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Tetrahydrofuran type-lignans from *Myristica fragrans* as AMP-activated protein kinase activators

Chosun University Graduate School

College of Pharmacy

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List of Abbreviations

$[\alpha]^{T}_{D}$: specific rotation

AMPK: AMP- activated protein kinase

ACC: Acetyl- CoA carboxylase

LKB1: Serine/threonine kinase 11

CaMKKs: calmodulin-dependent protein kinase kinases

DMSO: dimethyl sulfoxide

EtOH: Ethanol

MeOH: Methanol

HPLC: high performance liquid chromatography

HREIMS: high resolution electro impact mass spectroscopy

MS: mass spectrum

m/z: mass to charge ratio

NMR: nuclear magnetic resonance

ppm: parts per million

RP: reverse phase

UV: ultraviolet absorption

THF: tetrahydrofuran

HFD: high-fat diets

ND: normal fed diets

GPT: glutamate pyruvate transaminase

BUN: blood urea nitrogen

GLUC: glucose

CHOL: cholesterol

HDLC: high-density lipoprotein cholesterol

LDLC: low-density lipoprotein cholesterol

TRIG: triglyceride.

(국문 초록)

AMPK 활성화 물질로서 육두구로부터 분리한 테트라하이드로퓨란 형태의 리그난 화합물

레띠반뚜
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최근 고열량 음식의 소비와 생활습관에 기인한 비만의 급격한 증가는 당뇨병 및 대사증후군의 빠른 증가를 불러 왔다. AMP-activated protein kinase (AMPK) 효소는 세포 및 개체의 에너지 대사를 조절하는 효소로서 대사과정의 필수적인 조절자이다. 최근의 많은 연구는 운동을 모방한 효과를 보일 수 있는 AMPK 활성화 물질이 항비만, 항당뇨 및 대사증후군 질환의 강력한 약물 목표점으로 사용될 수 있음을 제시하고 있다.

본 연구는 사용한 육두구는 남아프리카, 인도, 적도지역에서 자라는 방향성 사철 관목이다. 육두구 (nutmeg)는 이러한 식물의 과실 핵의 껍질로서 12세기 유럽으로 향신료로서 도입된 이후 많은 서양 음식에서 사용되고 있다. 육두구는 또한 아시아에서 관절염, 근육경직, 식욕감퇴 및 장염치료제로서 오랫동안 처방되어 왔다. 본 실험실에서 새로운 AMPK 활성화 물질을 천연물로부터 발굴하려는 탐색과정에서 육두구 추출물이 분화된 근육전구세포인 C2C12 세포주에서 AMPK 효소를 활성화 함을 발견하였다.

활성물질로서 5-bis-aryl-3,4-dimethyltetrahydrofuran 리그난계 화합물 골격을 갖는 7종의 화합물을 크로마토그래피와 고압액체크로마토그래피 (HPLC)를 사용하여 분리하였다. 분광학적인 방법을 이용하여 결정한 화합물의 구조는 tetrahydrofuroguaiacin B (1), saucernetindiol (2), verrucosin (3), nectandrin

B (4), nectandrin A (5), fragransin C_1 (6)와 galbacin (7)로 확인되었다. 분리한 화합물 가운데 화합물 1, 4와 5는 AMPK 효소를 보다 강력히 활성화 함을 확인하였다. 또한, 넥탄드린 B를 주성분으로 함유한 리그난 분획의 항 비만효과가 C57BL6 마우스를 사용하여 연구되었다. 고지방식이를 먹인 마우스에서 테트라하이드로 퓨란 형태의 화합물을 포함한 추출물 분획은 체중의 증가와 지방조직의 증가를 저해함이 관찰되었으며, 이러한 효과는 AMPK 활성화 물질의 에너지대사과정의 조절에 기인한 것으로 판단된다.

육두구로부터 AMPK 활성화 물질로서 테트라하이드로 퓨란 형태의 화합물을 이용하는데 문제를 발생할 수 있는 것이 육두구에서 평균적으로 7 - 16% 정도 존재하는 희발성 오일속에 있는 미리스티신이다. 미리스티신은 희발성 오일의 4 - 8% 가량 존재하는 성분으로 섭취시 심각한 중독성과 환각증상을 유발한다. 최근의 보고에 의하면 육두구 5 g에 해당하는 kg당 1 - 2 mg의 미리스티신으로도 중독증상을 일으키는 예가 보고되었다. 따라서, 미리스티신의 함량을 줄이고 테트라하이드로 퓨란 형태의 화합물을 최적으로 포함하는 추출 및 분리방법의 개발이 또한 시도되었다.

ABSTRACT

Tetrahydrofuran type-lignans from *Myristica fragrans* as AMP-activated protein kinase activators

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In recent years, due to high fed diets and sedentary lifestyles, the upsurge in obesity has led to the increase in the prevalence of type 2 diabetes and other metabolic disorders. AMP-activated protein kinase (AMPK), a heterotrimeric enzyme complex that works as a fuel gauge to regulate cellular and whole body energy homeostasis, may act as a key player in metabolic control. Many studies indicate that AMPK activators, which mimic or potentiate the exercise-related effects, are regarded as potential candidates for development of anti-obesity, anti-diabetic agents as well as drugs for the treatment of other metabolic diseases.

Myristica fragrans Houtt. (Myristicaceae) is an aromatic evergreen tree cultivated in the South Africa, India and other tropical countries. After nutmeg, which refers to the dried kernels of this plant, was imported into Europe at the 12th century, it has long been indigenously used as a spice in many Western foods. Nutmeg is also prescribed for medicinal purposes in Asia to treat many diseases such as rheumatism, muscle spasm, decreased appetite, and diarrhea. In our program to search new AMPK activators from plants, we found that a total

extract of *M. fragrans* activated AMPK enzyme in differentiated C2C12 cells. As the active constituents, seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans, tetrahydrofuroguaiacin B (**1**), saucernetindiol (**2**), verrucosin (**3**), nectandrin B (**4**), nectandrin A (**5**), fragransin C_1 (**6**) and galbacin (**7**) were isolated from this extract. Among isolates, compounds **1**, **4**, and **5** showed a strong stimulation on AMPK enzyme. Moreover, anti-obesity property of this lignan-enriched extract was investigated by using C57BL/6 mice models. Our results revealed that tetrahydrofuran mixture extract prevented the increasing body weight and adipose tissue mass in mice fed with high fed diet (HFD). These effects may be obtained partly by whole-body energy regulation role of AMPK activators.

However, the seed of nutmeg yields average 7-16% volatile oil in which the content of myristicin is approximately 4-8%. Furthermore, severe intoxications and psychiatric effects were reported to occur after an ingestion of about 5 g of nutmeg corresponding to 1-2 mg myristicin/kg body weight. Hence, high contents of myristicin existed in nutmeg caused many difficulties in preparing extract to use as a functional food. For this reason, the processing conditions for preparing extract containing active compounds on AMPK from nutmeg and reducing myristicin contents were also suggested.

1. Introduction

1.1. Metabolic syndrome

The metabolic syndrome groups insulin resistance, hyperinsulinemia, hyperglycaemia, dyslipidaemia, high arterial blood pressure, central adiposity, and other abnormalities[1]. A variety of names have been associated with this condition: the pluri- metabolic syndrome, the insulin resistance syndrome, syndrome X, the dysmetabolic syndrome, the metabolic syndrome[1]… In 1988, Gerald Reaven was the first to give pathophysiological arguments for the existence of syndrome X. Following the description of the syndrome by Reaven, it has become a major theme of research and of public health interest.

In the absence of diagnostic test, a number of definitions of the syndrome have been proposed. Following criteria is the definition of the metabolic syndrome by World Health Organization.

WHO definition of the metabolic syndrome, 1999[2]
Glucose intolerance, IGT or diabetes mellitus and/or insulin resistance together with two or more of the other components listed below:
• Impaired glucose regulation or diabetes; • Insulin resistance (under hyperinsulinaemic, euglycaemic conditions, glucose uptake below quartile for background population under investigation); • Raised arterial pressure ≥ 140/90 mmHg; • Raised plasma triglycerides (≥ 1.7 mmol/l (1.5g/l) and/or low HDL-cholesterol (<0.9 mmol/l (0.35 g/l) men; < 1.0 mmol/l (0.39 g/l) women); • Central obesity (men: waist to hip ratio > 0.90; women: waist to hip ratio > 0.85 and/or BMI > 30 kg/m^2) • Microalbuminuria (urinary albumin excretion rate ≥ 20 µg/min or albumin : creatinine ratio ≥ 30 mg/g)

Consequences of the syndrome are diverse, including cardiovascular disease, hypertension, and chronic renal disease. Moreover, the metabolic disorders carry much higher risk for diabetes and obesity which are now becoming an epidemic in the developed and developing countries[1]. Today, more than 1.1 billion adults worldwide are overweight, and 312 million of them are obese. In the past 20 years, the rates of obesity have tripled in developing countries that have been adopting a Western lifestyle involving decreased physical activity and overconsumption of cheap, energy-dense food. In the developed world, 2 to 7% of total health care costs are attributable to obesity. In the United States alone, the combined direct and indirect costs of obesity were estimated to be $123 billion in 2001. The increase in the prevalence of type 2 diabetes is closely linked to the upsurge in obesity. About 90% of type 2 diabetes is attributable to excess weight. Furthermore, approximately 197 million people worldwide have impaired glucose tolerance, most commonly because of obesity and the associated metabolic syndrome. This number is expected to increase to 420 million by 2025[3].

The combination of the modern diet and sedentary lifestyle has resulted in an increase in obesity, type-2 diabetes, and other metabolic disorders. It is evident that if people would eat fewer calories and increase their activity level, this would reverse the abnormalities of the metabolic syndrome. However, compliance with such a strategy is clearly difficult. If society is not willing to make major lifestyle changes, are other interventions available? Some promising approaches have recently been introduced by the pharmaceutical industry. Recent studies indicate that AMPK may play an important role in exercise-related effects. Therefore, compounds activating AMPK, which have been claimed as “exercise in a pill”, are paid significant attention as potential drugs for the treatment of metabolic diseases.

1.2. AMP- activated protein kinase: a target for total metabolic control

AMP- activated protein kinase (AMPK) was originally discovered as an enzyme by its ability to inactivate HMG- CoA and acetyl- CoA carboxylase. When it became clear that the kinase had multiple physiolosical substrates, it was renamed AMPK after its allosteric activator.[4] AMPK is now known to exist as heterotrimeric complexes comprising a catalytic α subunit and regulatory β and γ subunits. The catalytic subunit contains a conventional serine/threonine protein kinase domain at the N- terminus and a C- terminal region that is required for the formation of the complex with the other two subunits. The β subunit contains a C-terminal domain that is required for complex formation and a central domain that is related to noncatalytic domains. The γ subunit contain variable N- terminal regions, followed by four tandem repeats of sequence known as a CBS motif, terms two Bateman domains, which bind ligans containing adenosine.[5]

AMPK is inactive unless phosphorylated by upstream kinases with the critical phosphorylation site being Thr^{172} within the activation loop of the kinase domain on the α subunit. As its name imply, AMP activates AMPK due to binding to the Bateman domains on the γ subunit via a complex mechanism involving three effects: (a) promotes phosphorylation by the upstream kinase; (b) causes allosteric activation of the phosphorylated kinase and (c) inhibits dephosphorylation of Thr^{172} by protein phosphatase. All three effects are also antagonized by binding of ATP, which binds to Bateman domains with a lower affinity than AMP and in a mutually exclusive manner. Because the adenylate kinase reaction is maintained close to the equilibrium in all eukaryotic cells, the cellular AMP: ATP ratio is a very sensitive indicator of compromised cellular energy status.[4]

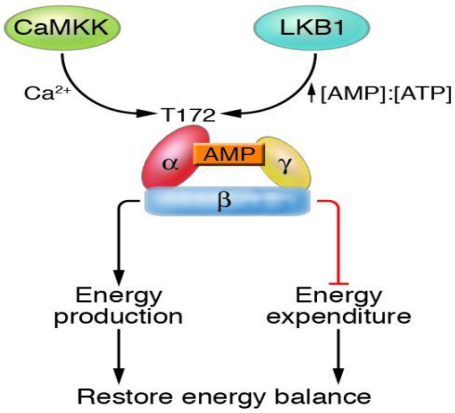


Fig. 1. Structure and regulation of AMPK[6]

The major upstream kinase of AMPK in most mammalian cells is a complex between the serine/threonine kinase 11 (LKB1) and two accessory subunits, STRAD and MO25. The LKB1 is a classical tumor suppressor and also acts upstream of at least 12 other AMPK-related kinase. The STRAD subunit is essential for the ability of the LKB1 complex to phosphorylate Thr^{172} on AMPK. The MO25 subunit contains a helical repeat that is distantly related to the armadillo proteins and appears to stabilize the LKB1-STRAD complex. Moreover, calmodulin-dependent protein kinase kinases (CaMKKs) which are upstream kinases for calmodulin-dephendent protein kinases I and IV would also phosphorylate and activate AMPK.[5]

Thus, phosphorylation of Thr^{172} in the activation loop of the catalytic α subunit is an absolute requirement for AMPK activity and is mediated by at least 2 upstream kinases: LKB1 and CaMKKs or by the increase of intracellular AMP: ATP ratio.[6] In general, activated AMPK switches off ATP-consuming anabolic pathways, switches on ATP-generating catabolic pathways, and reestablishes a proper energy balance in the cell.[5] Many downstream targets of AMPK have been identified to prove the role of AMPK in glucose and lipid metabolism, such as stimulating glucose uptake in muscle, suppressing hepatic glucose production, improving insulin sensitivity and reducing ectopic lipid accumulation.[7] Among them, AMPK regulation

on lipid homeostasis through acetyl- coA carboxylases (ACCs) is paid much attention because dys-regulation of fatty acid metabolism is strongly associated with the development of insulin resistance and type-2 diabetes.[8] AMPK-mediated phosphorylation and inactivation of ACC1 and ACC2 lead to the acute inhibition of fatty acid synthesis and increases fatty acid oxidation, respectively, and as a result, reduce triglyceride storage, lower plasma fatty acid and triglyceride levels.[7] AMPK not only plays an important role in mediating whole-body glucose and lipid homeostasis but also functions to regulate food intake, energy expenditure and control weight.[9] For all these reasons, AMPK activators are regarded as promising candidates for the discovery of anti-obesity, anti-diabetes agents as well as drugs for the treatment of other metabolic diseases.[5, 6, 7, 9]

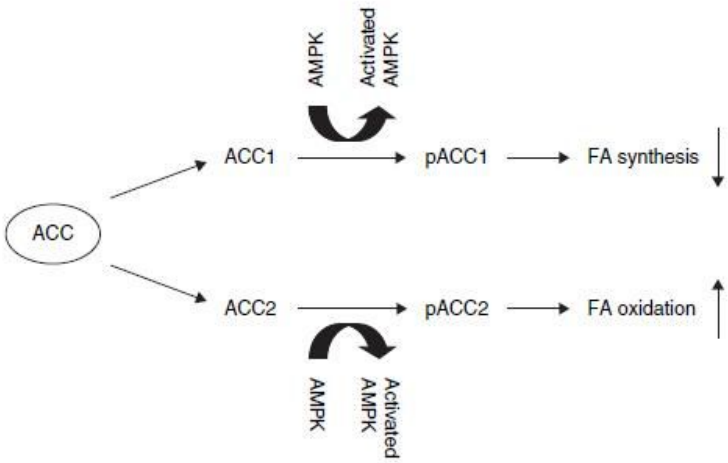


Fig. 2. Acetyl CoA carboxylase and fat metabolism[9]

1.3. *Myristica fragrans*

Myristica fragrans Houtt. belongs to the family Myristicaceae, with about 18 genera and 300 species. The genus *Myristica* is distributed from India and South-east Asia to North Australia and the Pacific Islands.[10] Nutmeg is a medium- sized tree reaching a height of 1-10 m. It is deoecious, with male and female flowers occurring on different trees. The fruit are pendulous, broadly pyriform, yellow smooth, 7-10 cm long freshly, splitting open into two halves when ripe, showing the avoid, 2-3 cm long, dark brown shining seeds, with hard seedcoat surrounded by a lanciate red aril attached to the base of the seed. The seed of nutmeg is large, with ruminative endosperm and is considered as the most primitive among the flowering plants. The first harvest of nutmeg trees is carried out 1-9 years after planting and the trees reach their full potential after 20 years.[11]

Two important spices are derived from the fruit - nutmeg and mace. Nutmeg is the dried kernel of the seed and mace is the dried aril surrounding it. Both the spices have similar flavour. However, nutmeg is reported to be slightly sweeter than mace and is more preferred in food. Besides nutmeg and mace, a number of other products, namely oleoresin, nutmeg butter and essential oils, are also derived from *M. fragrans*. These value-added products find varied use in the food, medicine, and perfume industries. World production of nutmeg is estimated to average between 10,000 and 12,000 ton per year, with annual world demand estimated at 9000 ton; production of mace is estimated at 1500–2000 ton. [10]

Nutmeg and mace has long been indigenously used as spice in many Western foods.[12] Traditionally, nutmeg has been used to treat digestive disorders, such as nausea, diarrhea, and kidney ailments. Southeast Asians also treat fevers, headaches and bronchial problems with nutmeg.[13] Pharmacological properties of nutmeg were reported as antimicrobial, insecticidal, hypolipidaemic, antioxidant, antiamoebic, nematicidal, antibacterial, antimicrobial, antifungal, anticancer, and anticariogenic activity.[10]

Nutmeg is reported to contain moisture, 14.3%; protein, 7.5%; ether extract, 36.4%; carbohydrates, 28.5%; fibre, 11.6%; and mineral matter, 1.7%; calcium, 0.12%; phosphorus, 0.24%; and iron, 4.6 mg/100g. It contains volatile oil (6-16%); starch (14.6- 24.2%); pentosans (2.25%); furfural (1.5%); and pectin (0.5- 0.6%), and other nutritional compositions.[10] The constituents of nutmeg can be classified broadly into terpenoids, aromaglycosides, sterol, fatty acids, phenolic acids, lignans, neolignans, and miscellaneous compounds. These secondary metabolites may account for the medicinal and pharmacological uses of nutmeg. However, the seed of *M. fragrans* yields average 7-16% volatile oil in which the content of myristicin is approximately 4-8%.[14, 15] It is well known that this natural accurring compound produced

neurotoxic effect. Severe intoxications and psychiatric effects were reported to occur after an ingestion of about 5 g of nutmeg corresponding to 1-2 mg myristicin/kg body weight.[16, 17] Hence, high contents of myristicin existed in nutmeg caused many difficulties in preparing extract containing active compounds to use as a functional food. This study concentrates on the isolation and structure determination of the AMPK activators from *Myristica fragrans*, and also simple processing procedure for reducing myristicin contents in the extract.

2. Materials and Methods

2.1. Materials

2.1.1. Plant

The dried semens of *Myristica fragrans* Houtt. (Myristicaceae) were purchased at a folk medicine market in Gwangju city, Republic of Korea. The sample was identified by Professor YH Moon at Chosun University, and its specimen (No. 0010) has been deposited at the Department of Pharmacy, Chosun University, Republic of Korea.

2.1.2. Chemicals, reagents, and chromatography

Column chromatography was conducted on silica gel (Merck, 63 – 200 μm particle size) and reversed phase (ODS-A, Merck, 120 μm particle size) from Merck. TLC was carried out with silica gel 60 F254 plates from Merck. The solvent for NMR analysis was purchased from CIL (Cambridge Isotope Lab., USA). Myristicin standard compound for HPLC analysis was purchased from Fluka (09237), and HPLC solvents were from Burdick & Jackson, USA.

AICAR was purchased from Sigma Chemical Company (St Louis, MO, USA), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA). Antibodies against phospho AMPKα Thr^{172}, phosphospecific ACC Ser^{79}, *β*-actin, anti-mouse, and anti-rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA)

2.1.3. General experimental procedures

UV spectra were taken in MeOH using a Shimadzu spectrometer, and the optical rotations were obtained in MeOH using a Rudolph Autopol IV polarimeter. The nuclear

magnetic resonance (NMR) spectra were obtained on Varian Unity Inova 500 MHz spectrometer at Korea Basic Science Institute (KBSI, Gwangju Center, Korea). EIMS and HREIMS data were performed on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. HPLC was carried out using a Gilson System with UV detector and an RP-C18 column (10 × 250 mm, 10 μm particle size, RS Tech Optima Pak C18 column, Korea).

2.2. Methods

2.2.1. Cell culture

Mouse C2C12 skeletal myoblasts were maintained in DMEM supplemented with 10% fetal bovine serum in an atmosphere of 95% air and 5% CO_2 at 37 ^{o}C. To prepare for each assay, cells were seeded in 12-well plates, 10^5 cells/well in 2 mL growth medium. Differentiation of C2C12 myoblasts was induced by replacing growth medium with DMEM containing 5% horse serum when the cells were confluent. The medium were changed every 48 h until the formation of myotubes. Cells were used in experiments at 4-5 days after differentiation.

2.2.2. AMPK assay by Western blot analysis

C2C12 myotubes were incubated with appropriate concentration of compounds for 30 minutes and then lysed in EBC lysis buffer [50 mM Tris-HCl (pH 7.6), 120 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% NP-40, and 50 mM sodium fluoride]. Cell debris was removed by centrifugation at 12,000 rpm for 15 min, at 4 ^{o}C. Protein concentrations in the cell lysates were determined using a Bio-rad protein assay kit. About 30 μg proteins of total cell extracts were subjected to western blot analysis using anti-phosphospecific AMPKα Thr^{172}, anti-phosphospecific ACC Ser^{79}. β-Actin protein levels were used as a control for equal protein

loading. The immunoreactive antigen was then recognized by using a horseradish peroxidase-labeled anti-rabbit IgG and an enhanced chemiluminescence detection kit.[18]

2.2.3. HPLC analysis of active compounds and myristicin under different extraction conditions

2.2.2.1. Characterization of major constituents and myristicin

The major compounds and myristicin in each extract were analyzed by a Gilson HPLC with the 321-pumps systems; UV/Vis-155; 234-autoinjector; an OptimaPak C18 column (4.6 × 250 mm, partical size 5 μm), using a gradient of MeOH (solvent A) and 0.1% formic acid in H_2O (solvent B) as mobile phase. The gradient program was set as follows: flow rate 1 ml/min, 0–40 min (50→70% A), 40–52 min (70→100% A), 52–60 min (100% A). The UV absorptions were detected at 205 and 280 nm. Retention time (t_R, min) for the major compounds: **1** (macelignan, 50.3), **2** (*meso*-dihydroguaiaretic acid, 39.8), **3** [(±)-*trans*-dehydrodiisoeugenol, 34.6)], **4** (nectandrin B, 23.1), **5** (licarin A, 36.9), **6** (otobaphenol, 54.7) and **7** (myristicin, 29.6). (see Supporting Information).

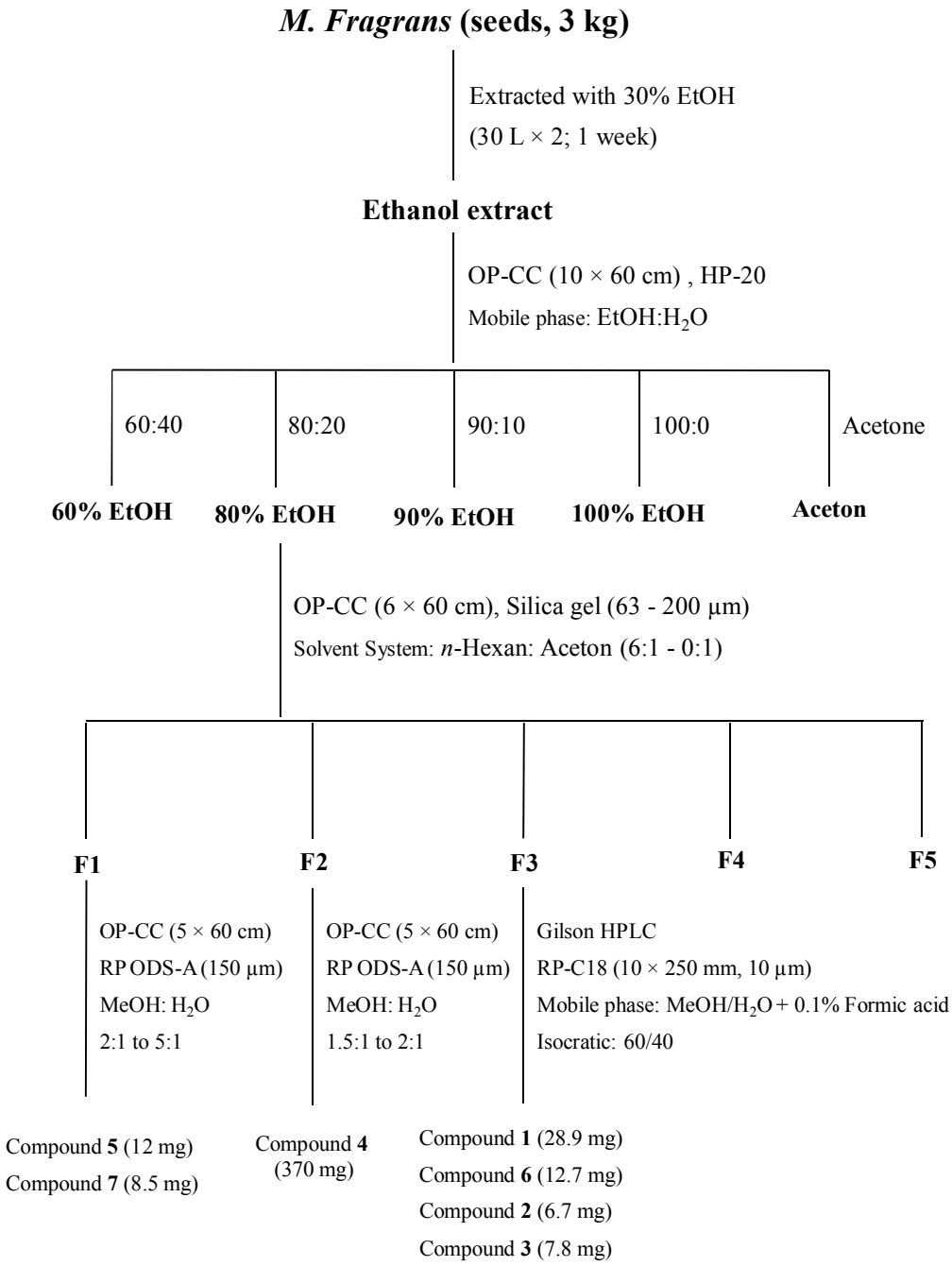
2.2.2.1. Characterization of tetrahydrofuran-type compounds

The tetrahydrofuran-type compounds were analyzed by a Gilson HPLC using a gradient of MeOH (solvent A) and 0.1% formic acid in H_2O (solvent B) as mobile phase, with an OptimaPak C18 column (4.6 × 250 mm, partical size 5 μm), flow rate 1 mL/min. The UV absorptions were detected at 205 and 280 nm. The gradient program was set as follows: 0–35 min (60% A), 35–60 min (60→100% A), 60–66 min (100% A). Retention time (t_R, min) for compounds **1** (tetrahydroguaiacin B, 23.9), compound **2** (saucernetindiol, 27.5), compound **3** (verrucosin, 28.9), compound **4** (nectandrin B, 31.8), compound **5** (nectandrin A, 47.5), compound **6** (fragransin C_1, 25.3), and compound **7** (galbacin, 58.3).

2.2.4. Extraction and isolation of active compounds on AMPK from *Myristica fragrans*

The seeds of *M. fragrans* (3 kg) were extracted with 30% EtOH at room temperature for 7 days. The 30% ethanol-soluble extract was filtered, and then directly subjected on a Diaion HP-20 column (10 × 60 cm), eluted with H_2O/EtOH (40:60, 20:80, 10:90, 0:100, each 3 L), and finally washed by acetone (2 L) to give five fractions. Bioassay of five fractions on AMPK revealed that the 80% ethanol-eluted fraction was most active. This was further chromatographed over silica gel (6 × 60 cm; 63–200 μm particle size) using a gradient of *n*-hexane/acetone (from 6:1 to 0:1), to yield five fractions (F.1 – F.5) according to their TLC profiles. Compound **4** (370 mg) was purified from a part of fraction 2 by chromatography on a reversed phase ODS-A column (5.0 × 60 cm, 150 μm particle size) eluted with MeOH/H_2O (1.5:1, to 2:1, each 3 L). Further chromatography of fraction 1 on a reversed phase ODS-A column (5.0 × 60 cm, 150 μm particle size) eluted with MeOH/H_2O (2:1, 3:1 to 5: 1, each 2.5 L), resulted in the isolation of compound **5** (12 mg) and compound **7 (**8.5 mg). Purification of fraction 3 by semi-preparative Gilson HPLC systems [using RS Tech OptimaPak C18 column (10 × 250 mm, 10 μm particle size); mobile phase MeOH/H_2O (60:40); flow rate 2 mL/min; UV-detections at 205 and 280 nm] resulted in the isolation of compound **1** (28.9 mg, t_R 33.4 min), compound **6** (12.7 mg, t_R 37 min), compounds **2** (6.7 mg, t_R 39.5 min), and compound **3** (7.8 mg, t_R 43.5 min), respectively.

Scheme 1. Isolation of compounds (**1** – **7**) from the *M. fragrans*



Tetrahydrofuroguaiacin B (**1**): Colorless oil; HREIMS *m/z* 344.1624 (calcd for $C_{20}H_{24}O_5$, 344.1614); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

Saucernetindiol (**2**): Colorless oil; $[\alpha]_D^{25}$ +14.8° (*c* 1.38, $CHCl_3$); HREIMS *m/z* 344.1624 (calcd for $C_{20}H_{24}O_5$ 344.1616); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

Verrucosin (**3**): Colorless oil; $[\alpha]_D^{25}$ +14.8° (*c* 1.38, $CHCl_3$); HREIMS *m/z* 344.1676 (calcd for $C_{20}H_{24}O_5$ 344.1622); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

Nectandrin B (**4**): Colorless oil; $[\alpha]_D^{25}$ –2° (*c* 0.3, MeOH); HREIMS *m/z* 344.1610 (calcd for $C_{20}H_{24}O_5$, 344.1624); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

Nectandrin A (**5**): Colorless oil; $[\alpha]_D^{25}$ +14.8° (*c* 1.38, $CHCl_3$); HREIMS *m/z* 358.1715 (calcd for $C_{21}H_{26}O_5$ 358.1728); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

Fragransin C_1 (**6**): Colorless oil; $[\alpha]_D^{25}$ +3.7° (*c* 0.4, MeOH); HREIMS *m/z* 377.1715 (calcd for $C_{21}H_{26}O_6$ 374.1728); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

Galbacin (**7**): Colorless oil; HREIMS *m/z* 340.1311 (calcd for $C_{20}H_{20}O_5$ 340.1322); ^{1}H NMR (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$) data, see **Table 1** and **2**.

2.2.5. Processing procedures for reducing myristicin contents from extracts of *Myristica fragrans*

The crunched seeds of *M. fragrans* were extracted and fractionated in different conditions. The concentration of active substance and myristicin content of every fraction obtained in each condition was analyzed using Gilson HPLC system as described above. Processing procedure to reduce myristicin content in nutmeg extract was set up according to the result of HPLC analysis of chemical substances and bioassay-guided fractionation.[13]

First of all, the crunched seeds of *M. fragrans* were extracted with 30%, ethanol in water. This 30% ethanol soluble extract was applied to a Diaion HP-20 column, and then eluted with 80% ethanol. Bioassay on AMPK activity revealed that the 80% ethanol-eluted fraction, which contained high content of active substance and low level of myristicin, exhibited strongest activity.

2.2.6. Animal experiments

Thirty of eight-week-old male C57BL/6 mice were housed in plastic cages in a temperature-controlled (22 ± 1 °C) room and maintained on a reverse 12h light/dark cycle. The mice were randomly divided into three groups: normal diet group (ND), high-fat diet group (HFD) with daily gavage of vehicle as control; and HFD plus 200 mg/kg, i.g. tetrahydrofuran mixture-fed group (HFD + THF). The HFD (DIO series diets, Research Diets Inc, USA) was based on a modified Western diet and contained 21% (*w/w*) lard and 0.15% (*w/w)* cholesterol.[19] Tetrahydrofuran mixture was administered for 6 weeks, and weight gain was measured once a week. Food intake was measured for three consecutive days per week by subtraction of food jar pre- and post-weights for 6 weeks, and the mean values were used as the daily food intake. At necropsy, both sides of the inguinal and epididymal adipose tissues were removed and weighed,

and the relative adipose tissue weight to body weight was calculated. All animal experiments were approved by the Institutional Animal Care and Use Committee, and were performed in accordance with the institutional guidelines at the AmorePacific Corporation, Korea.

3. Results

3.1. Structure determination of tetrahydrofuran type-lignans from *M. fragrans*

3.1.1. Structure determination of compound 1

Compound **1** was obtained as colorless oil with the molecular formula $C_{20}H_{24}O_5$, as determined by the high resolution mass spectrum [HREIMS *m/z* 344.1624 (calcd for $C_{20}H_{24}O_5$, 344.1614)]. The ^{1}H and ^{13}C NMR spectra of compound **1** showed the presence of twelve protons and ten carbon atoms (**Table 1** and **2**). This suggested the symmetric nature of the molecular structure of compound **1**, with each signal in the ^{1}H and ^{13}C NMR spectra corresponding to two identical sets of protons and carbon atoms, respectively. Furthermore, the ^{1}H NMR spectral data of compound **1** showed characteristic signals arising from the tetrasubstituted tetrahydrofuran ring system comprising of two secondary methyl groups at δ_H 0.61 (2H, d, J=6.0 Hz), a two proton multiplet at δ_H 2.65 (2H, m) due to the H-3, H-4 methine protons and two protons doublet at δ_H 5.12 (2H, d, J=6.6 Hz) due to the two oxymethine protons, H-2 and H-5.[20] The coupling constant 6.0 Hz of the doublet at δ_H 0.61 for 3-methyl and 4-methyl suggested that these two methyl groups are in a *cis*-configuration with the adjacent protons H-2 and H-5, respectively[21]. The chemical shifts observed for aromatic hydrogen along with the presence of one singlet at δ_H 3.87 (6H, s) corresponding to two aromatic methoxyl groups and one broaden peak at δ_H 5.59 (2H, br, s) of two hydrogen of phenyl groups. The remaining six aromatic protons indicated the presence of two sets of 3-methoxy-4-hydroxyphenyl systems. ^{13}C NMR spectrum corroborated the assignments made for the structural determination of both aromatic rings. Comparison of spectroscopic data and physiochemical values between compound **1** and published data[22] led the structure of compound **1** to be tetrahydrofuroguaiacin B.

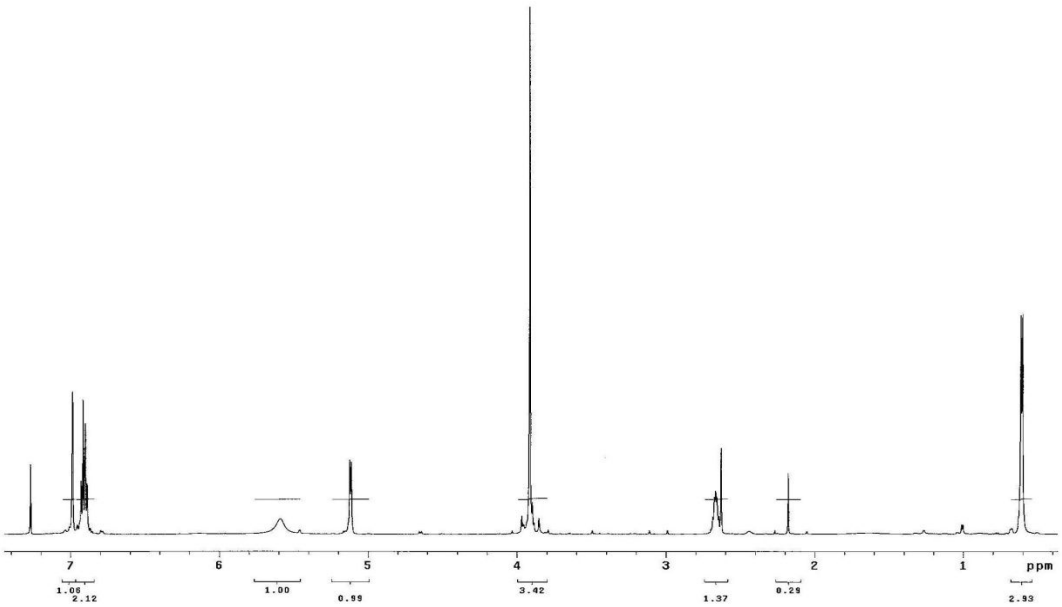


Fig. 3. ^{1}H-NMR spectrum of compound **1** (600 MHz, $CDCl_3$)

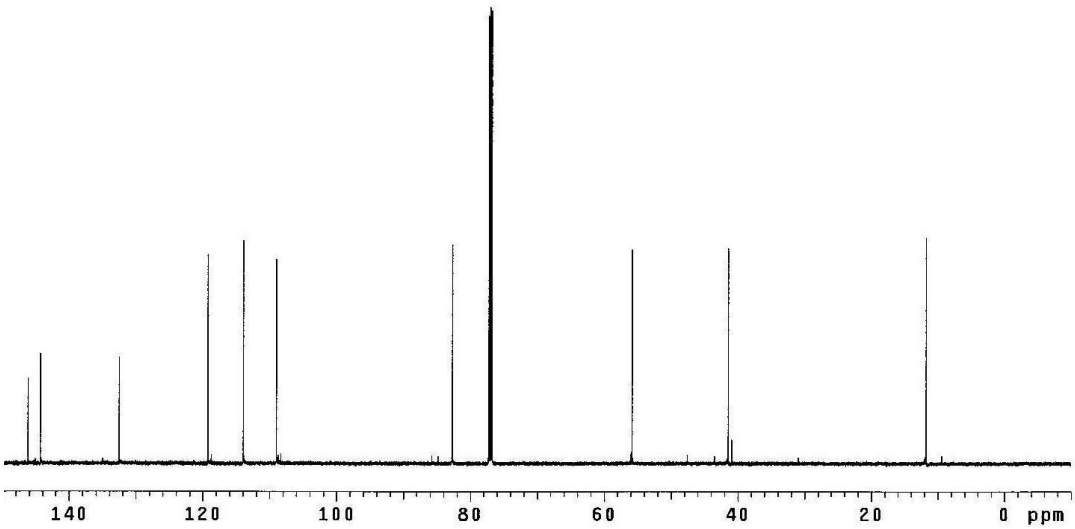


Fig. 4. ^{13}C-NMR spectrum of compound **1** (150 MHz, $CDCl_3$)

3.1.2. Structure determination of compound 2

Compound **2** was purified as colorless oil with the molecular formula $C_{20}H_{24}O_5$, as determined by the high resolution mass spectrum [HREIMS *m/z* 344.1624 (calcd. for $C_{20}H_{24}O_5$, 344.1614)]. Comparison of its ^{1}H NMR and ^{13}C NMR spectral data with saucerneol D and saucerneol D indicated that **2** is a tetrahydrofuran-type sesquineolignan.[23] The ^{1}H NMR spectrum of **2** showed two methine groups at δ_H 2.33 (2H, m) coupled with oxymethine gropus at δ_H 4.65 (1H, d, J=6.6) and δ_H 5.46 (1H, d, J=6.6) as well as with methyl groups at δ_H 0.63 (1H, d, J=6.6) and δ_H 1.01 (1H, d, J=6.6), respectively. These signals were assigned to those protons in the asymmetric 3,4-dimethyl-2,5-disubstituted tetrahydrofuran moiety of **2**. Since the two methyl groups at the 3,4 positions appeared at two different chemical shift values in ^{1}H NMR spectrum, it was deduced that the two methyls are in the trans-configuration. The ^{1}H and ^{13}C NMR spectroscopic data showed the presence of two 1,3,4 trisubstituted benzene rings. The presence of two aromatic methoxy groups was established by the appearance of two sharp singlets at δ_H 3.90 (s, 3H) and δ_H 3.92 (s, 3H). The remaining aromatic protons indicated the presence of two sets of 3-methoxy-4-hydroxyphenyl systems. ^{13}C NMR spectrum corroborated the assignments made for the structural determination of both aromatic rings. Compound **2** was determined as saucernetin diol by ^{1}H and ^{13}C NMR spectroscopic data analyses, and comparison with those published in literatures.[24]

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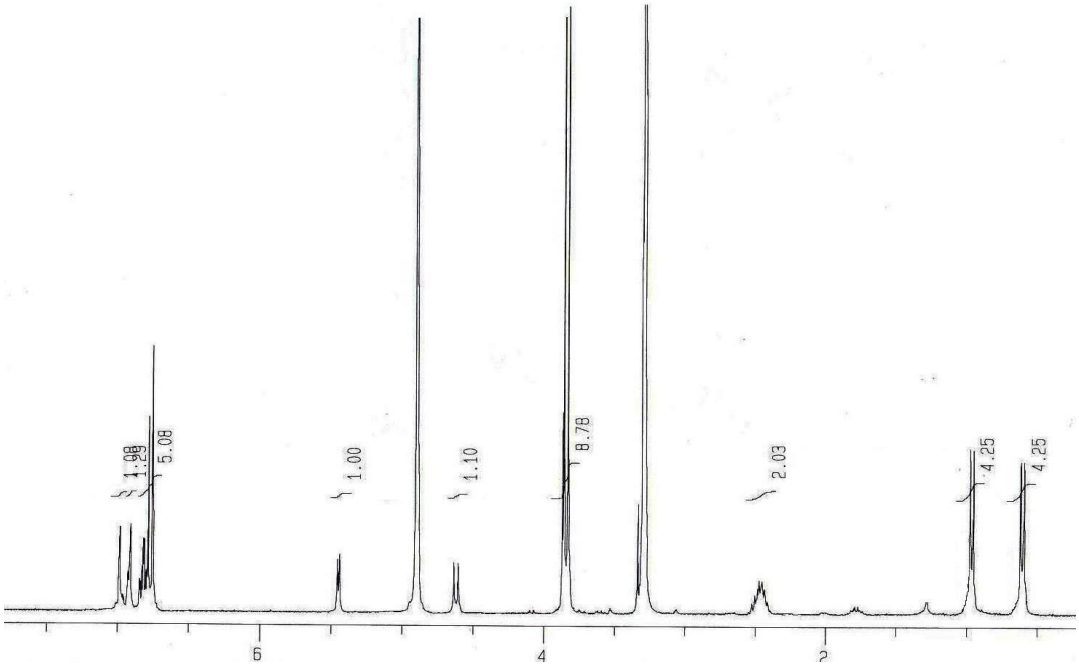


Fig. 5. ^{1}H-NMR spectrum of compound **2** (300 MHz, MeOH)

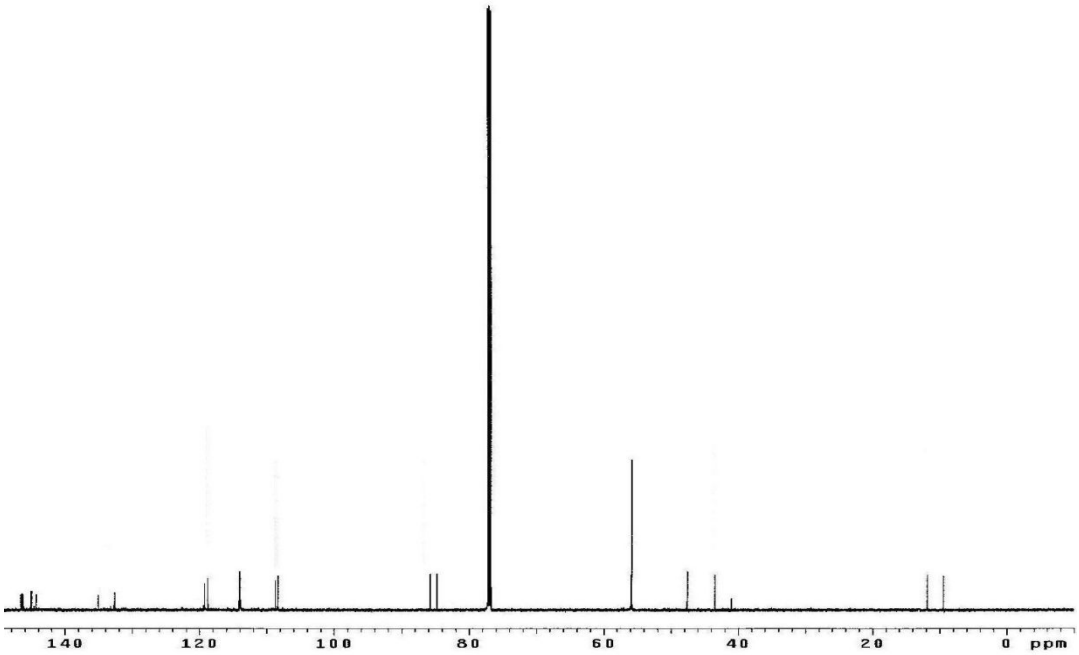


Fig. 6. ^{13}C-NMR spectrum of compound **2** (150 MHz, $CDCl_3$)

3.1.3. Structure determination of compound 3

Compound **3** was isolated as an oily substance with the molecular formula $C_{20}H_{24}O_5$, isomeric with **1** and **2**. The ^{1}H-NMR spectra of compound **3** was characteristic of non-symmetric tetrahydrofuran lignans since it exhibited a pair of doublets at δ_H 0.65 (3H, d, J=6.6) and δ_H 1.01 (3H, d, J=6.6) corresponding to methyl groups, a second set of doublets at δ_H 5.10 (1H, d, J=9.0) and δ_H 4.36 (1H, d, J=9.6) that were assignable to oxybenzyl methines, and multiplets at δ_H 2.24 (1H, m) and δ_H 1.78 (1H, m) associated with the H-3 and H-4 protons respectively. As previously reported, the coupling constants of 9.0 and 9.6 Hz for the doublets at δ_H 5.10, and δ_H 4.36 for H-2 and H-5 indicated that these hydrogen are in *trans* configuration with the adjacent H-3 and H-4 in a tetrahydrofuran ring. The ^{13}C NMR spectroscopic data also corroborated the presence of the tetrahydrofuran system. Analysis of the ^{1}H-NMR spectrum suggested that compound **3** exhibited two aromatic methoxyl groups at δ_H 3.87. The remaining six aromatic protons indicated the presence of two sets of 3-methoxy-4-hydroxyphenyl systems. Thus, the ^{1}H-NMR spectrum was indicative of a veraguensin type of tetrahydrofuran lignan but with two 4-hydroxy-3-methoxyphenyl groups.[25] Compound **3** was determined as verrucosin by ^{1}H and ^{13}C NMR spectroscopic data analyses, and comparison with those published in literatures.[26]

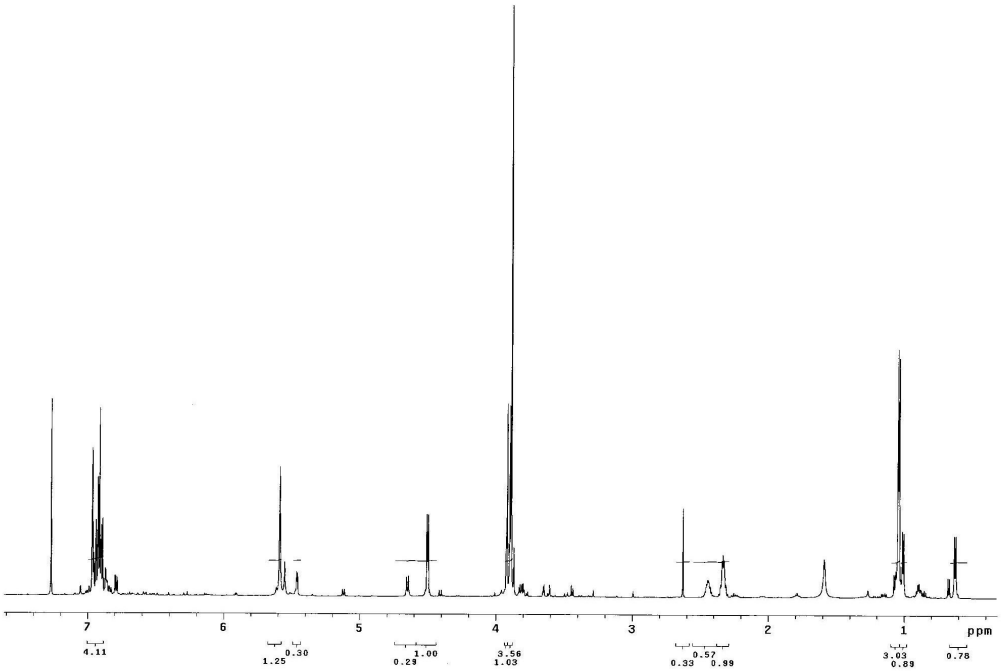


Fig. 7. ^{1}H-NMR spectrum of compound **3** (600 MHz, $CDCl_3$)

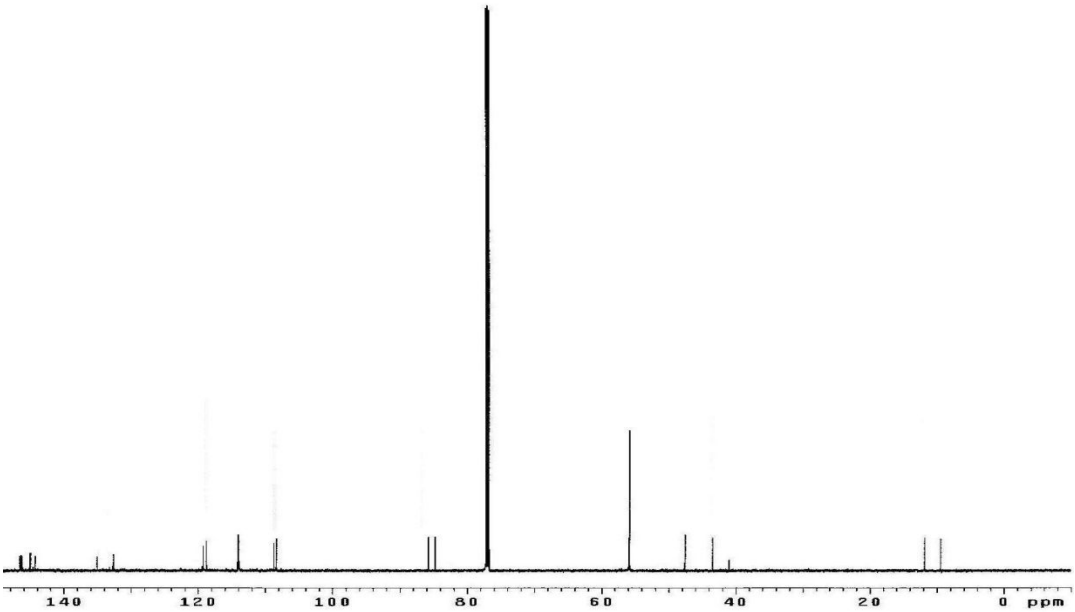


Fig. 8. ^{13}C-NMR spectrum of compound **3** (150 MHz, $CDCl_3$)

3.1.4. Structure determination of compound 4

Compound **4** was isolated as an oily substance with the molecular formula $C_{20}H_{24}O_5$ by the HREIMS [*m/z* 344.1610 (calcd for $C_{20}H_{24}O_5$, 344.1624)]. The ^{1}H and ^{13}C NMR spectra of compound **4** showed the presence of only twelve protons and ten carbon atoms also suggested the symmetric structure. Thus each signal in the ^{1}H and ^{13}C NMR spectra must represent two identical atoms. The presence of a 3, 4- dimethyl tetrahydrofuran moiety in **4** was indicated by signals at δ_H 4.53 (2H, d, J=6.6), δ_H 2.35 (2H, m) and δ_H 1.05 (6H, d, J=6.6). These signals due to the methyl, methine, and benzylic methine protons were essentially similar in chemical shifts and coupling constants to those of galgravin-type lignan.[25] The coupling constant J = 6.6 Hz of the doublet at δ_H 4.53 showed that the protons at 2, 5 were in *trans* configuration with the protons at 3 and 4, respectively. However, the two methyl at C-3 and C-4 were shifted to down field [δ_H 1.05 (6H, d, *J*=6.6 Hz)] and two protons at C-2 and C-5 were shifted to up field [δ_H 4.53 (2H, d, *J*=6.6 Hz)] compared with those of **1**. These observations further confirmed that H-2 and H-5 are in a *trans*-configuration with the adjacent protons H-3 and H-4, respectively. In addition, the ^{13}C NMR data also gave additional evidence for the presence of tetrasubstituted tetrahydrofuran moiety with trisubstituted benzene rings in compound **4.** The aromatic signals of ^{1}H NMR also supported 1,3,4 substitution of the benzene rings. The sharp singlet at δ_H 3.85 (6H) and δ_C 55.6 showed the presence of two methoxy groups in identical environments attached to aromatic rings. Phenolic hydroxyl groups were appeared as a broad single peak at δ_H 5.96. From the spectroscopic data and physiochemical values between **4** and published data[26], compound **4** was determined to be nectandrin B.

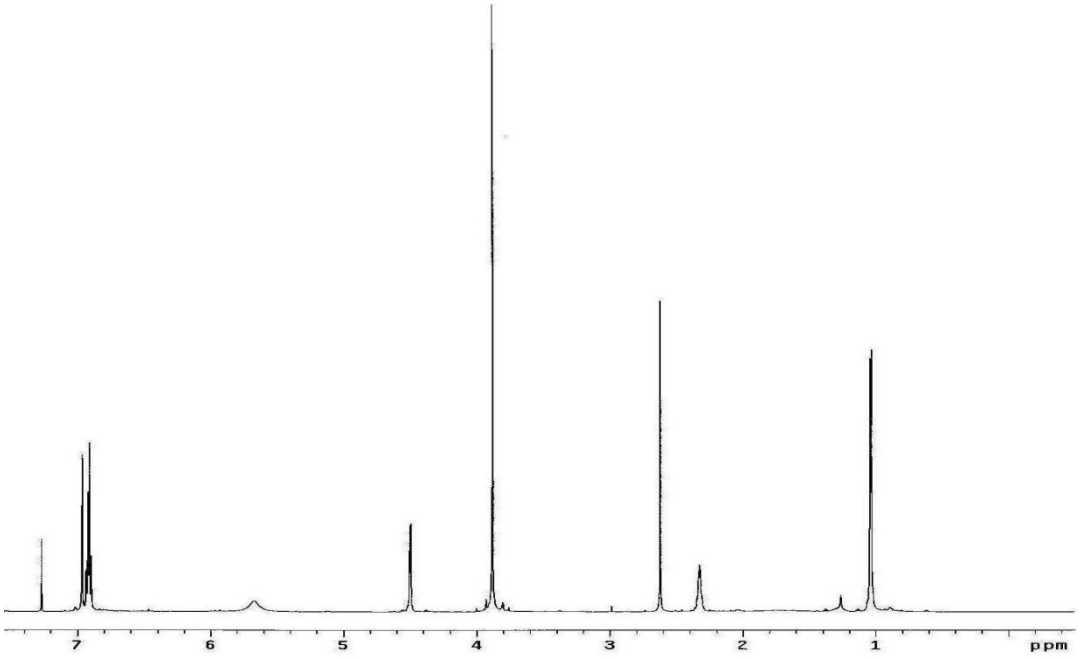


Fig. 9. ^{1}H-NMR spectrum of compound **4** (600 MHz, $CDCl_3$)

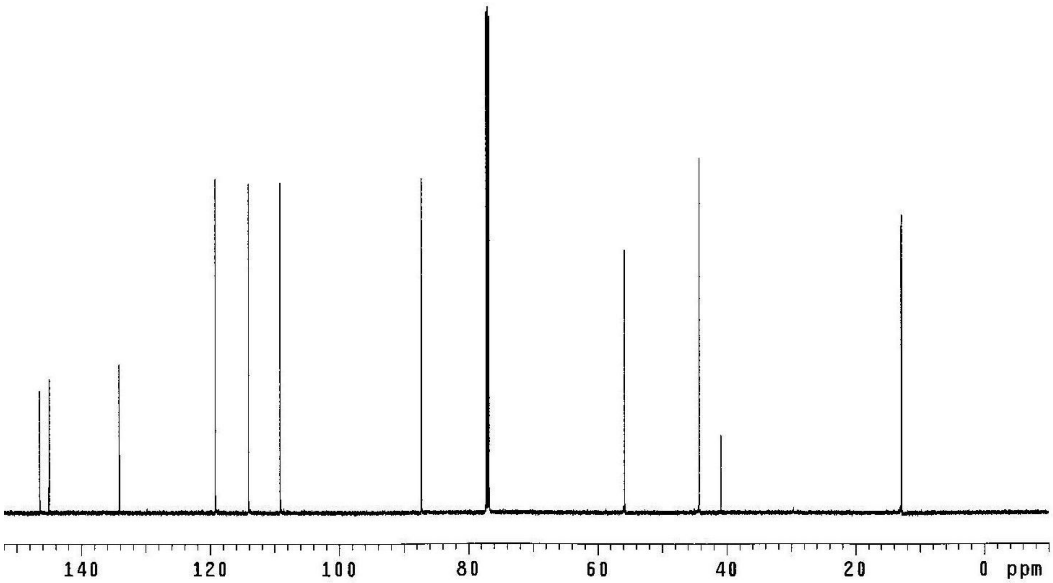


Fig. 10. ^{13}C-NMR spectrum of compound **4** (150 MHz, $CDCl_3$)

3.1.5. Structure determination of compound 5

Compound **5** was isolated as colorless oil with the molecular formula $C_{21}H_{26}O_5$ by the high resolution mass spectrum [HREIMS *m/z* 358.1715 (calcd for $C_{21}H_{26}O_5$ 358.1728)]. Comparison of the ^{1}H and ^{13}C NMR spectra of compound **5** with those of compound **4** indicated the presence of additional methoxyl group in compound **5** [δ_H 3.82 (3H, s); δ_C 56.2]. By the comparison of the ^{1}H and ^{13}C NMR spectral data of compound **5** with those published in literature[27], it was identified as nectandrin A.

3.1.6. Structure determination of compound 6

Compound **6** had the molecular formula $C_{21}H_{26}O_6$. The mass spectrum showed a molecular ion peak at *m/z* 374 and intense fragment ions at *m/z* 192 and 122. The ^{13}C NMR spectrum showed the presence of 4-hydroxy-3-methoxyphenyl and 4-hydroxy-3,5-dimethoxyphenyl groups. The ^{1}H NMR chemical shifts of methyl, methine and benzylic methine protons were similar to those of galgravin-type lignan.[25] The signal patterns, however, were rather complex due to presence of two different aryl substituents in the molecule. Signals due to two *sec*-methyls [δ_H 1.04 (3H, d, J = 6.6) and δ_H 1.06 (3H, d, J = 7.2)], two methines [δ_H 2.32 (1H, m); δ_H 2.34 (1H, m)]; and two benzylic methines substituted by oxygen [δ_H 4.50 (1H, d, J = 7.2) and δ_H 4.52 (1H, d, J = 7.2)] were seen. This known compound was characterized as fragransin C_1 by comparing their physicochemical values, ^{1}H and ^{13}C NMR spectra, and MS data with those published in literature.[22, 26, 27]

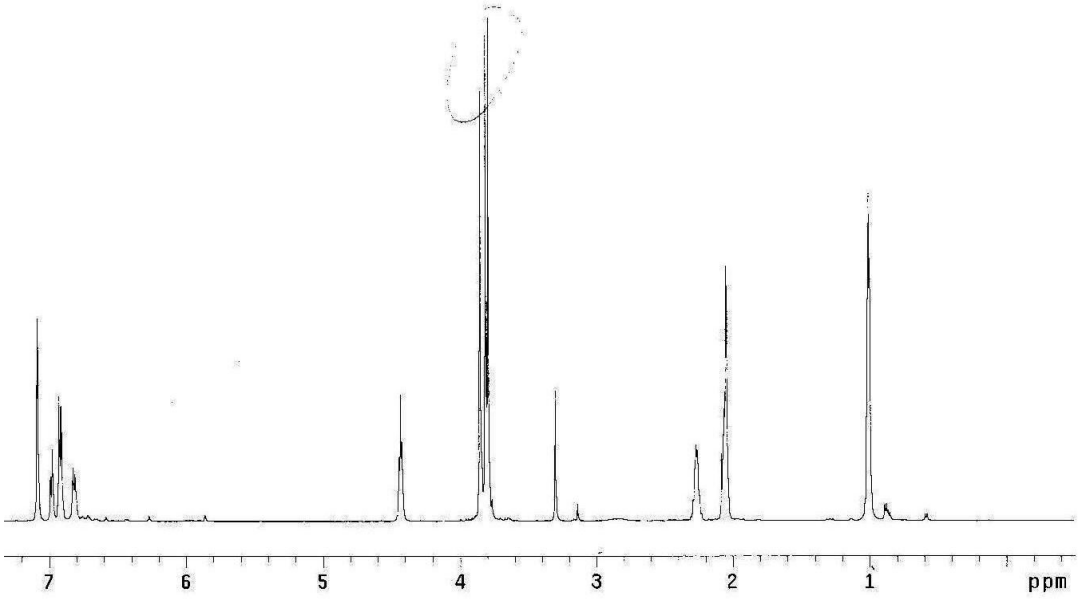


Fig. 11. ^{1}H-NMR spectrum of compound **5** (500 MHz, $CDCl_3$)

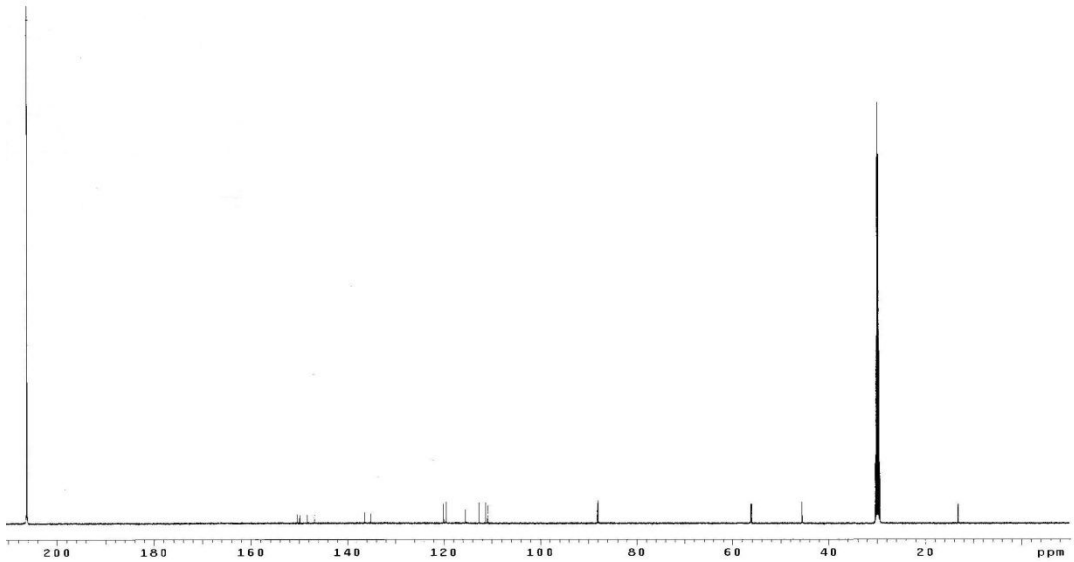


Fig. 12. ^{13}C-NMR spectrum of compound **5** (125 MHz, $CDCl_3$)

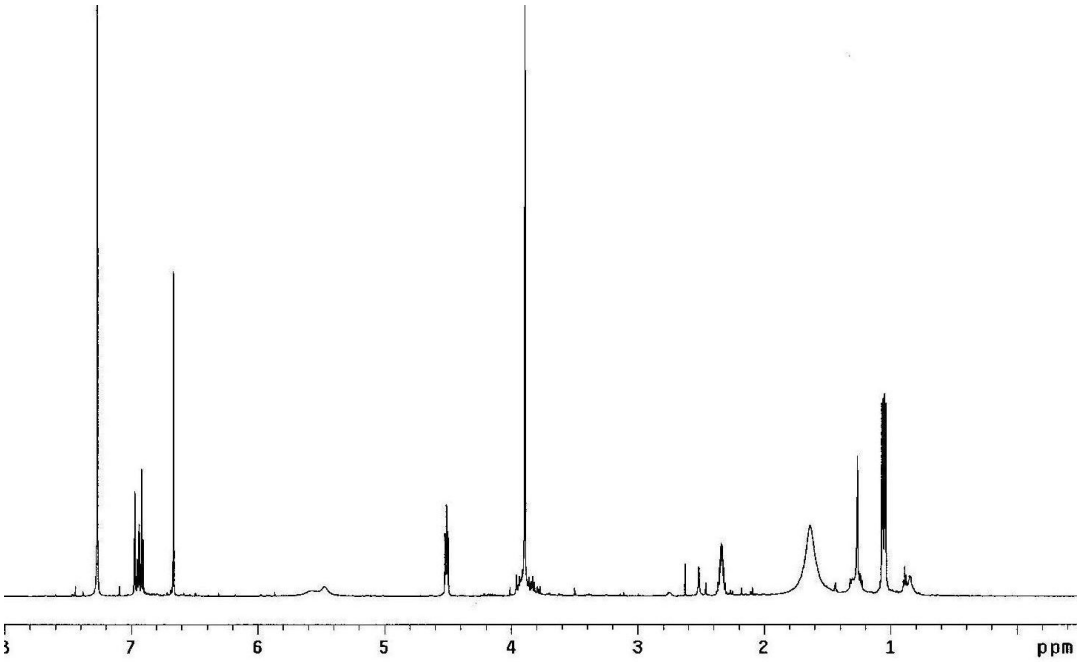


Fig. 13. ^{1}H-NMR spectrum of compound **6** (600 MHz, $CDCl_3$)

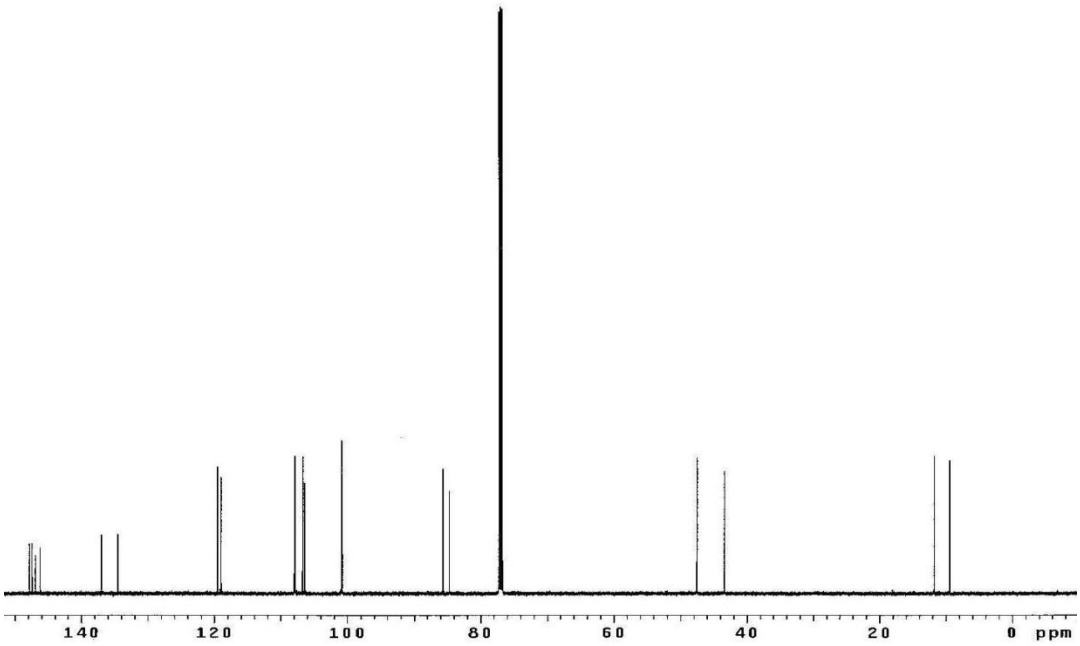


Fig. 14. ^{13}C-NMR spectrum of compound **6** (125 MHz, $CDCl_3$)

3.1.7. Structure determination of compound 7

Compound **7** had the molecular formula $C_{20}H_{20}O_5$. The ^{1}H NMR spectra of **7** showed absorption of oxybenzylic protons H-2 and H-5 [δ_H 5.45 (1H, d, J= 9.0) and δ_H 4.61 (1H, d, J=9.6)], methyl protons 3-Me and 4- Me [δ_H 0.97 (1H, d, J=6.6) and δ_H 0.6 (1H, d, J=7.2)], and methine protons [δ_H (2H, m)] related to a tetrahydrofuran moiety. The coupling constants of 9.0 and 9.6 Hz for the doublets at δ_H 5.45, and δ_H 4.61 for H-2 and H-5 indicated that these hydrogen are in *trans* configuration with the adjacent H-3 and H-4 in a tetrahydrofuran ring. Since the two methyl groups at 3, 4 positions appeared at two different chemical shift values in ^{1}H NMR spectrum of **7**, it was deduced that the two methyls were in *trans*-configuration. The presence of two methylenedioxy groups was established by the appearance of two sharp singlet at δ_H 5.93 (2H, s) and δ_H 5.94 (2H, s) in the ^{1}H NMR. This known compound was characterized as galbacin by comparing their physicochemical values, ^{1}H and ^{13}C NMR spectra, and MS data with those published in literature.[22, 27]

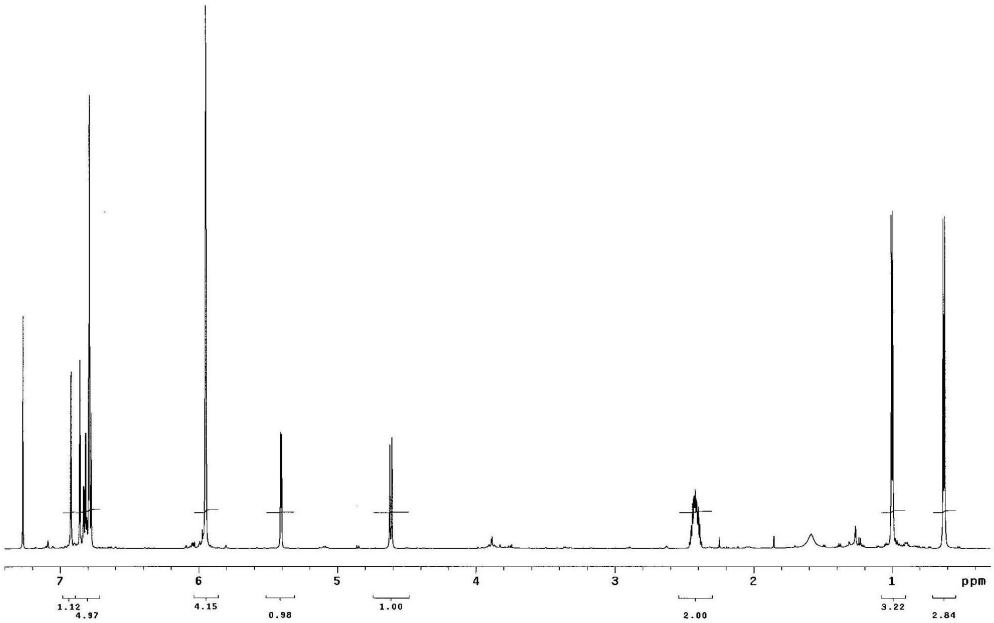


Fig. 15. ^{1}H-NMR spectrum of compound **7** (500 MHz, $CDCl_3$)

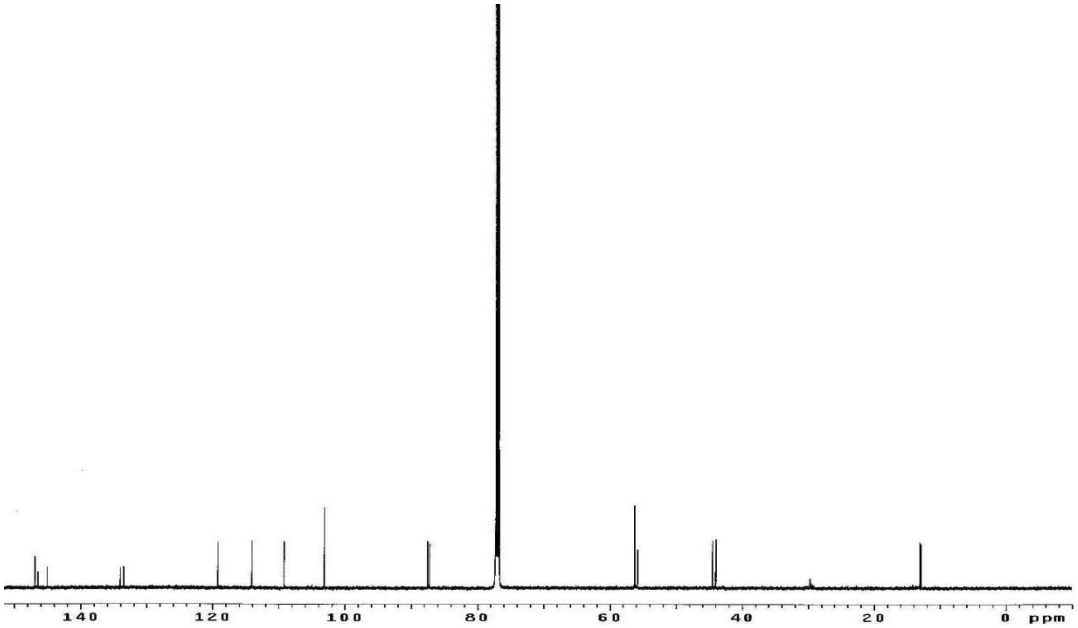


Fig. 16. ^{13}C-NMR spectrum of compound **7** (125 MHz, $CDCl_3$)

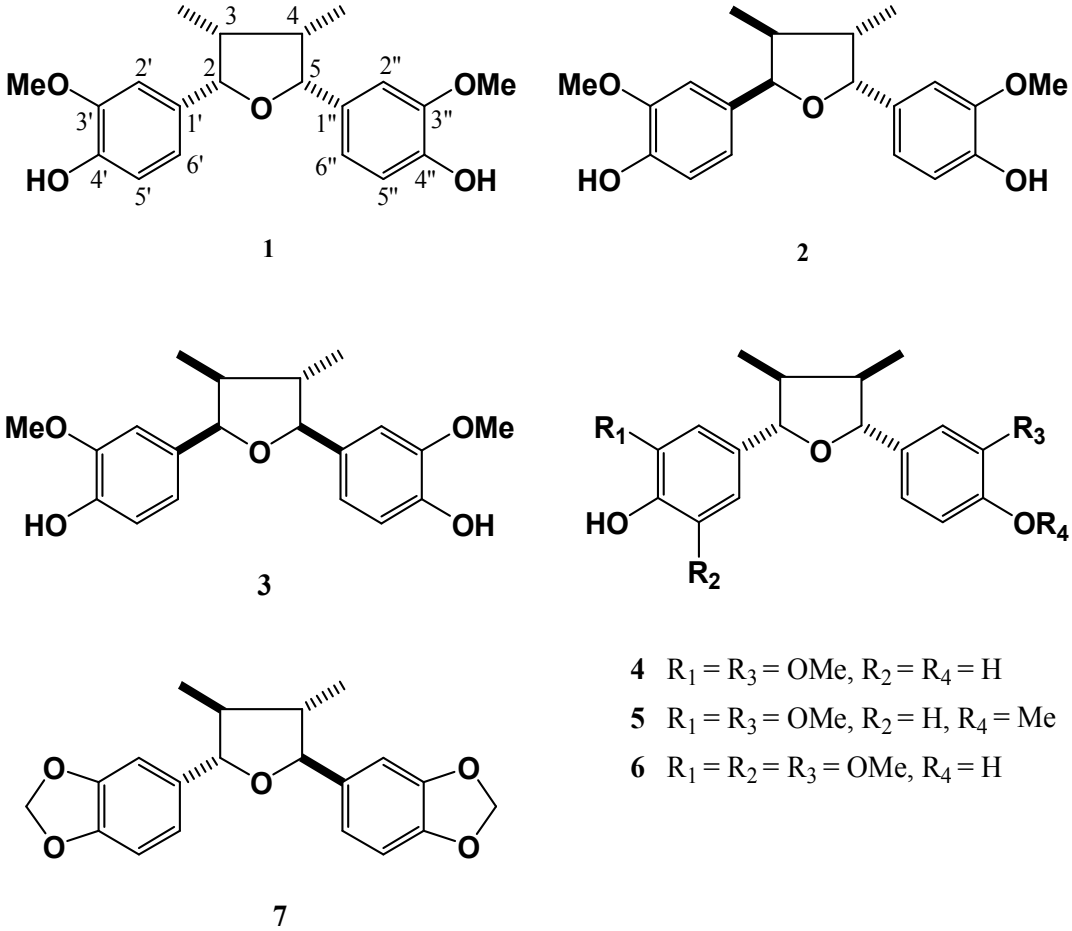


Fig. 17. Chemical structures of compounds **1–7** isolated from *M. fragrans*.

Table 1. ^{1}H (600 MHz) data of isolated compounds **1−7** from *M. fragrans* in $CDCl_3$

No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)	δ_H (*J* in Hz)
1							
2	5.12, d, 6.6	4.65, d, 6.6	5.10, d, 9.0	4.53, d, 6.6	4.43, d, 7.8	4.50, d, 7.2	5.45, d, 9.0
3	2.65, m	2.33, m	2.24, m	2.35, m	2.27, m	2.32, m	2.43, m
4	2.65, m	2.33, m	1.78, m	2.35, m	2.27, m	2.34, m	2.43, m
5	5.12, d, 6.6	5.46, d, 6.6	4.36, d, 9.6	4.53, d, 6.6	4.44, d, 7.2	4.52, d, 7.2	4.61, d, 9.6
1′							
2′	6.99, d, 1.8	6.92, d, 2.4	7.07, d, 1.8	6.99, d, 1.8	7.09, br, s	6.67, br, s	6.93, d, 1.8
3′							
4′							
5′	6.90, d, 8.4	6.77, d, 8.4	6.78, d, 8.4	6.92, d, 8.4	6.92, d, 8.4	6.91, d, 7.8	6.78, d, 8.4
6′	6.92, dd, 1.8, 8.4	6.80, dd, 2.4, 8.4	6.93, dd, 1.8, 8.4	6.98, dd, 1.8, 7.8	6.98, dd, 1.8, 9.0	6.70, br, s	6.84, dd, 1.8, 8.4
1″							
2″	6.99, d, 1.8	6.92, d, 2.4	7.07, d, 1.8	6.99, d, 1.8	7.09, br, s	6.97, d, 1.2	6.93, d, 1.8
3″							
4″							
5″	6.90, d, 8.4	6.77, d, 8.4	6.78, d, 8.4	6.91, d, 7.8	6.92, s, 8.4	6.91, d, 7.8	6.78, d, 8.4
6″	6.92, dd, 1.8, 8.4	6.80, dd, 2.4, 8.4	6.93, dd, 1.8, 8.4	6.93, dd, 1.8, 7.8	6.81, dd, 1.2, 8.4	6.94, dd, 1.2, 7.8	6.84, dd, 1.8, 8.4
3-Me	0.61, d, 6.0	1.01, d, 6.6	1.01, d, 6.6	1.05, d, 6.6	1.02, d, 3.6	1.04, d, 6.6	0.97, d, 6.6
4-Me	0.61, d, 6.0	0.63, d, 6.6	0.65, d, 6.6	1.05, d, 6.6	1.00, d, 3.6	1.06, d, 7.2	0.60, d, 7.2
3′-OMe	3.91, s	3.90, s	3.87	3.85, s	3.80, s	3.88, s	
4′-OH	5.59, br, s			5.96, br, s		5.58, br, s	
5′-OMe						3.88, br, s	
3″-OMe	3.91, s	3.92, s	3.87, s	3.85, s	3.82, s	3.88, s	
4″-OH	5.59, br, s			5.96, br, s		5.47, br, s	
4″-OMe					3.86, s		
$-OCH_2O-$							5.93, s 5.94, s

Table 2. ^{13}C NMR (150 MHz) data of isolated compounds **1−7** from *M. fragrans* in $CDCl_3$

No.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)		δ_C (ppm)	δ_C (ppm)
1							
2	82.7	84.8	84.6	87.2	88.2	87.2	87.4
3	41.5	43.4	43.5	44.1	45.5	44.0	44.7
4	41.5	47.6	47.8	44.1	45.6	44.5	44.7
5	82.7	85.8	89.0	87.2	88.0	87.5	86.3
1′	132.5	132.6	133.0	133.9	136.4	134.0	138.0
2′	109.0	108.3	115.7	114.1	115.5	109.2	108.8
3′	146.2	146.6	148.6	146.4	150.4	146.9	149.3
4′	144.3	146.3	146.8	144.9	146.9	145.1	148.9
5′	113.9	113.9	111.9	109.2	111.3	146.9	107.8
6′	119.3	119.3	120.9	119.1	111.9	103.1	121.0
1″	132.5	135.0	133.8	133.9	135.1	133.5	135.8
2″	109.0	108.7	116.1	114.1	112.7	109.2	108.8
3″	146.2	145.0	149.0	146.4	149.9	146.9	147.9
4″	144.3	144.3	147.5	144.9	148.3	146.4	148.6
5″	113.9	114.0	111.6	109.2	110.9	114.1	107.8
6″	119.3	118.8	120.7	119.1	120.0	119.3	120.2
3-Me	11.8	9.4	14.8	12.7	13.1	12.9	11.9
4-Me	11.8	11.8	15.2	12.7	13.2	13.1	9.8
3′-OMe	55.8	56.0	56.3	55.6	56.2	56.3	
3″-OMe	55.8	55.8	56.4	55.6	56.3	55.8	
4″-OMe					56.1		
5″-OMe						56.3	
$-OCH_2O-$							102.3 102.5

3.2. Processing procedures for reducing myristicin contents from extracts of *Myristica fragrans*

Because the bioactivities of *M. fragrans* have been linked to its major compounds[28, 29, 30, 31], we evaluated the difference of major constituents of nutmeg by using various solvent extraction. Thus, 100 g crushed nutmeg was extracted three times in an ultrasonic extractor with 500 mL of different solvent systems for 2 hours. Eight different solvent systems were used to give different extracts: water extract, 10%, 20%, 30%, 40%, 50%, 75% ethanol-soluble extract and 75% methanol-soluble extract. The concentrations of active substances of each extract were analyzed using Gilson HPLC system. According to the data obtained (**Table 3**), the 30% ethanol-soluble extract (500 g) of nutmeg, which contains the highest concentration of nectandrin B and lowest level of myristicin, was then applied to a Diaion HP-20 column (6.0 × 60 cm), eluted with H_2O/EtOH (40:60, 20:80, 10:90, 0:100, each 1 L), and finally washed by acetone (2 L) to separated into five fractions. Immunoblot analysis on phospho-AMPK Thr^{172} revealed that 80% ethanol-eluted fraction was most active. This result was well correlated with HPLC analysis which showed that fraction eluted with 80% ethanol contains high concentration of nectandrin B (26.01%) without myristicin (0% found). However, fraction eluted with 90% ethanol contains highest concentration of nectandrin B (61%) and low content of myristicin (0.61%).

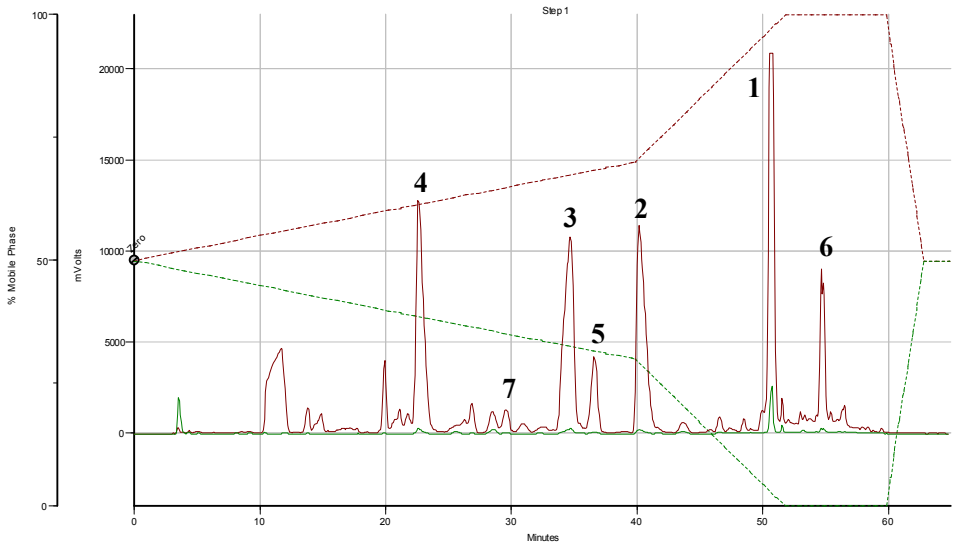


Fig. 18. A representative HPLC profile of some major lignans (**1**–**6**) and myristicin from the total MeOH extract of *Myristica fragrans* with detections at 205 and 280 nm.

Key to peak identity: (**1**) macelignan, (**2**) *meso*-dihydroguaiaretic acid, (**3**) (±)-*trans*-dehydrodiisoeugenol, (**4**) nectandrin B, (**5**) licarin A, (**6**) otobaphenol, and (**7**) myristicin. (For chromatographic protocol see Experiments and Methods).

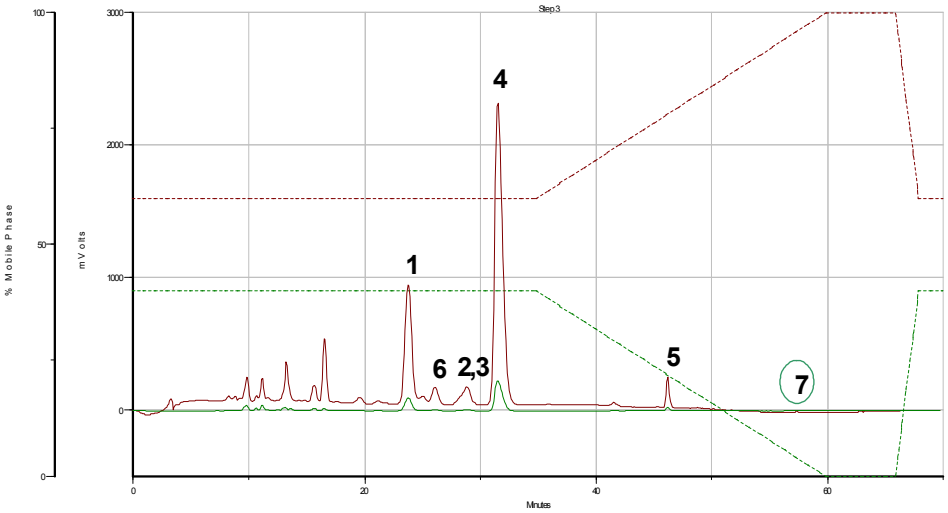


Fig. 19. A representative HPLC profile of isolated lignans **1**−**7** from the 80% EtOH fraction of the 30% EtOH-soluble extract of *Myristica fragrans* with detections at 205 and 280 nm. Key to peak identity: (**1**) tetrahydrofuroguaiacin B, (**2**) saucernetindiol, (**3**) verrucosin, (**4**) nectandrin B, (**5**) nectandrin A, (**6**) fragransin C1, and (**7**) galbacin. (For chromatographic protocol see Experiments and Methods).

Table 3. Contents of nectandrin B and myristicin in each solvent extract of *M. fragrans*

Compounds	**Fractions**							
	Water	10% EtOH	20% EtOH	30% EtOH	40% EtOH	50% EtOH	75% EtOH	75% MeOH
nectandrin B	0.62%	0.94%	2.98%	8.69%	1.95%	2.77%	2.95%	7.47%
myristicin	0.19%	0.31%	0.33%	0.51%	1.34%	1.48%	1.27%	1.80%

Table 4. Contents of nectandrin B and myristicin in each solvent fraction from 30% EtOH-soluble extract of *M. fragrans*

Compounds	**Fractions**			
	80% EtOH	90% EtOH	100% EtOH	100% Acetone
nectandrin B	26.01%	61.02%	14.52%	–
meso-dihydro guaiaretic acid	–	2.99%	62.49%	13.78%
macelignans	–	–	–	16.57%
myristicin	–	0.61%	3.61%	21.66%

3.3. Effect of tetrahydrofuran type-lignans from *M. fragrans* on AMPK activation

Compounds **1**−**7** were tested using an *in vitro* assay to investigate their stimulation effects on AMPK. Among them, compounds **1**, **4**, and **5** showed the most potential activation, exhibiting strong stimulation at the concentration of 5 μg/mL, the others showed remarkable activation at the concentration of 20 μg/mL on this enzyme assay. Detailed investigation of the chemical and biological properties of the isolates indicated that tetrahydrofuran lignans with symmetric structures (compound **1**, **4**, and **5**) exhibited higher stimulation effects on AMPK activity rather than the asymmetrical ones (compound **2**, **3**, and **6**; see **Fig. 17** and **20** for more information). Furthermore, chemical structure investigations of the isolates showed that all of the compounds had (4′- and 4″-OH), and or (3′- and 3″-OCH_3) in two aromatic rings (compounds **1**−**6**), excepted for compound **7**, which were replaced by two methylenedioxyl moieties in the structure. Although chemical structure of compound **7** was symmetric, the stimulation effect was shown to be lowest effect at the concentration of 20 μg/mL (**Fig. 20A**). These findings suggested that the methylenedioxyl groups may be decreases the activity, and the methoxyl moieties may produce significant increase of the activation on AMPK activity of these tetrahydrofuran lignans. The stereochemistry of the methyl groups, and of the protons at position C-3, and C-4 with the adjacent protons H-2 and H-5, respectively are also important to the AMPK activity of tetrahydrofuran-type lignans. Indeed, except for compound **7**, compounds **4** and **5** which had *trans*-configuration showed higher activity than the others with *cis*-configuration (compound **2** and **3**) in the structures at both concentrations of 20 and 5 μg/mL (**Fig. 20A** and **20B**).

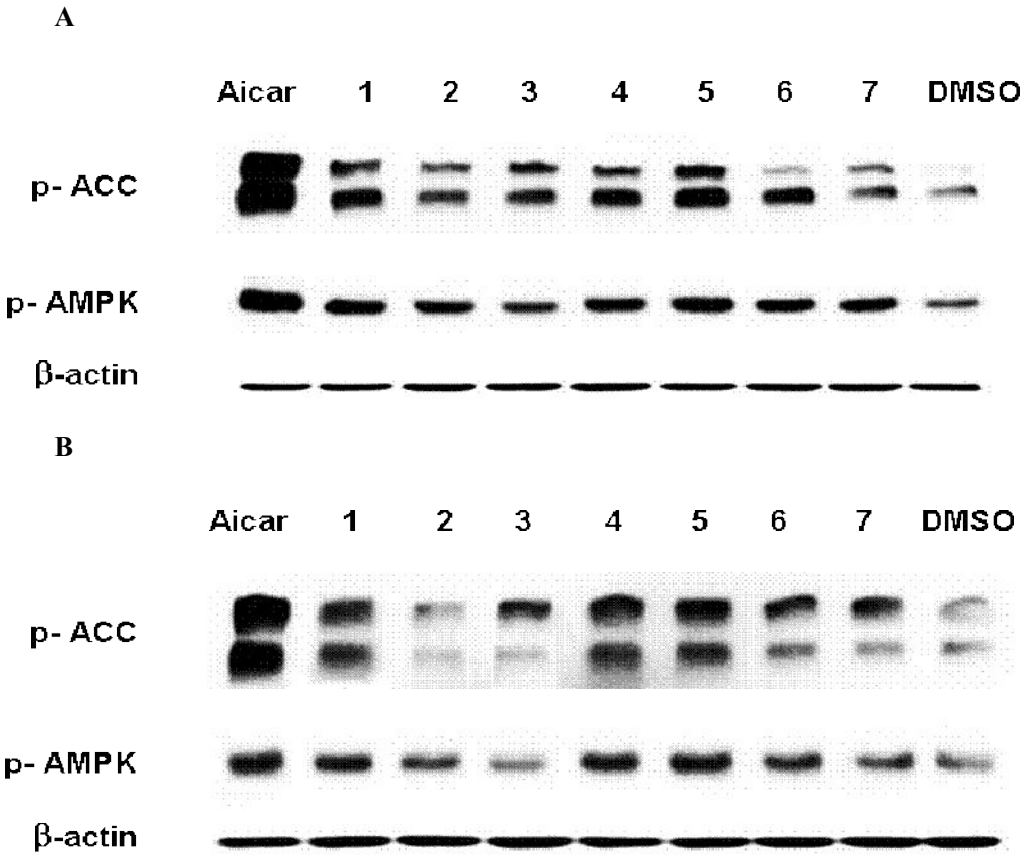


Fig. 20. Stimulatory effects of isolated lignans **1**−**7** on AMPK activity.

(**A**) The stimulatory effects of isolated compounds **1**−**7** on AMPK activity at the concentration of 20 μg/mL.

(**B**) The stimulatory effects of isolated compounds **1**−**7** on AMPK activity at the concentration of 5 μg/mL.

3.4. Animal experiment

The phenotypic characterizations of HFD-induced C57BL/6 mice treated with or without THF mixture and ND mice are shown in **Table 5**. Blood chemistry revealed that THF had effect in lowering the level of plasma glucose and LDL in diet induced obesity mice. All group of mice started with similar mean body weights (20.37 ± 0.21 g), and average body weight of HFD mice after six weeks treatment were shown to increase of nearly 7.8 g more than those of ND mice. The tetrahydrofuran mixture administration groups significantly lowered the body weights than those of the HFD group (**Fig. 21A**) without any difference in food intakes between the two groups. These results suggested that tetrahydrofuran mixture from *M. fragrans* could switch on ATP-generating catabolic pathways by fatty acid oxidation for a proper energy supply in the cell. After 6-week treatment, the contents of epididymis fat on mice of HFD and HFD + THF were compared. **Fig. 21B** illustrated that relative epididymis fat weight of THF fed mice decreased 22% compared to those of HFD mice. These results suggested that AMPK activators from *M. fragrans* exerted preventive effect in diet-induced obesity in vivo through regulating glucose and lipid homeostasis.[5, 9]

Table 5. Characterization of ND mice as control, and HFD mice treated with or without THF for six weeks.

Blood chemistry	ND (n = 10)	HFD (n = 10)	HFD + THF (n = 10)
GPT	48.14 ± 15.22	81.84 ± 67.63	68.06 ± 56.29
BUN	25.93 ± 3.09	23.89 ± 1.77	22.74 ± 1.89
GLUC	159.33 ± 28.44	232.7 ± 23.39*	199.10 ± 29.89**
CHOL	115.73 ± 17.16	170.91 ± 15.35*	153.46 ± 22.06
HDLC	90.64 ± 14.52	125.48 ± 5.40*	125.98 ± 12.39
LDLC	22.78 ± 2.54	32.20 ± 3.97*	25.90 ± 2.47**
TRIG	99 ± 15.64	88.40 ± 14.83	79.70 ± 9.88
Kidney weight (g)	0.29 ± 0.03	0.31 ± 0.02	0.29 ± 0.02***
Liver weight (g)	1.04 ± 0.10	1.01 ± 0.08	0.95 ± 0.12***
Epididymis fat (g)	0.46 ± 0.06	1.74 ± 0.53	1.24 ± 0.40**
Food intake (g/day)	2.71 ± 0.08	2.24 ± 0.05	2.20 ± 0.07***

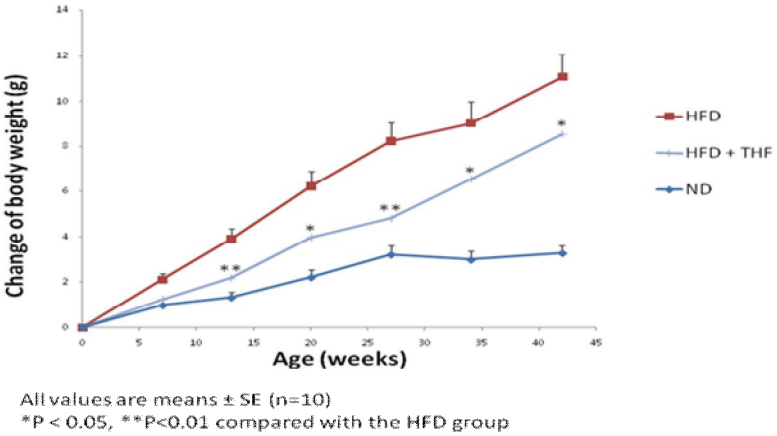
Data were expressed as means ± SD.

* $P < 0.0001$ vs ND.

** $P < 0.05$ vs HFD.

*** $P > 0.05$ vs HFD.

A



B

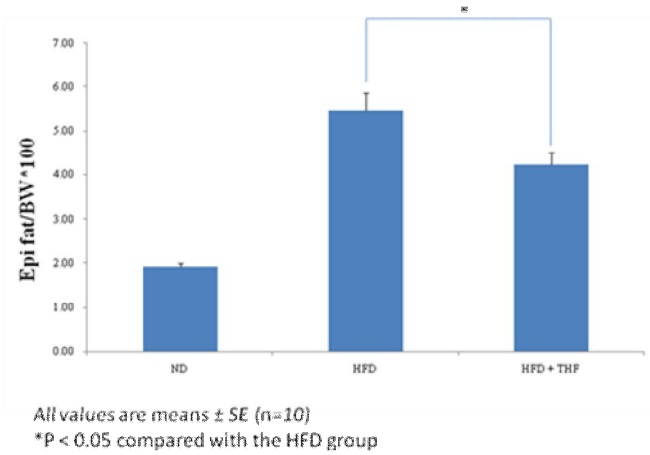


Fig. 21. Change in body weight (A), relative fat mass (B) in C57BL/6 normal diet mice (ND) or high-fat diet mice treated with vehicle (HFD) or high-fed diet mice administered 200 mg/kg tetrahydrofuran mixture (HFD + THF).

3.5. Discussions

The species *M. fragrans* has been used traditionally for spices and various medicinal purposes such as stomachic, carminative, tonic, aphrodisiac and nervous stimulant. Because AMPK has recently drawn attention as a next generation target for total metabolic control[9], it has been suggested that AMPK activators can be used for treating not only type-2 diabetes but also obesity. In the course of our screening efforts on new AMPK activators from natural foods by a cell-based assay on AMPK phosphorylation, seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans were isolated from *M. fragrans*.

The effect of individual tetrahydrofuran-type lignans on AMPK and its downstream target ACCs in culture skeletal cells were examined. Western blot analysis results indicated that the phosphorylation level of not only AMPKα Thr^{172} but also its intracellular substrate, ACC2 Ser^{79}, was increased after treatment of these compounds. Although partial correlation between chemical structure and biological activity of individual compounds were discussed, the use of lignan-rich extract may provide better benefits for food industry to develop nutraceuticals for preventing metabolic syndrome. Generally, consumption of high levels of dietary fat is thought to be a major factor in promoting obesity in human and animals. To further confirm the possible existence of anti-obesity function of tetrahydrofuran-type lignan rich fraction from *M. fragrans*, we used C57BL/6 models to investigate its protective effect against weight gain in high-fat diet induced obesity. Our result revealed that final body weights and weight gain in HFD mice were significantly higher than those of mice with normal fed diet. Feeding tetrahydrofuran mixture prevented the increasing body weight and adipose tissue mass in mice fed with HFD. Glucose and LDL levels in the HFD + THF group were also lower than those of the HFD group. These effects may be obtained partly by whole body energy regulation role of AMPK activators existed in this extract.

Finally, processing procedure to prepare a myristicin-free, lignan enriched fraction was established. Solvent containing ethanol and water are very familiar with traditional medicine extraction technique. Furthermore, HPLC analysis proved that processing procedure effectively reduced myristicin content in final extracted mixture. Thus the mixture itself could be considered as safe nutraceuticals based on the potential uses in folk medicine. From the above data obtained, tetrahydrofuran lignans could be promised to be a new class of AMPK activators. Therefore, it is strongly suggested that tetrahydrofuran-type lignans and its enriched extract from *M. fragrans* can be used not only for development of agents for the treatment of type-2 diabetes and possibly obesity, but also be beneficially used for metabolic disorders as functional food.

4. Conclusions

AMP-activated protein kinase (AMPK) has been considered as a therapeutic target for the treatment of metabolic syndrome including obesity and type-2 diabetes. In our program to search new AMPK activators from plants, we found that a total extract of *M. fragrans* activated AMPK enzyme in differentiated C2C12 cells. As the active constituents, seven 2,5-bis-aryl-3,4-dimethyltetrahydrofuran lignans, tetrahydrofuroguaiacin B (**1**), saucernetindiol (**2**), verrucosin (**3**), nectandrin B (**4**), nectandrin A (**5**), fragransin C_1 (**6**) and galbacin (**7**) were isolated from this extract. Compounds **1**, **2**, and **7** were isolated from this plant for the first time. Among isolates, compounds **1**, **4** and **5** showed a strong stimulation on AMPK enzyme. These lignans significantly prevented the increasing body weight and adipose tissue mass in high fat diet induced obesity C57BL/6 mice compared to control groups. Finally, because myristicin as principal aromatic constituent of *M. fragrans* causes severe psychopharmacological effects by overdose intake in human, a processing procedure was established following bioassay guided fractionation and HPLC analysis to prepare a myristicin-free and lignan enriched extract which can be beneficially used as functional food to prevent metabolic syndrome.

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SUPPORTING INFORMATION

List of Figures and Tables

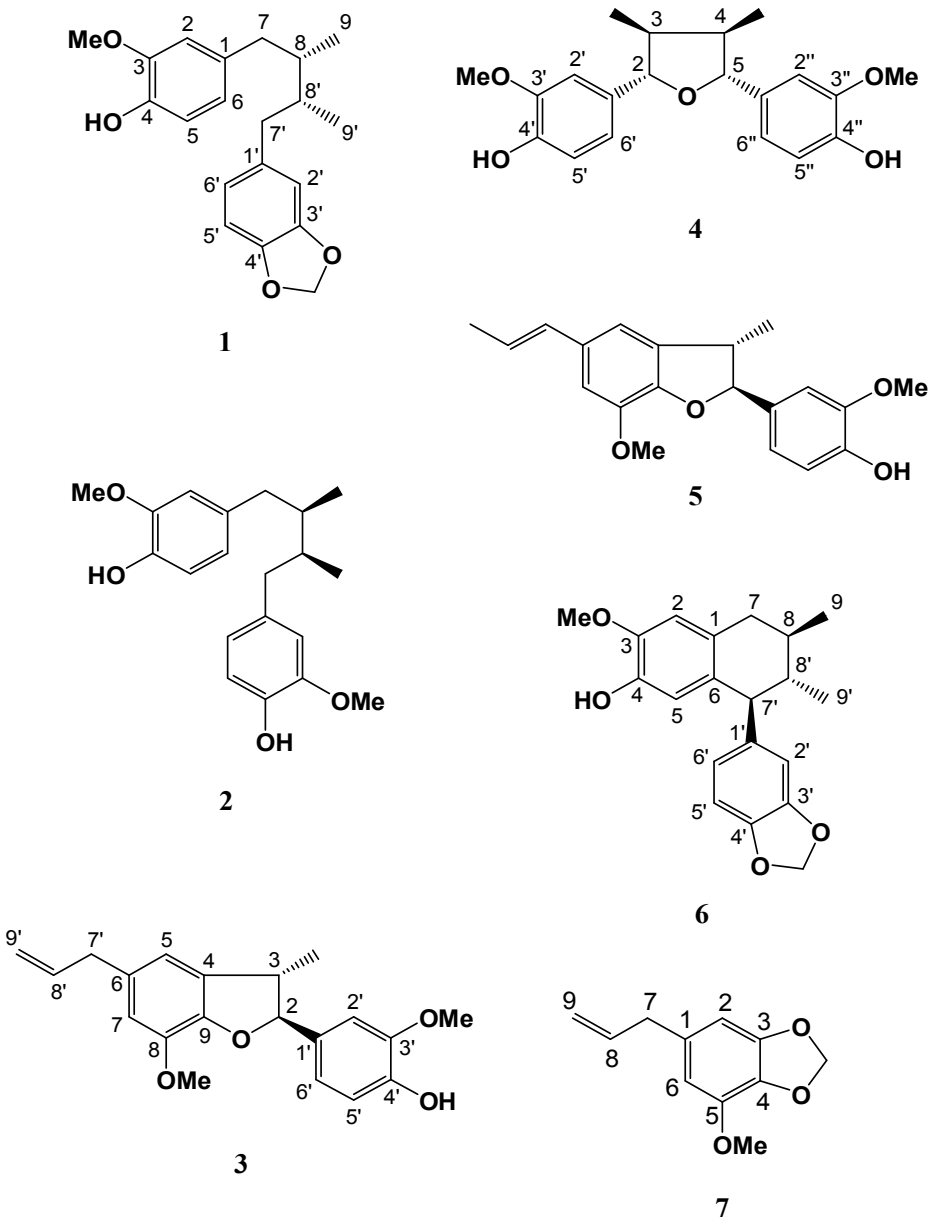

Figure S.1. Chemical structures of major phenolics **1–3**, **5–6,** nectandrin B (**4**), and myristicin (**7**) from *M. fragrans*.

(**1**) macelignan, (**2**) *meso*-dihydroguaiaretic acid, (**3**) (±)-*trans*-dehydrodiisoeugenol, (**5**) licarin A, (**6**) otobaphenol,

Table S.1. ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR data of macelignan (**1**), *meso*-dihydroguaiaretic acid (**2**), and myristicin (**7**) in $CDCl_3$

position	**1**		**2**		**7**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	136.9		134.6		133.5	
2	122.9	6.63, dd, 1.8, 8.4	115.8	6.57, dd, 1.8, 8.4	107.7	2.38, d, 1.8
3	115.9	6.70, d, 8.4	122.5	6.68, d, 8.4	137.4	
4	146.9		145.4		148.9	
5	148.9		148.7		143.5	
6	110.2	6.57, d, 1.8	113.5	6.63, d, 1.8	102.7	6.35, d, 1.8
7	40.5	2.68, dd, 5.4, 13.2	39.6	2.70, dd, 5.4, 13.2	40.2	3.29, m
		2.50, dd, 9.0, 13.2		2.26, dd, 9.0, 13.2		
8	39.9	1.70, m	40.1	1.72, m	134.6	5.93, m
			16.6	0.82, d, 6.6	115.9	5.06, d, 10.2
9	16.7	0.81, d, 6.6				5.07, d, 16.8
1′	134.6		134.6			
2′	122.5	6.63, dd, 1.8, 8.4	115.8	6.57, dd, 1.8, 8.4		
3′	113.5	6.70, d, 8.4	122.5	6.68, d, 8.4		
4′	148.7		145.4			
5′	145.4		148.7			
6′	108.8	6.57, d, 1.8	113.5	6.63, d, 1.8		
7′	40.3	2.68, dd, 5.4, 13.2	39.6	2.70, dd, 5.4, 13.2		
		2.50, dd, 9.0, 13.2		2.26, dd, 9.0, 13.2		
8′	39.6	1.70, m	40.1	1.72, m		
9′	16.5	0.79, d, 6.0	16.6	0.82, d, 6.6		
5-OMe	56.3	3.75, s	56.3	3.78, s	56.6	3.88, s
5-OH		5.65, br, s		5.25, br, s		
3′-OMe						
4′-OMe						
5′-OMe			56.3	3.78, s		
-					101.2	5.94, s
OCH_2O-	101.9	5.85, s				

Table S.2. ^{1}H- (600 MHz) and ^{13}C- (150 MHz) NMR data of (±)-*trans*-dehydrodiisoeugenol (**3**), licarin A (**5**), and otobaphenol (**6**) in $CDCl_3$

position	**3**		**5**		**6**	
	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1					132.4	
2	94.9	5.02, d, 9.6	94.9	5.05, d, 9.6	109.1	6.55, s
3	47.1	3.37, m	46.8	3.37, m	148.7	
4	135.2		133.7		145.7	
5	116.2	6.67, s	114.7	6.83, s	110.9	6.22, s
6	133.5		133.2		130.4	
7	113.9	6.62, s	111.0	6.77, s	30.4	2.88, dd, 5.4, 16.2 2.45, dd, 7.2, 16.2
8	149.3		149.1		42.1	2.04, m
9	145.3		145.3		16.2	0.90, d, 6.6
1′	134.9		134.7		140.0	
2′	111.0	7.00, d, 1.8	110.8	6.99, d, 1.8	115.8	6.58, d, 1.8
3′	148.0		147.9		147.2	
4′	147.1		147.7		147.3	
5′	120.6	6.79, d, 8.4	120.5	6.79, d, 7.8	122.8	6.69, d, 8.4
6′	117.2	6.86, dd, 1.8, 8.4	116.0	6.85, dd, 1.8, 7.8	113.6	6.42, dd, 1.8, 8.4
7′	41.2	3.40, m	132.3	6.34, dd, 15.6, 1.8	52.3	3.60, d, 6.6
8′	139.6	5.96, m	123.9	6.11, dd, 15.6, 6.6	36.6	1.92, m
9′	115.7	5.05, dd, 2.4, 10.2 5.02, dd, 2.4, 10.2	18.1	1.84, dd, 1.8, 6.6	16.0	0.89, d, 6.6
3-Me	18.2	1.35, d, 6.6	18.5	1.35, d, 7.2		
3-OMe					56.3	3.76, s
8-OMe	56.4	3.83, s	56.4	3.83, s		
3′-OMe	56.6	3.84, s	56.6	3.84, s		
$-OCH_2O-$					101.8	5.81, s

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Le Thi Van Thu

저작물 이용 허락서

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논문제목	한글: AMPK 활성화 물질로서 육두구로부터 분리한 테트라하이드로퓨란 형태의 리그난 화합물 영문: Tetrahydrofuran type-lignans from *Myristica fragrans* as AMP-activated protein kinase activators				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 -조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집・형식상의 변경을 허락함.
다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

2009 년 11월 30일

동의 여부: 동의 (0) 조건부 동의( ) 반대 ( )

저작자: LE THI VAN THU (서명 또는 인)

조선대학교 총장 귀하