, 2012). The stimulatory or inhibitory effects of tested compounds on the specified genes expressions may provide effective information to further examination of therapeutics and synthesis of promising drugs as well. For example, Li et al. (2015) found that andrographolide derivative AL-1 could improve insulin resistance

of rats with high-fat diet/STZ-induced diabetes via NF-κB p65 phosphorylation signaling pathway. In particular, "AL-1 alleviated lipid metabolism, promoted glycogen synthesis and maintained the normal structure of islet and β-cells" (Li et al., 2015). The results both suggested antidiabetic effect of AL-1 and provided the insight for prevention and treatment of the disease through molecular and biochemical mechanisms simultaneously.

In term of drug development, in vitro assays are indispensable steps among primary tests. The inhibitions of MA and MB on various enzyme reactions by in vitro assays help partly evaluate the potential ability of these compounds in controlling the relevant diseases. In the next step, in vivo models and clinical trials should be further implemented to affirm the medicinal properties and bioapplicability of MA and MB. However, in case of the screening potentials of several single compounds, in vitro models help save time and cost for the future exams. Particularly, by in vitro assays, the most promising candidate metabolites could be obtained for in vivo and clinical tests, whereas the unreasonable compounds would be denied.

6.4. Potential sources of momilactones A and B

Previous chapters exposed valuable biological activities of MA and MB which are prospective in management and control the risks from diabetes, obesity as well as skin diseases. Also, the use of various advanced spectroscopic methods helped determine exactly the amount of these compounds in rice. Moreover, knowledge on the genetic information of gene cluster encoding MA and MB described in chapter 1 suggested to future studies that towards to the mutation approach creating new rice varieties enriched with momilactones.

Besides, the natural source, the moss *Hypnum plumaeforme* should be paid more considerations as a promising source of MA and MB. Researches on cloning and cultivation of the moss together with the new purification method in order to get more amount of MA and MB should be conducted. Aside from these approaches, the synthetic production of

momilactones could be widely applied at the larger scales and beyond that confirming such compounds as the pharmaceutical product. Nonetheless, medical trials should be carried out under a strict management together with safety, moral and human right certificates.

SUPPLEMENTARY DATA

Table S1. The fragmentation patterns and intensity data of momilactone A

Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity	Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity
1	29.13	21.92	155949.10	82	136.40	7.08	50387.20
2	39.13	23.86	169761.76	83	137.38	8.58	61067.64
3	40.14	7.56	53771.94	84	141.40	13.24	94220.79
4	41.15	84.62	601975.69	85	142.40	11.43	81336.00
5	42.13	4.31	30639.69	86	143.41	27.59	196281.84
6	42.16	6.10	43422.10	87	144.42	12.24	87041.62
7	43.14	14.86	105744.85	88	145.43	33.22	236323.80
8	43.17	11.21	79721.02	89	146.44	12.74	90649.82
9	44.11	2.01	14318.28	90	147.45	19.91	141634.95
10	51.16	6.01	42770.76	91	148.43	8.28	58871.09
11	52.17	3.89	27673.41	92	149.44	8.29	58947.08
12	53.18	45.10	320831.78	93	150.41	6.99	49701.97
13	54.19	5.71	40602.19	94	151.42	6.96	49543.00
14	55.16	22.41	159393.50	95	152.42	4.87	34639.45
15	55.20	58.06	413048.53	96	153.42	5.16	36676.51
16	56.21	3.07	21815.81	97	154.43	3.39	24138.81
17	57.18	3.51	24982.46	98	155.44	11.07	78765.00
18	57.22	2.88	20512.19	99	156.45	7.79	55396.87
19	65.21	23.86	169748.99	100	157.46	23.95	170372.08
20	66.22	9.82	69825.32	101	158.47	9.80	69738.01
21	67.23	53.80	382724.15	102	159.48	20.95	149038.77
22	68.20	3.77	26821.67	103	160.45	3.73	26568.96
23	68.23	14.37	102248.88	104	160.49	5.71	40653.62
24	69.21	8.06	57363.93	105	161.46	15.08	107279.19
25	69.25	10.81	76901.74	106	162.47	5.33	37898.79

Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity	Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity
26	77.23	51.52	366475.96	107	162.50	3.17	22559.26
27	78.24	13.64	97064.75	108	163.48	6.51	46286.97
28	79.25	74.82	532220.17	109	165.45	3.60	25641.83
29	80.26	14.85	105606.64	110	167.46	3.15	22439.70
30	81.27	100.00	711378.71	111	169.48	10.26	72961.77
31	82.28	8.63	61374.82	112	170.49	5.96	42416.07
32	83.22	4.92	35019.92	113	171.50	19.28	137173.16
33	83.25	3.03	21585.51	114	172.51	7.36	52340.88
34	83.29	3.49	24802.88	115	173.49	18.60	132304.97
35	89.26	2.43	17303.54	116	174.49	8.66	61583.81
36	91.28	88.12	626895.60	117	175.50	15.45	109879.62
37	92.28	13.45	95648.47	118	176.51	6.21	44146.60
38	93.30	35.37	251644.15	119	177.49	10.92	77678.72
39	94.30	9.00	64016.23	120	183.53	9.04	64324.66
40	95.28	5.16	36724.36	121	184.53	4.87	34633.34
41	95.32	29.91	212803.54	122	185.55	15.22	108288.25
42	96.32	2.69	19162.76	123	186.52	2.68	19061.10
43	97.26	2.77	19708.39	124	186.55	4.27	30381.33
44	97.30	3.20	22740.45	125	187.53	21.93	156017.39
45	102.29	2.44	17378.63	126	188.54	9.51	67646.72
46	103.30	13.14	93478.42	127	189.55	16.48	117207.09
47	104.31	6.43	45713.91	128	190.52	3.26	23175.01
48	105.32	51.05	363144.81	129	190.56	5.19	36913.76
49	106.33	12.80	91061.47	130	192.51	3.51	24967.78
50	107.30	3.66	26061.82	131	197.57	4.24	30190.81
51	107.34	20.50	145854.91	132	198.58	5.08	36143.67
52	108.31	4.27	30361.90	133	199.59	94.19	670069.40
53	108.35	5.29	37632.60	134	200.58	21.03	149620.70

Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity	Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity
54	109.32	9.10	64745.74	135	201.57	14.38	102311.84
55	109.36	4.24	30151.68	136	202.58	11.09	78896.30
56	110.33	4.32	30766.11	137	213.63	31.30	222681.58
57	111.31	3.93	27947.45	138	214.64	9.36	66613.44
58	115.33	33.93	241361.78	139	217.56	5.92	42078.07
59	116.34	14.78	105139.90	140	223.64	3.68	26193.83
60	117.35	36.34	258546.65	141	227.66	16.17	115022.23
61	118.35	12.89	91694.09	142	228.66	9.42	67046.42
62	119.37	37.97	270075.96	143	229.66	8.28	58922.01
63	120.37	10.58	75287.47	144	232.61	17.62	125323.91
64	121.35	8.16	58065.78	145	233.62	3.08	21931.57
65	121.38	10.20	72560.31	146	241.68	8.12	57779.21
66	122.36	8.17	58129.18	147	242.69	5.32	37847.55
67	122.39	2.81	19994.57	148	243.70	8.53	60673.48
68	123.37	23.63	168081.69	149	255.73	39.06	277846.13
69	124.38	10.02	71312.31	150	256.73	7.80	55473.33
70	127.35	7.49	53285.42	151	257.71	6.31	44893.35
71	128.36	26.81	190715.16	152	258.72	5.74	40835.30
72	129.37	30.94	220112.47	153	259.73	4.28	30431.47
73	130.38	12.58	89459.41	154	270.78	24.40	173608.38
74	131.39	41.00	291634.31	155	271.75	35.80	254646.40
75	132.40	16.81	119599.81	156	272.76	11.55	82142.84
76	133.41	57.11	406256.05	157	286.80	8.47	60244.49
77	134.38	2.82	20029.66	158	299.80	29.08	206867.80
78	134.42	10.48	74535.18	159	300.81	6.01	42747.39
79	135.39	6.34	45075.48	160	314.85	97.49	693541.65
80	135.43	6.92	49245.99	161	315.86	20.88	148502.57
81	136.37	7.70	54755.48				

Table S2. The fragmentation patterns and intensity data of momilactone B

Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity	Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity
1	29.01	2.89	14790.27	84	154.11	7.11	36391.31
2	29.05	21.18	108463.98	85	155.12	29.24	149731.29
3	31.03	2.87	14672.55	86	156.13	14.24	72902.55
4	39.03	23.25	119048.10	87	157.13	45.01	230483.38
5	40.04	7.83	40088.47	88	158.14	16.61	85049.95
6	41.05	90.58	463789.86	89	159.15	24.57	125797.38
7	42.05	6.61	33849.35	90	160.12	8.70	44542.20
8	43.03	37.04	189653.34	91	161.17	20.54	105146.81
9	43.06	12.45	63758.81	92	163.11	24.64	126139.99
10	44.00	3.82	19540.20	93	164.12	9.03	46212.52
11	45.01	4.61	23608.27	94	165.11	7.95	40721.53
12	45.04	3.04	15546.07	95	167.12	9.00	46083.67
13	51.04	5.93	30354.96	96	168.13	4.86	24910.37
14	52.04	3.66	18725.85	97	169.14	27.09	138708.38
15	53.05	44.08	225710.93	98	170.10	4.52	23157.42
16	54.06	5.88	30114.55	99	170.14	9.33	47780.04
17	55.03	21.88	112037.96	100	171.15	42.43	217252.28
18	55.07	47.41	242744.55	101	172.12	13.24	67774.60
19	57.05	5.77	29530.08	102	173.13	21.55	110335.75
20	65.05	24.53	125604.19	103	174.14	7.01	35871.33
21	66.06	9.12	46684.66	104	175.11	5.92	30317.80
22	67.07	43.71	223826.58	105	175.15	4.65	23819.02
23	68.04	5.07	25984.53	106	176.12	17.57	89951.33
24	68.08	11.38	58277.88	107	177.13	6.55	33514.14
25	69.05	12.07	61808.91	108	179.12	4.85	24841.77
26	69.09	10.29	52705.20	109	181.14	8.84	45255.03
27	71.06	4.25	21783.00	110	183.12	48.60	248855.28

Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity	Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity
28	77.06	54.13	277146.92	111	184.13	19.22	98391.16
29	78.06	16.38	83882.04	112	185.17	36.79	188396.34
30	79.07	67.05	343323.17	113	186.16	11.60	59380.41
31	80.08	11.60	59417.03	114	187.15	10.54	53975.46
32	81.05	3.70	18921.49	115	189.13	14.26	72994.85
33	81.09	60.89	311769.87	116	190.14	6.76	34600.65
34	82.09	5.10	26116.42	117	195.16	9.50	48633.69
35	83.03	3.89	19940.43	118	197.14	37.70	193038.93
36	83.07	4.48	22921.92	119	198.14	17.49	89535.27
37	89.06	3.42	17507.47	120	199.15	18.38	94086.63
38	91.07	100.00	512035.48	121	200.16	8.84	45289.42
39	92.08	16.48	84402.12	122	201.13	25.41	130084.44
40	93.09	28.88	147854.22	123	202.14	15.58	79796.57
41	94.10	4.72	24163.73	124	203.15	10.40	53270.69
42	95.07	6.82	34920.63	125	209.14	3.35	17143.44
43	95.11	7.44	38094.92	126	209.17	6.10	31243.79
44	97.05	8.09	41439.53	127	211.19	23.59	120769.47
45	97.09	4.18	21413.02	128	213.16	7.39	37834.50
46	98.06	4.96	25405.56	129	213.20	5.82	29817.68
47	103.08	17.42	89220.66	130	215.15	14.98	76705.22
48	104.08	15.13	77448.59	131	216.16	17.14	87760.43
49	105.09	58.27	298384.27	132	217.17	9.28	47530.58
50	106.10	9.54	48833.50	133	223.16	8.54	43741.62
51	107.07	5.81	29736.56	134	225.21	15.26	78153.66
52	107.11	10.68	54698.20	135	226.17	21.33	109234.81
53	109.09	4.64	23751.78	136	227.19	14.69	75221.90
54	111.07	5.53	28302.59	137	229.17	13.37	68447.53
55	115.08	46.10	236057.94	138	231.18	8.01	41006.01

Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity	Peak	Fragmentation pattern (m/z)	Relative intensity (%)	Intensity
56	116.09	18.63	95398.13	139	232.19	7.88	40371.66
57	117.10	51.57	264037.45	140	237.18	6.73	34466.45
58	118.10	14.87	76162.78	141	239.19	52.13	266913.86
59	119.11	28.07	143740.50	142	240.20	10.69	54741.98
60	120.12	4.46	22811.48	143	241.18	7.64	39117.49
61	121.09	14.64	74984.38	144	243.17	11.35	58091.09
62	121.13	3.43	17547.24	145	244.17	21.20	108542.62
63	122.10	6.27	32124.70	146	251.19	8.80	45059.77
64	123.11	4.46	22821.91	147	253.21	11.24	57559.82
65	127.08	12.18	62373.54	148	255.19	8.92	45690.92
66	128.09	43.37	222054.34	149	256.20	9.77	50035.95
67	129.10	59.83	306335.36	150	257.21	57.09	292340.22
68	130.10	20.88	106927.74	151	258.21	13.14	67287.28
69	131.11	42.69	218591.83	152	262.17	10.36	53047.31
70	132.12	10.83	55431.24	153	266.22	10.87	55681.63
71	133.09	6.28	32145.29	154	267.23	5.59	28644.31
72	133.13	10.22	52319.58	155	268.24	6.90	35352.70
73	135.11	6.59	33763.51	156	269.21	8.55	43799.24
74	141.10	29.11	149058.13	157	270.21	6.89	35261.99
75	142.11	24.58	125872.34	158	271.22	6.54	33512.39
76	143.12	52.61	269401.06	159	284.23	14.14	72416.01
77	144.12	19.96	102202.93	160	285.24	13.61	69702.70
78	145.13	36.27	185713.95	161	294.22	9.80	50185.53
79	146.10	5.43	27808.54	162	297.21	5.41	27686.34
80	146.14	5.82	29780.10	163	312.24	21.54	110307.30
81	147.11	12.65	64796.96	164	313.24	4.65	23824.56
82	152.09	6.21	31784.72	165	330.25	14.86	76102.35
83	153.10	9.90	50697.82				

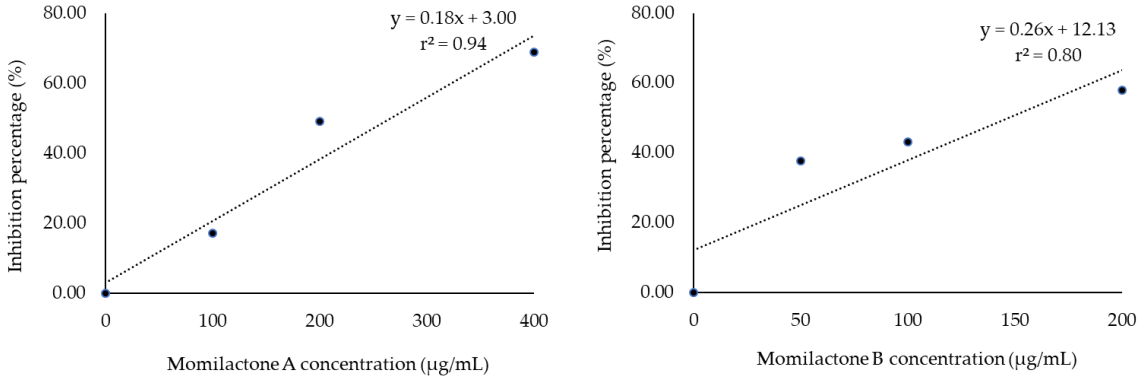


Figure S1. Inhibition of momilactones A and B on α-amylase activity.

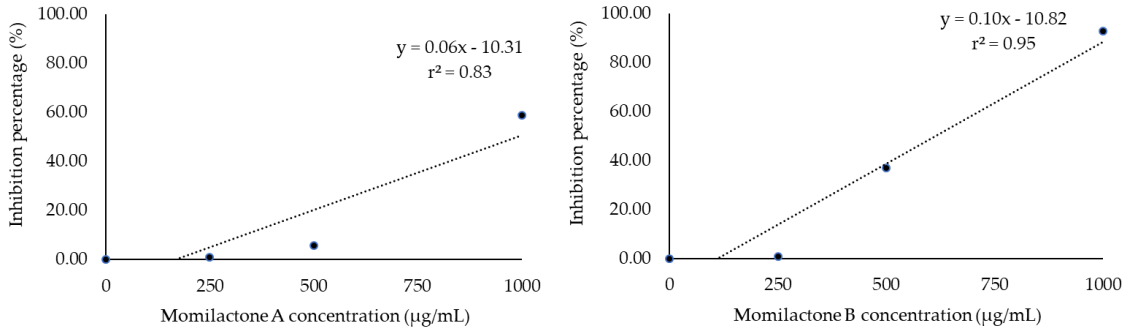


Figure S2. Inhibition of momilactones A and B on α-glucosidase activity.

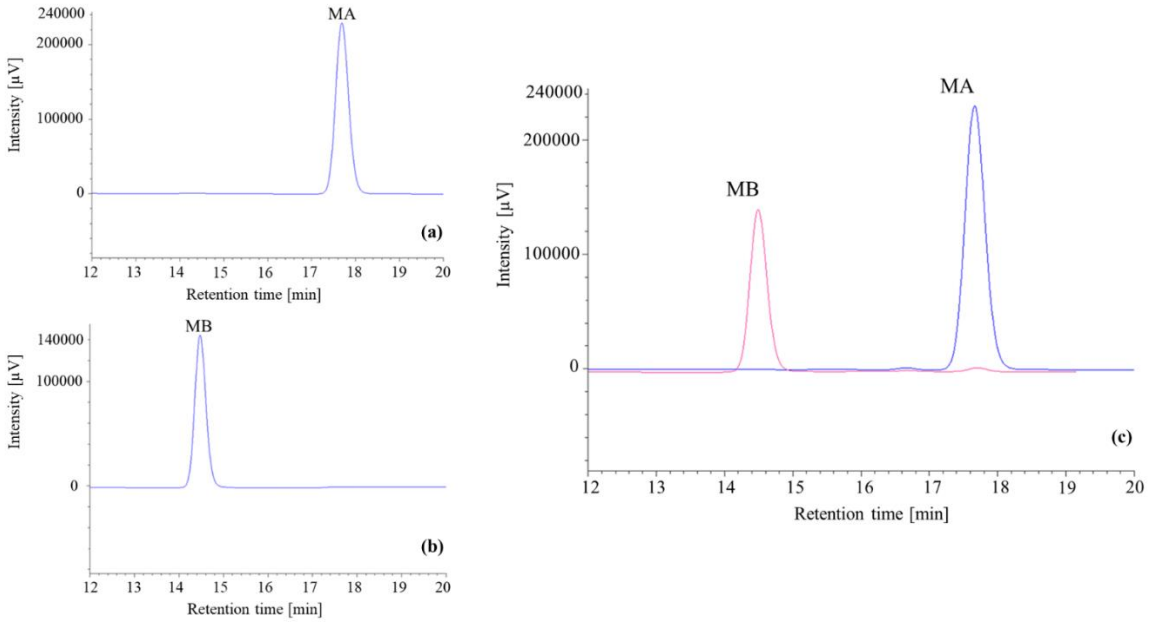


Figure S3. HPLC chromatograms of momilactones A and B: **(a)** standard momilactone A (MA); **(b)** standard momilactone B (MB); **(c)** isolated momilactones A and B.

Figure S4. TLC chromatogram of momilactones: **S** standard momilactones A and B; **MA** momilactone A; **MB** momilactone B.

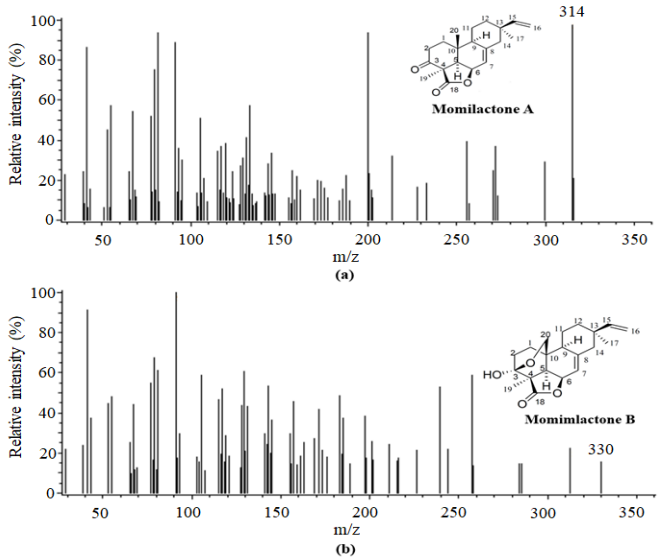


Figure S5. Mass spectra of the isolated **(a)** momilactone A and **(b)** momilactone B.

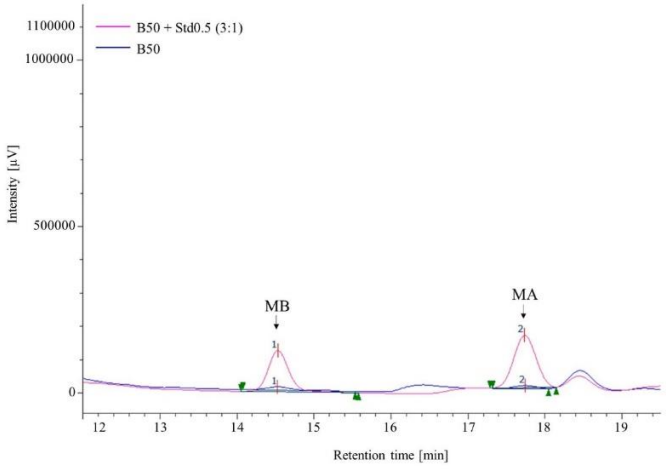
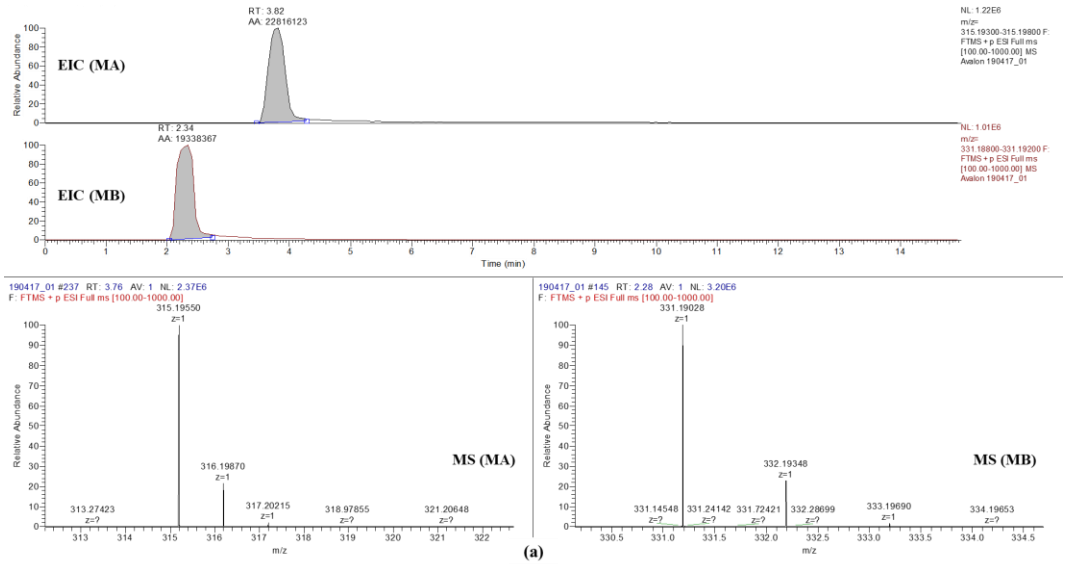
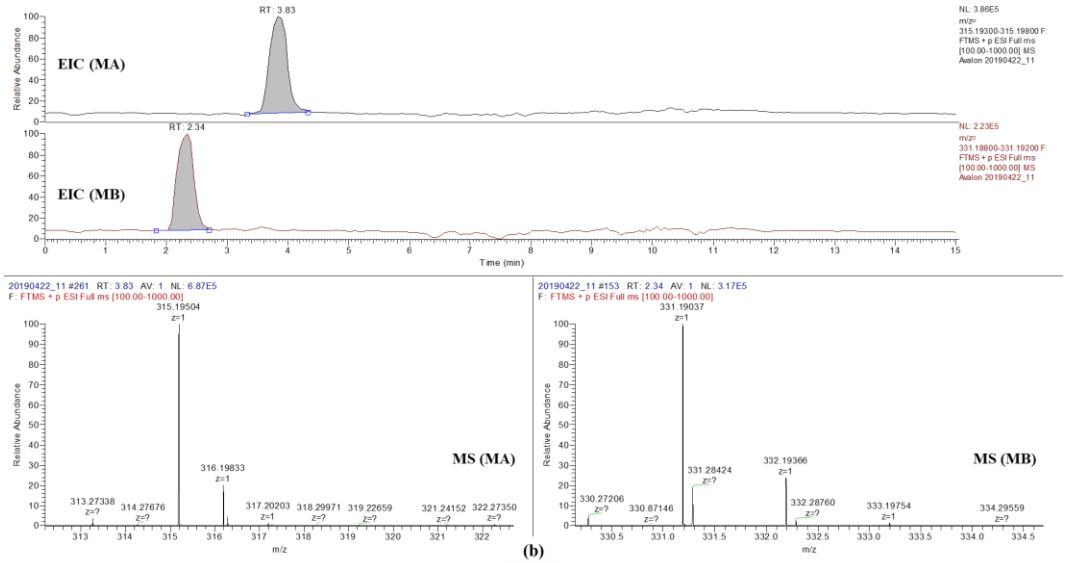


Figure S6. HPLC chromatogram of momilactones A and B detected in rice bran.

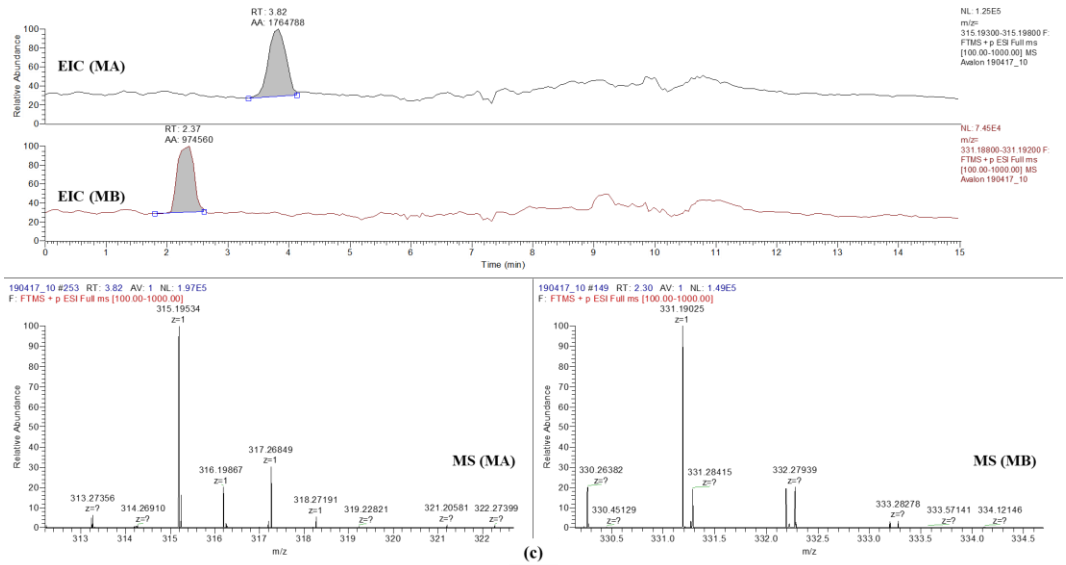
B50 was defatted bran extract at 50 mg/mL; B50 + Std0.5 (3:1) was defatted bran extract (50 mg/mL) mixed with pure MA and MB (0.5 mg/mL) at ratio 3:1 (v/v).


RT: 3.82
AA: 22816123
EIC (MA)
NL: 1.22E6
m/z= 315.19300-315.19800 F: FTMS + p ESI Full ms [100.00-1000.00] MS Avalon 190417_01
RT: 2.34
AA: 19338367
EIC (MB)
NL: 1.01E6
m/z= 331.18800-331.19200 F: FTMS + p ESI Full ms [100.00-1000.00] MS Avalon 190417_01
Relative Abundance
Time (min)
190417_01 #237 RT: 3.76 AV: 1 NL: 2.37E6
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19550 z=1
316.19870 z=1
317.20215 z=1
313.27423 z=?
318.97855 z=?
321.20648 z=?
MS (MA)
190417_01 #145 RT: 2.28 AV: 1 NL: 3.20E6
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19028 z=1
332.19348 z=1
331.14548 z=?
331.24142 z=?
331.72421 z=?
332.28699 z=?
333.19690 z=1
334.19653 z=?
MS (MB)
m/z

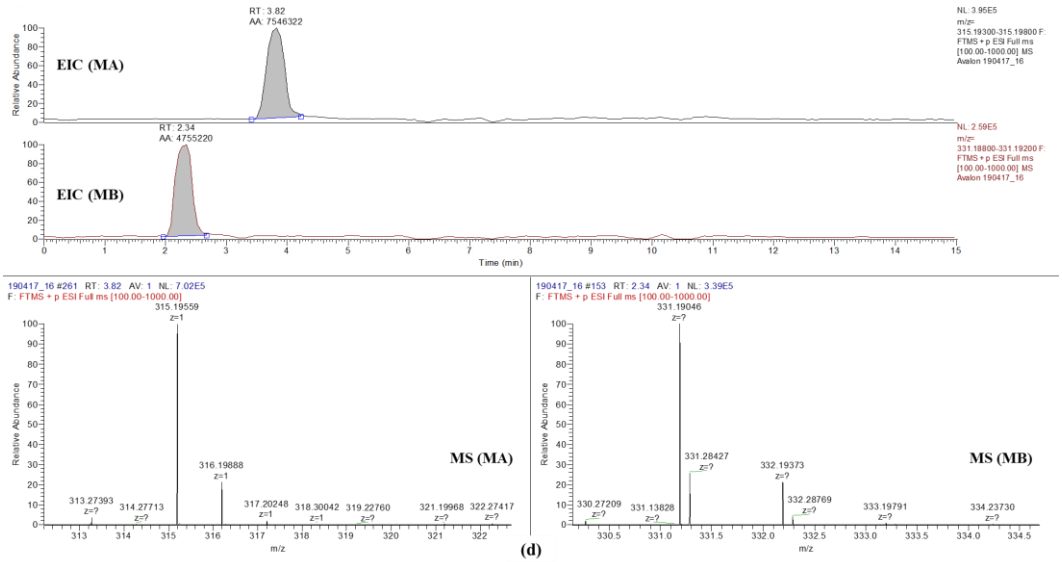
(a)


RT: 3.83
EIC (MA)
NL: 3.86E5
m/z= 315.19300-315.19800 F: FTMS + p ESI Full ms [100.00-1000.00] MS Avalon 20190422_11
RT: 2.34
EIC (MB)
NL: 2.23E5
m/z= 331.18800-331.19200 F: FTMS + p ESI Full ms [100.00-1000.00] MS Avalon 20190422_11
Relative Abundance
Time (min)
20190422_11 #261 RT: 3.83 AV: 1 NL: 6.87E5
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19504 z=1
316.19833 z=1
313.27338 z=?
314.27676 z=?
317.20203 z=1
318.29971 z=?
319.22659 z=?
321.24152 z=?
322.27350 z=?
MS (MA)
20190422_11 #153 RT: 2.34 AV: 1 NL: 3.17E5
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19037 z=1
331.28424 z=?
332.19366 z=1
330.27206 z=?
330.87146 z=?
332.28760 z=?
333.19754 z=1
334.29559 z=?
MS (MB)
m/z

(b)

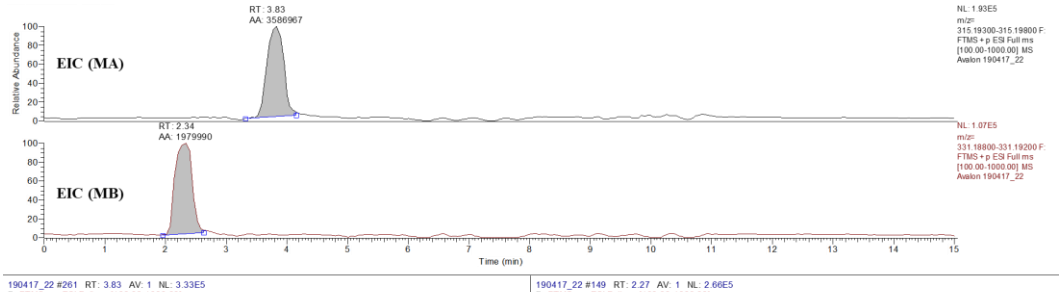

RT: 3.82
AA: 1764788
EIC (MA)
NL: 1.25E5
m/z= 315.19300-315.19800 F: FTMS + p ESI Full ms [100.00-1000.00] MS Avalon 190417_10
RT: 2.37
AA: 974560
EIC (MB)
NL: 7.45E4
m/z= 331.18800-331.19200 F: FTMS + p ESI Full ms [100.00-1000.00] MS Avalon 190417_10
Relative Abundance
Time (min)
190417_10 #253 RT: 3.82 AV: 1 NL: 1.97E5
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19534 z=1
317.26849 z=1
316.19867 z=1
318.27191 z=1
313.27356 z=?
314.26910 z=?
319.22821 z=?
321.20581 z=?
322.27399 z=?
MS (MA)
190417_10 #149 RT: 2.30 AV: 1 NL: 1.49E5
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19025 z=1
330.26382 z=?
331.28415 z=?
332.27939 z=?
330.45129 z=?
333.28278 z=?
333.57141 z=?
334.12146 z=?
MS (MB)
m/z

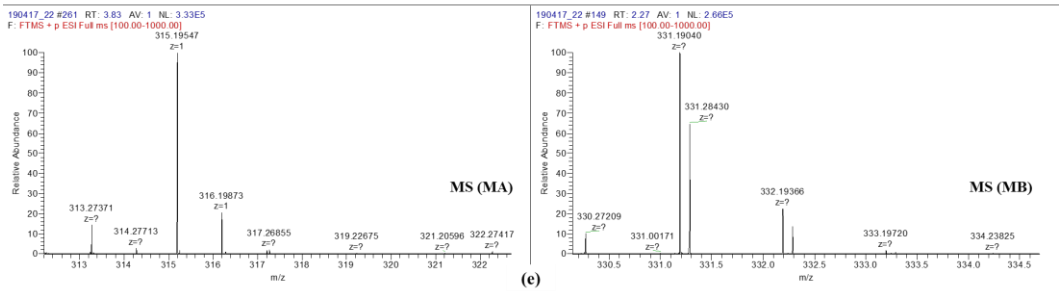
(c)


RT: 3.82
AA: 7546322
EIC (MA)
RT: 2.34
AA: 4755220
EIC (MB)
Relative Abundance
Time (min)
NL: 3.95E5
m/z=
315.19300-315.19800 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_16
NL: 2.59E5
m/z=
331.18800-331.19200 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_16

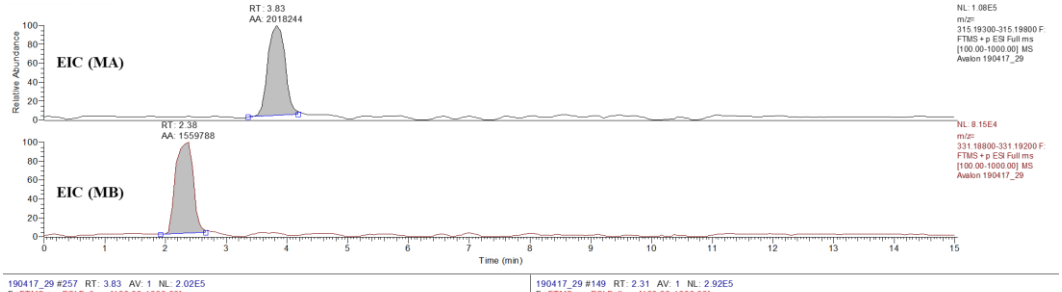
190417_16 #261 RT: 3.82 AV: 1 NL: 7.02E5
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19559 z=1
316.19888 z=1
313.27393 z=?
314.27713 z=?
317.20248 z=1
318.30042 z=1
319.22760 z=?
321.19968 z=?
322.27417 z=?
MS (MA)
190417_16 #153 RT: 2.34 AV: 1 NL: 3.39E5
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19046 z=?
331.28427 z=?
332.19373 z=?
330.27209 z=?
331.13828 z=?
332.28769 z=?
333.19791 z=?
334.23730 z=?
MS (MB)
Relative Abundance
m/z

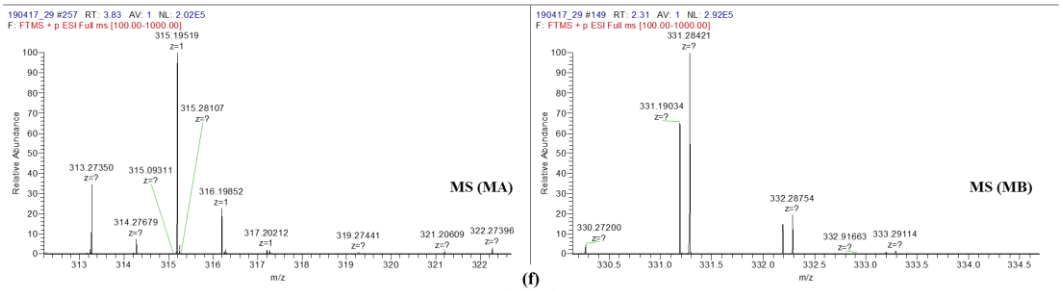
(d)


RT: 3.83
AA: 3586967
EIC (MA)
RT: 2.34
AA: 1979990
EIC (MB)
Relative Abundance
Time (min)
NL: 1.93E5
m/z=
315.19300-315.19800 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_22
NL: 1.07E5
m/z=
331.18800-331.19200 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_22

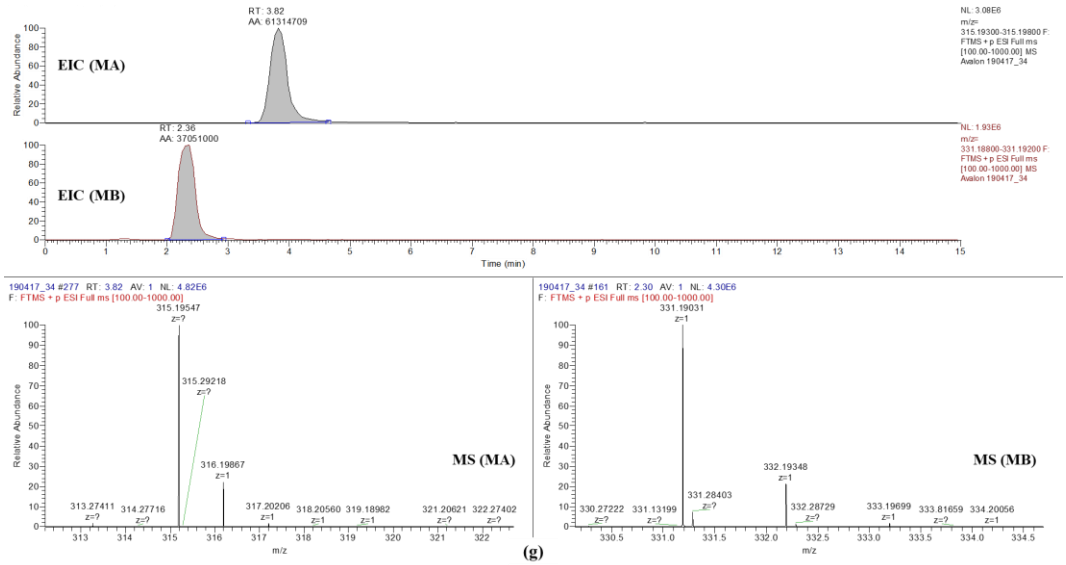

190417_22 #261 RT: 3.83 AV: 1 NL: 3.33E5
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19547 z=1
316.19873 z=1
313.27371 z=?
314.27713 z=?
317.26855 z=?
319.22675 z=?
321.20596 z=?
322.27417 z=?
MS (MA)
190417_22 #149 RT: 2.27 AV: 1 NL: 2.66E5
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19040 z=?
331.28430 z=?
332.19366 z=?
330.27209 z=?
331.00171 z=?
333.19720 z=?
334.23825 z=?
MS (MB)
Relative Abundance
m/z

(e)

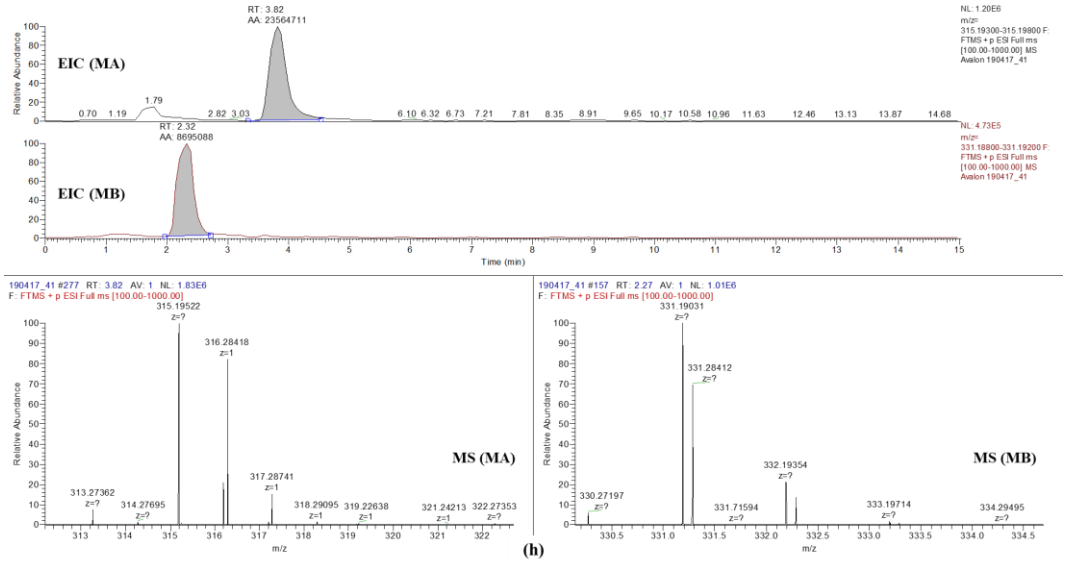

RT: 3.83
AA: 2018244
EIC (MA)
RT: 2.38
AA: 1559788
EIC (MB)
Relative Abundance
Time (min)
NL: 1.08E5
m/z=
315.19300-315.19800 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_29
NL: 8.15E4
m/z=
331.18800-331.19200 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_29


190417_29 #257 RT: 3.83 AV: 1 NL: 2.02E5
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19519 z=1
315.28107 z=?
313.27350 z=?
315.09311 z=?
316.19852 z=1
314.27679 z=?
317.20212 z=1
319.27441 z=?
321.20609 z=?
322.27396 z=?
MS (MA)
190417_29 #149 RT: 2.31 AV: 1 NL: 2.92E5
F: FTMS + p ESI Full ms [100.00-1000.00]
331.28421 z=?
331.19034 z=?
332.28754 z=?
330.27200 z=?
332.91663 z=?
333.29114 z=?
MS (MB)
Relative Abundance
m/z

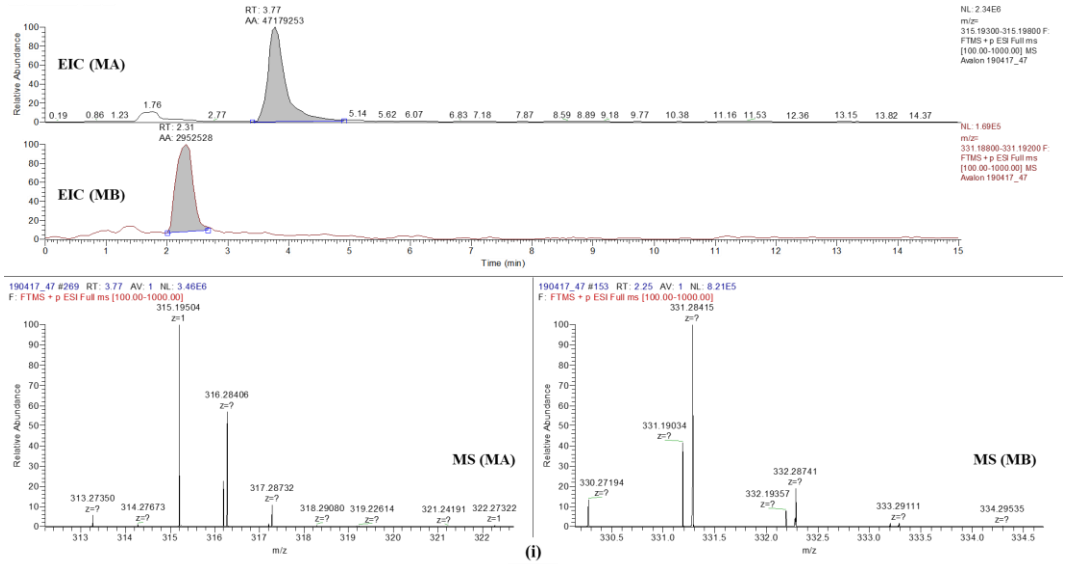
(f)


RT: 3.82
AA: 61314709
EIC (MA)
NL: 3.08E6
m/z=
315.19300-315.19800 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_34
RT: 2.36
AA: 37051000
EIC (MB)
NL: 1.93E6
m/z=
331.18800-331.19200 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_34
Relative Abundance
Time (min)
190417_34 #277 RT: 3.82 AV: 1 NL: 4.82E6
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19547 z=?
315.29218 z=?
316.19867 z=1
313.27411 z=?
314.27716 z=?
317.20206 z=1
318.20560 z=1
319.18982 z=1
321.20621 z=?
322.27402 z=?
MS (MA)
190417_34 #161 RT: 2.30 AV: 1 NL: 4.30E6
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19031 z=1
332.19348 z=1
331.28403 z=?
330.27222 z=?
331.13199 z=?
332.28729 z=?
333.19699 z=1
333.81659 z=?
334.20056 z=1
MS (MB)
m/z

(g)


RT: 3.82
AA: 23564711
EIC (MA)
NL: 1.20E6
m/z=
315.19300-315.19800 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_41
RT: 2.32
AA: 8695088
EIC (MB)
NL: 4.73E5
m/z=
331.18800-331.19200 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_41
Relative Abundance
Time (min)
190417_41 #277 RT: 3.82 AV: 1 NL: 1.83E6
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19522 z=?
316.28418 z=1
317.28741 z=1
313.27362 z=?
314.27695 z=?
318.29095 z=1
319.22638 z=1
321.24213 z=1
322.27353 z=?
MS (MA)
190417_41 #157 RT: 2.27 AV: 1 NL: 1.01E6
F: FTMS + p ESI Full ms [100.00-1000.00]
331.19031 z=?
331.28412 z=?
332.19354 z=?
330.27197 z=?
331.71594 z=?
333.19714 z=?
334.29495 z=?
MS (MB)
m/z

(h)


RT: 3.77
AA: 47179253
EIC (MA)
NL: 2.34E6
m/z=
315.19300-315.19800 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_47
RT: 2.31
AA: 2952528
EIC (MB)
NL: 1.69E5
m/z=
331.18800-331.19200 F:
FTMS + p ESI Full ms
[100.00-1000.00] MS
Avalon 190417_47
Relative Abundance
Time (min)
190417_47 #269 RT: 3.77 AV: 1 NL: 3.46E6
F: FTMS + p ESI Full ms [100.00-1000.00]
315.19504 z=1
316.28406 z=?
317.28732 z=?
313.27350 z=?
314.27673 z=?
318.29080 z=?
319.22614 z=?
321.24191 z=?
322.27322 z=1
MS (MA)
190417_47 #153 RT: 2.25 AV: 1 NL: 8.21E5
F: FTMS + p ESI Full ms [100.00-1000.00]
331.28415 z=?
331.19034 z=?
332.28741 z=?
332.19357 z=?
330.27194 z=?
333.29111 z=?
334.29535 z=?
MS (MB)
m/z

(i)

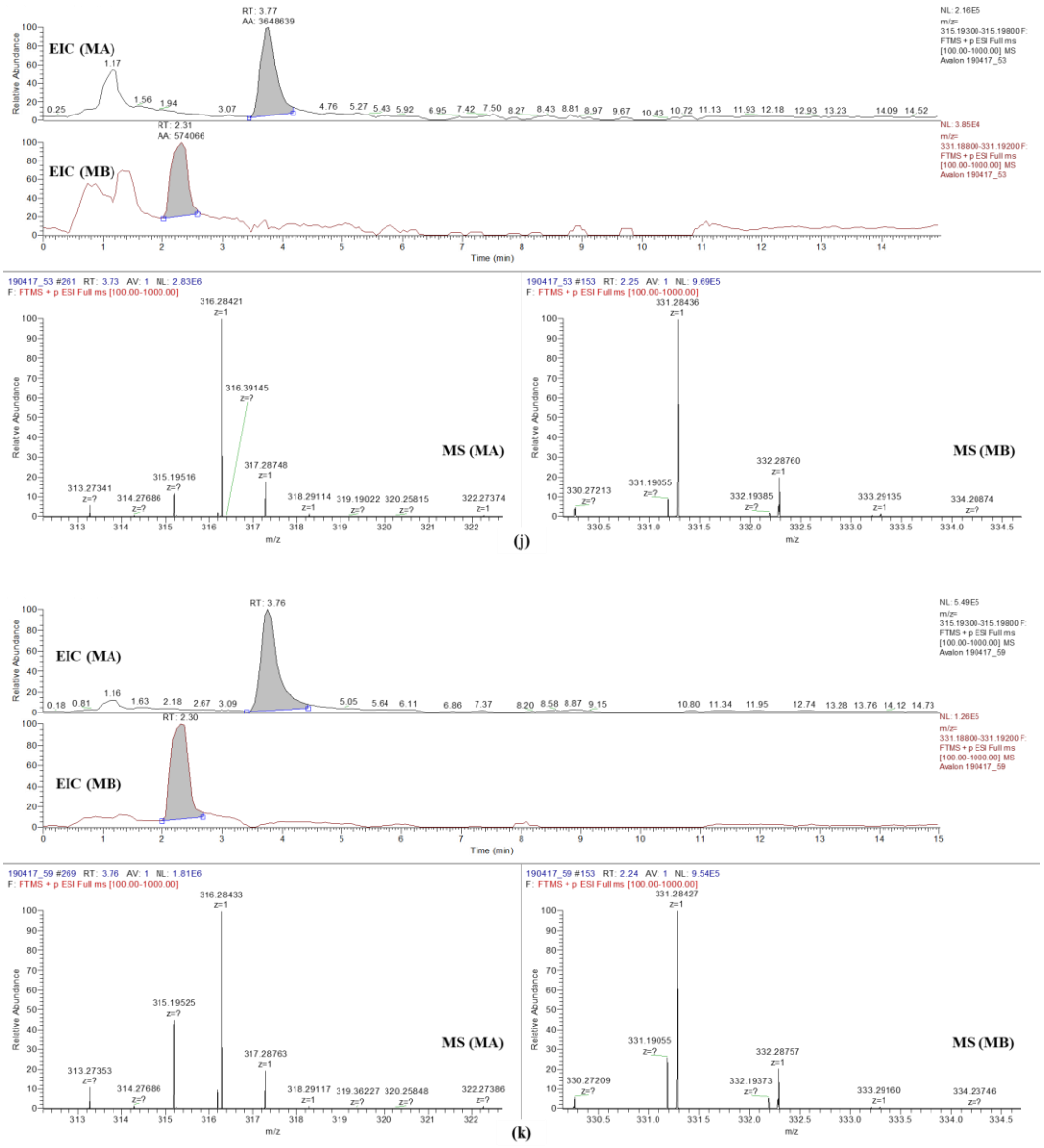


Figure S7. Extracted ion chromatograms (EIC) and mass spectra (MS) of MA and MB in SEP-PAK samples by UPLC-ESI-MS.

(a), standard MAB; (b), Ko: Koshihikari; (c), KoCo: cooked Koshihikari; (d), KT1: shinnosuke rice; (e), KT2: seiten no hekireki rice; (f), KT3: ginga no shizuku rice; (g), KT4: ho no mai; (h), Bin 9; (i), KD18: Khang dan 18; (j), ST24; (k), RVT.

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