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Thesis for Master Degree

Isolation and identification of increasing materials on insulin sensitivity from *Tilia amurensis*

Chosun University Graduate School

Department of Pharmacy

Do Tuan Phong

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피나무로부터 인슐린 작용을 증가시키는 화합물의 분리 정제

August 24th, 2012

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2012 년 05 월

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List of Abbreviations

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

MeOH, Me: methanol

EtOAc, EA: ethyl acetate

BuOH: n-butyl alcohol

CH_2Cl_2: methylene chloride

Hx: n-hexane

m/z: mass to charge ratio

t_R: retention time

MW: molecular weight

ppm: parts per million

RP: reverse phase

NP: normal phase

CC: column chromatography

TA: *Tilia amurensis*

UV: ultraviolet absorption

IR: infrared spectroscopy

NMR: nuclear magnetic resonance

MS: mass spectrum

EIMS: impact mass spectroscopy

HREIMS: high resolution electro impact mass spectroscopy

NOE: nuclear overhauser effect

HPLC: high performance liquid chromatography

TLC: thin layer chromatography

J: spin-spin coupling constant [Hz]

m: multiplet (in connection with NMR data)

s: singlet

q: quartet

t: triplet

br: broad

MHz: mega hertz

DMSO: dimethyl sulfoxide

2-NBDG: 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2-deoxyglucose

DMEM: dulbecco's modified eagle medium

HBSS: hanks' balanced salt solution

FBS: fetal bovine serum

CS: calf serum

PS: penicillin streptomycin

(국문초록)

피나무로부터 인슐린 작용을 증가시키는 화합물의 분리 정제

도 뚜안 펑

지도교수 : 오원근

약학과

조선대학교대학원

당뇨병은 췌장이 충분한 양의 인슐린을 생산하지 않거나 생산된 인슐린이 효과적으로 사용되지 않은 때 발생하는 만성질환이다. 인슐린은 혈중의 당을 세포 속으로 영양분을 공급하기 위하여 작용하는 일종의 호르몬이다. 혈당치가 상승하는 고혈당증은 당뇨병의 가장 일반적인 모습이며 신체 시스템, 특히 신경계나 혈관에 심각한 손상을 일으킨다. 그러므로, 인슐린과 비슷한 생화학적인 기전을 따르는 화합물에 의하여 세포 속으로 당의 흡수를 증가시키는 약물의 개발이 필요하다.

이번 연구에서, 피나무 껍질을 메탄올로 추출한 후 얻어진 에틸아세테이트와 부탄올 층으로부터 13 개의 화합물이 활성 추적 검사법에 의하여 분리 되었다. 그들의 구조는 4 개의 갈릭 유도체 (**1**−**4**), 8 개의 플라반 3−올인 (+)−catechin (**6**), (−)−epicatechin (**7**), (+)−gallocatechin (**8**), (−)−epigallocatechin (**9**), (+)−catechin−3−O− β −D−glucopyranoside (**10**), (−)−epigallocatechin−3−O−gallate (**11**), gallocatechin−3−O−gallate (**12**), epicatechin−3−O−gallate (**13**)과 하나의 코마린 화합물인 fraxin (**5**) 임을 분광학적인 방법과 기존 화합물의 결과 비교를 통하여 확인 하였다.

분리된 화합물은 3T3-L1 지방세포를 분화시킨 뒤 2-NBDG 활성 측정을 통하여 세포 내 인슐린 자극된 당 흡수 능력을 측정하였다. 이러한 3T3-L1 지방세포를 이용한 당 흡수 측정은 인슐린 모방 화합물의 활성검색에 일반적으로 사용되고 있다 (보기 38, 39). 그 결과는 분리된 화합물이 인슐린 작용을 증가시키는 것으로 측정되는데, 특히 화합물은 **1** 은 강한 인슐린 효과의 증강작용을 보였다. 활성의 차이는 (+)-gallocatechin-3-O-gallate (**12**), gallic acid derivatives (**2-4**), 와 (-)-epigallocatechin (**9**) 순으로 측정되었다.

구조 활성간의 관계에서 플라보놀의 C-3 에 당이나 갈로일 기의 존재는 활성의 감소와 관련되는 것으로 추정되었다. 이러한 결과는 피나무의 활성분획물이나 화합물 **1** 을 포함한 분획은 강한 인슐린 작용 강화 성질을 갖고 있으므로 향후 추가적인 연구를 통하여 이러한 식물의 이용성에 대한 검토가 필요하다.

1. Introduction

1.1. Insulin and diabetes

Diabetes mellitus is one of the most prevalent and serious metabolic diseases and the principal cause of morbidity and mortality in the human. In diabetes, there is a failure to increase glucose uptake into peripheral tissues in response to insulin, leading to chronically elevated levels of glucose in the circulation. Diabetes may be divided into three main types (**Table 1**). First, in type I diabetes, the immune system attacks the insulin-producing β cells in the pancreas, and then the pancreas produces little or no insulin. It occurs mainly in children and young adults (**Fig. 1, 2**). Second, in type II diabetes, the insulin does not produce expected results - a phenomenon known as insulin resistance (**Fig. 3**). Most of its patients are over age 40 and overweight. Third, gestational diabetes occurs during pregnancy. Although it usually disappears after delivery, the mother will more likely develop type II diabetes later.

Among three types of diabetes, type II diabetes, known as non-insulin-dependent diabetes mellitus (NIDDM), accounts for > 90% of all diabetes cases. With diabetes prevalence rates doubling between 1990 and 1995, the American Centers for Disease Control and Prevention (CDC) has just declared that we are facing a diabetes epidemic. The diabetes epidemic has struck worldwide. More than 285 million people across the world suffer from diabetes, 90% of whom have Type II Diabetes. It is estimated that by the year 2030 438 million people will be inflicted with diabetes worldwide, profoundly increasing the need for treatment for type II diabetes. In the United States, CDC projects that by 2050 one in three individuals will be diagnosed with diabetes. In Europe, there are 55 million diabetics, and by 2030 this number is expected to grow to 66 million. Russia and Germany lead in European

diabetes prevalence with 9.6 million and 7.5 million diabetics respectively. Diabetes is also becoming a major health concern in Asia with over 90 million diabetics in China and 50 million in India.

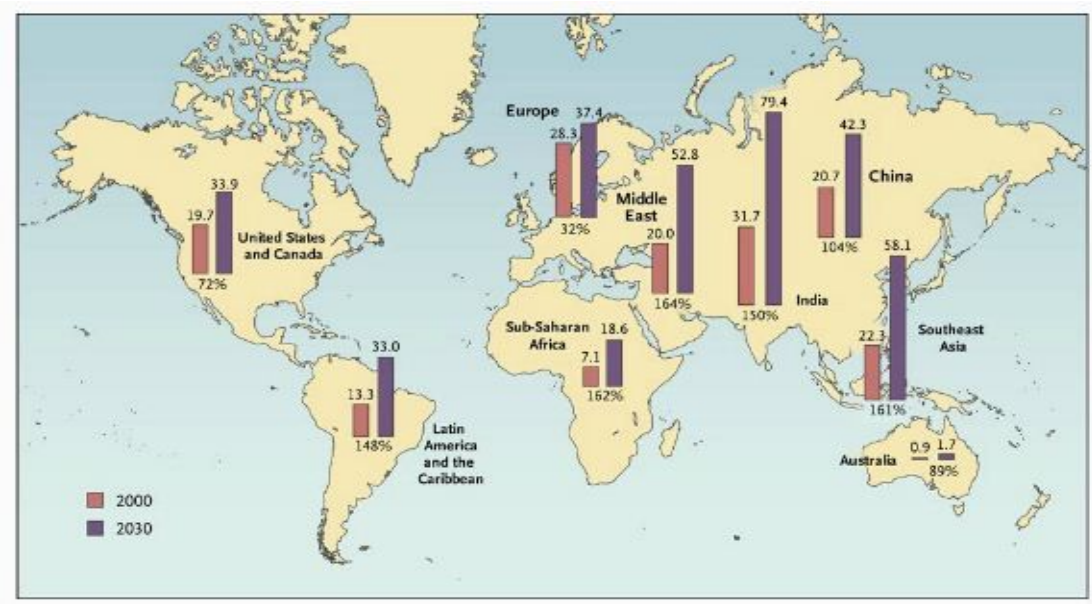


Fig. 1: Worldwide prevalence of diabetes 2000-2030

In type II diabetes, either the body does not produce enough insulin or the cells ignore the insulin (**Fig. 4**). Insulin is necessary for the body to be able to use glucose for energy. When we eat food, the body breaks down all of the sugars and starches into glucose, which is the basic fuel for the cells in the body. Insulin takes the sugar from the blood into the cells. When glucose builds up in the blood instead of going into cells, it can lead to diabetes complications.

Insulin is the only agent developed for the treatment of either type I diabetes or severe type II diabetes. A number of synthetic small molecules, such as zinc (II) complexes and vanadium compounds, have been shown to mimic the action of insulin in cell culture and

animal models of diabetes. In addition, many natural products, such as antibiotics (e.g. anisomycin), fungal metabolites (e.g. L-783,281), plant extracts (e.g. leaf alcoholic extract from the tropical herbaceous perennial *Catharanthus roseus*) and animal constituents (e.g. dried chrysalis of the silkworm *Bombyx batryticatus*) also promote glucose uptake in cells. However, clinical tests have shown that none of these compounds or extracts can replace insulin in the treatment of diabetes. Therefore, there is a need to search for new anti-diabetic agents that can mimic the effect of insulin. In addition, the characterization of new insulin mimetic agents can promote the discovery of new drug targets that further our understanding of the biochemical mechanisms producing diabetes and insulin resistance.

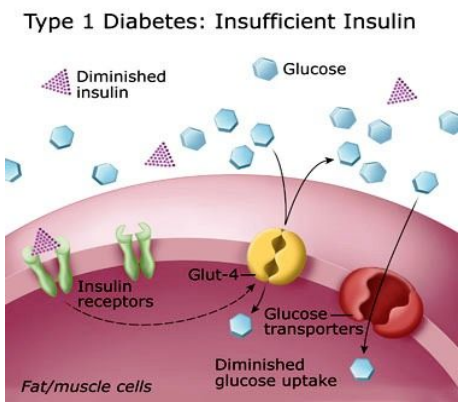


Fig. 2: Type I diabetes

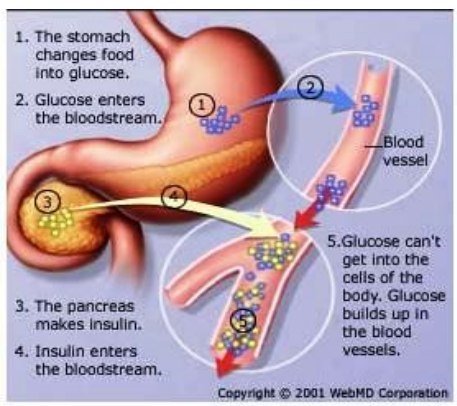


Fig. 3: Type II diabetes

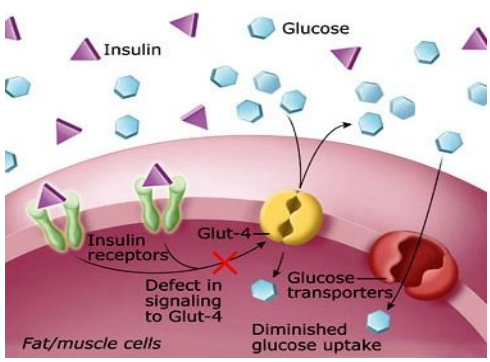
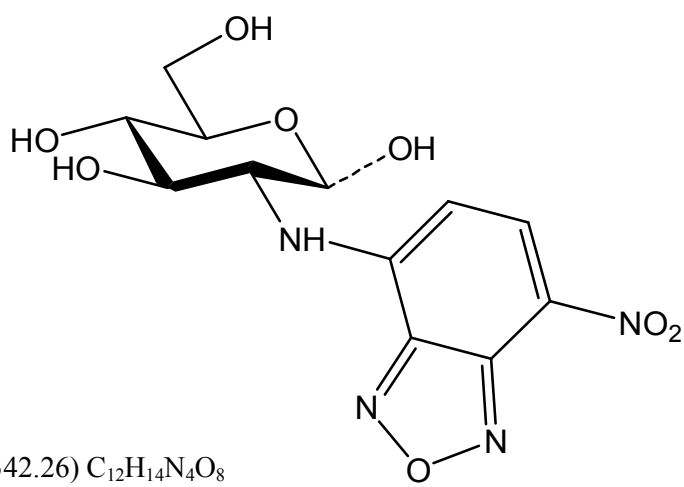


Fig. 4: Type II diabetes: Insulin resistance

Table 1. Classification of diabetes

	Type I (IDDM)	Type II (NIDDM)
Distribution	< 10%	> 90%
Cause	Autoimmune disease	Insulin resistance (obese)
		Insulin secretion defect (nonobese)
Age	< 30 (mainly 11 - 14)	> 45
Clinical	Fast progress	Slow progress
	Hypoinsulinemia	Hyperinsulinemia (obese)
	Ketonemia	Normal insulin level (non-obese)
Treatment	Injection of insulin	Die, Glucose-lowing agent, linsulin



(MW=342.26) $C_{12}H_{14}N_4O_8$

Fig. 5: 2-NBDG [2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose]

1.2. Fluorescent D-glucose analog [2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2 deoxy-d-glucose (2-NBDG)]

A focus of current anti-diabetic medicine research is the development and screening of compounds with potential insulin-mimetic effects to stimulate rate of cell glucose uptake. Most studies on glucose uptake are commonly carried out using radio-tracers such as 2-deoxy-d-[^{14}C] glucose or 2-deoxy-d-[^{3}H] glucose. The high signal to noise ratio of these isotopes is favorable for kinetic studies of glucose transport. However, there are several disadvantages associated with the radio-tracer such as disposal of radioactive waste or radioactive cleanup. More importantly, it cannot directly measure glucose uptake in single and living cells. Thus, an assay was reported that it is based on direct incubation of adipose cells with a fluorescent D-glucose analog, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG), followed by flow cytometric detection of fluorescence produced by the cells. 2-NBDG (Molecular Probes) is a new fluorescent derivative of glucose modified with a 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino group at the C-2 position (MW = 342.26) (**Fig. 5**). This product showed intense fluorescence at 542 nm when excited at 467 nm. [1]

In this study, we describe a cell-based screening that employs 2-NBDG and can be used to discover new compounds which regulate glucose uptake. 3T3-L1 adipocytes cell line was chosen for 2-NBDG assay, which represents the major body tissue type that is sensitive to the action of insulin (fat). 3T3-L1 adipocytes showed the increase in 2-NBDG uptake after insulin treatment.

1.3. *Tilia amurensis*

Fig. 6. *Tilia amurensis*: 1. Flowing branch, 2. Flower, 3. Leaves.

Tilia species are large deciduous trees, typically 20-40 m tall, belonging to the family Tiliaceae, and are native throughout most of the temperate northern hemisphere, in Asia, Europe, and eastern North America.[2] *Tilia* species are traditional medicinal plants which have been used as sedatives, tranquilizers, diuretics, expectorants, and diaphoretics.[3, 4] Some reports indicated that *Tilia* also represent other activities such as anxiolytic [5] and antistress activity.[6] Previous chemical studies of this species have shown the presence of coumarin[7-9], flavonoid[7, 9-11], triterpenes[7], and hydrocarbons.[7]

Most medicinal research has focused on *Tilia amurensis*, although other species are also used medicinally and somewhat interchangeably. The dried flowers are mildly sweet and sticky, and the fruit is somewhat sweet and mucilaginous. The flowers, leaves, wood, and charcoal (obtained from the wood) are used for medicinal purposes. Active ingredients in the *Tilia* flowers include flavonoids (which act as antioxidants), volatile oils, and mucilaginous

constituents (which soothe and reduce inflammation). The plant also contains tannins that can act as an astringent. *Tilia* flowers are used medicinally for colds, cough, fever, infections, inflammation, high blood pressure, headache (particularly migraine), and as a diuretic (increases urine production), antispasmodic (reduces smooth muscle spasm along the digestive tract), and sedative. New evidence shows that the flowers may be hepatoprotective. The flowers were added to baths to quell hysteria, and steeped as a tea to relieve anxiety-related indigestion, irregular heartbeat, and vomiting. The leaves are used to promote sweating to reduce fevers. The wood is used for liver and gallbladder disorders and cellulitis (inflammation of the skin and surrounding soft tissue). That wood burned to charcoal is ingested to treat intestinal disorders and used topically to treat edema or infection such as cellulitis or ulcers of the lower leg.

In this study, the barks of *Tilia amurensis* were extracted with methanol and then partitioned successively with *n*-hexane, EtOAc and BuOH. The *n*-hexane, ethyl acetate and butanol fractions were then concentrated to yield the dried residues. These crude fractions were tested for their insulin-stimulated glucose uptake activity in cells using 2-NBDG assay on 3T3-L1 adipocyte cells. The result showed that the EtOAc and BuOH fractions were active (**Fig. 36**). Activity-guided fractionation of this combined fraction led to the isolation of a series of as four gallic acid derivatives (**1-4**), eight flavan-3-ols (**6-13**), and one coumarin glycoside, fraxin (**5**). In this thesis, the isolation, structural elucidation of the isolated compounds and the evaluation of their insulin-stimulated glucose uptake activity in 3T3-L1 adipocyte cells using a fluorescent D-glucose analog, 2-[N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl) amino]-2-deoxy-d-glucose (2-NBDG) assay, are described.

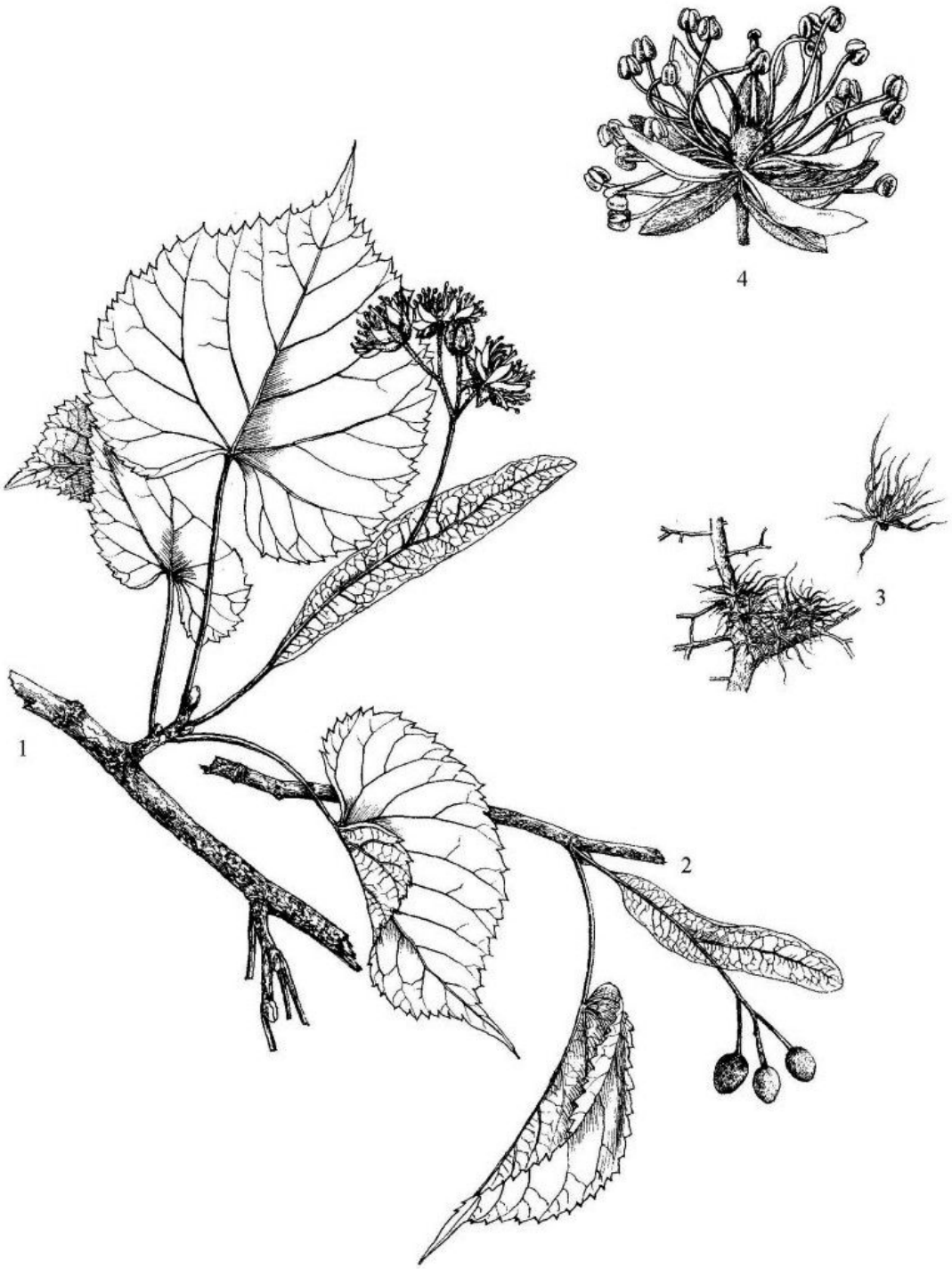


Fig. 7. ***Tilia amurensis***: 1. Flowing branch, 2. Fruiting, 3. Hairs, 4. Flower.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

The bark of *Tilia amurensis* was collected at Gangwon province in May 2010, Republic of Korea. This material was taxonomically confirmed by Professor Won Keun Oh, Chosun University, Gwangju city, in Republic of Korea. A voucher specimen (CHU 20-2010) has been deposited at the College of Pharmacy, Chosun University.

2.1.2. Chemicals, reagents, and chromatography

Column chromatography was conducted on silica gel (63 - 200 μm particle size) and RP-18 (40 - 63 μm particle size) from Merck. TLC was carried out with silica gel 60 F_{254} plates from Merck. HPLC was carried out using a Gilson System 321 pump equipped with a model UV/VIS-155 UV detectors and an Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech Korea). Baker analyzed HPLC solvents MeCN was purchased from Mallinckrodt Baker, Inc. USA; deuterated solvent for NMR analysis Me_2CO-d_6, D_2O, methanol-d_4 was purchased from CIL (Cambridge Isotope Lab., USA).

2-NBDG [2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-2-deoxyglucose] was purchased from Invitrogen (OR, USA). For comparison and validation of our new screening based on NBDG uptake, we used a commercial, enzyme-based glucose assay provided by Biovision Inc., CA, USA. Dulbecco's mofified Eagle's medium (DMEM), fetal bovine serum (PBS), and trypsin were purchased from GIBCO-BRL (Grand Island, NY, USA).

Insulin from bovine pancreas was provided by Sigma-Aldrich (SL, USA). Rosiglitazine was purchased from Cayman Chemicals (MI, USA).

2.1.3. General experimental procedures

The optical rotations were determined on an Autopol IV A7040–12 automatic polarimeter using a 100 mm glass microcell. The ultraviolet (UV) spectra were obtained in MeOH using an Optizen 3220UV spectrophotometer. The CD spectra were recorded in MeOH on a JASCO J-715 spectrometer. ^{1}H nuclear magnetic resonance (NMR, 500 MHz) and ^{13}C NMR (75 MHz) spectra were recorded on an YH300-OXFORD NMR spectrometer. The ^{1}H NMR (500 MHz) and ^{13}C NMR (75 MHz) spectra were measured on a Unity INOVA 500 FT–NMR spectrometer with TMS as the internal standard at Korea Basic Science Institute (KBSI, Gwangju Center, Korea). Electron ionization (EI)-mass spectroscopy (MS) and high-resolution ESI-MS spectra were recorded on a Micromass ESI–Tof II (Micromass, Wythenshawe, UK) mass spectrometer. Column chromatography was carried out using silica gel 60 (40–63 and 63–200 μm particle size) and RP-18 (40–63 μm particle size) from Merck. Precoated TLC silica gel 60 F_{254} plates from Merck were used for thin-layer chromatography. The spots were visualized using UV light or 10% sulfuric acid. The HPLC runs were carried out using a Gilson System LC-321 pump with a UV/vis–155 UV detectors, and an Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea) for semi-preparative runs.

The 3T3-L1 adipocyte cell used in the present study was obtained from the American Type Culture Collection (ATCC) through Highveld Biological (Johannesburg, South Africa). 3T3-L1 adipocyte were seeded at 5×10^4 cells per well in a 96-well culture plate. 24 h later,

culture media was changed and adipocytes were cultured in serum-free low glucose DMEM for 3 h prior to drug treatment. Cells were lysed with 100 μL Cell Lytic M (Sigma-Aldrich, SL, USA) and 50 μL lysate was used for assay. All other chemicals and solvents were analytical grade and used with further purification, if necessary.

2.2. Methods

2.2.1. Cell culture and induction of 3T3-L1 adipocytes

3T3-L1 adipocytes were maintained in proliferation media, consisting of DMEM supplemented with 10% calf serum (CS) and antibiotic PS 1% (Penicillin and Streptomycin) in an atmosphere of 95% air and 5% CO_2 at 37 ^{o}C. To prepare for each assay, cells were seeded in 96-well plates with 3×10^3 cells/well (v = 200 μL/well) in 2 mL growth medium. Differentiation of 3T3-L1 adipocyte was induced by replacing growth medium with DMEM containing 10% calf serum (CS) when the cells were confluent. The medium were changed every 24 h until the formation of 3T3-L1 adipocytes. Cells were used in experiments at 4-5 days after differentiation. To induce adipogenesis, the media was changed to DMEM supplemented with 10% fetal bovine serum (FBS), 0.5 mM 3-isobutyl-1-methyl-xanthine, 2 μg/mL dexamethasone, 1 μg/mL insulin, 1 μM rosiglitazone, and pen/strep and further cultured for 48 h. Every 2 days thereafter, the cells were incubated with fresh DMEM supplemented with 10% FBS, 1 μM rosiglitazone, 1 μg/mL insulin, and pen/strep. 3T3-L1 adipocytes were used for experiments 7 days after the induction of differentiation.

2.2.2. 2-NBDG assay for measuring glucose uptake

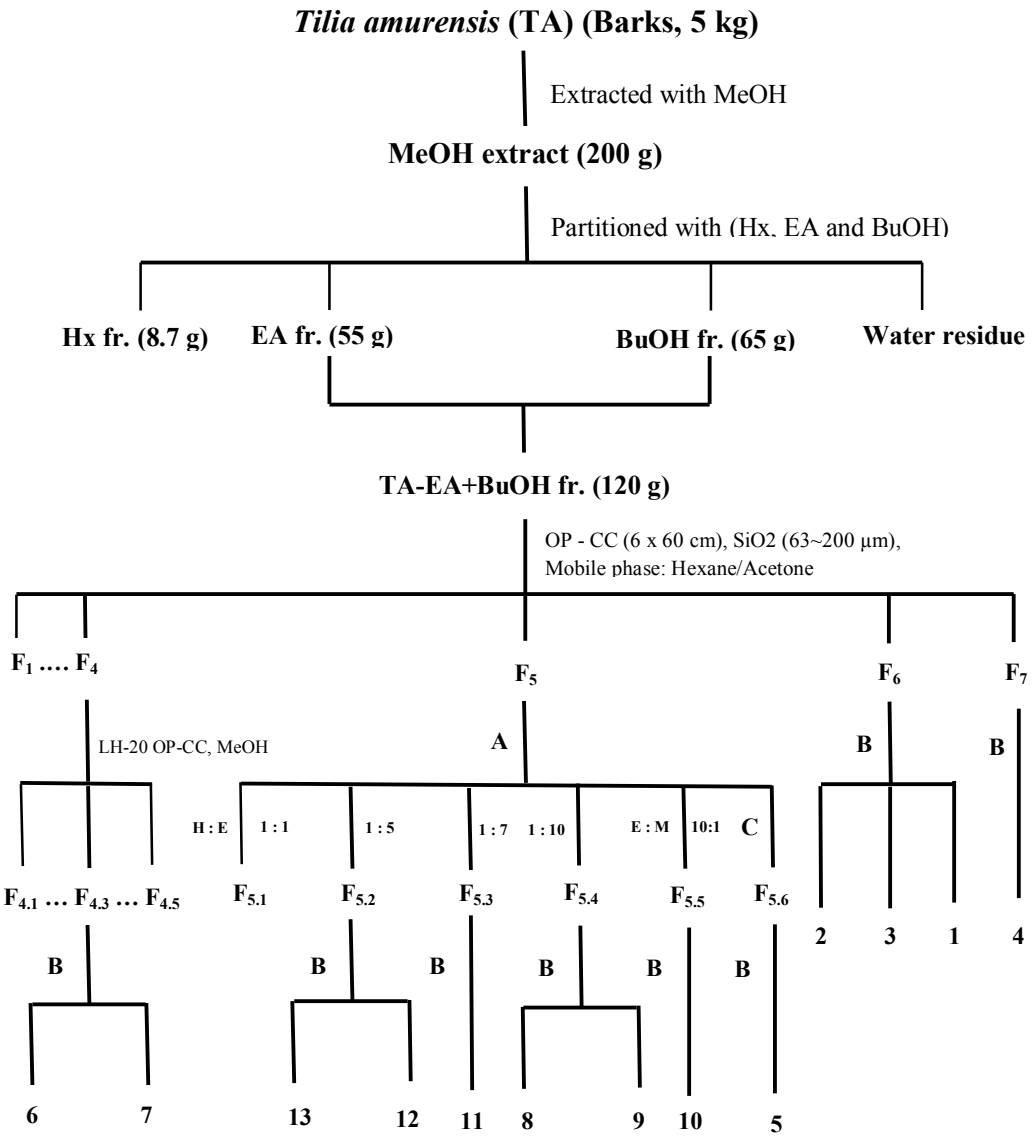
NBDG assay was performed as previously described with some modifications [12]. Briefly, differentiated 3T3-L1 adipocytes (2×10^4 cells/well) were seeded in a 24-well tissue culture plate (BD Falcon, NJ,USA) and cultured in glucose-free culture media supplemented with 10% FBS and pen/strep for treating NBDG with or without compounds of interest. Compounds were treated at a concentration of 10 μM. 2 μg/mL insulin served as a positive control. After 90 min, cells were rinsed with 1 mL of PBS prior to measuring fluorescent signals (Excitation 450 nm and Emission 535 nm) using the VICTOR™ X3 Multilabel Plate Reader fluorescent reader (PerkinElmer).

2.2.3. Extraction and isolation of active compounds from *Tilia amurensis*

The bark of *Tilia amurensis* (5 kg) was extracted with MeOH three times by refluxing for 24 h at the room temperature and the MeOH solution was evaporated to dryness (1 kg). The MeOH extract was suspended in H_2O (1 L) then partitioned successively with n-hexane, EtOAc and BuOH (each 1 L × 3). These crude extracts were tested 2-NBDG assay for their insulin-sensitizing glucose uptake activity. The result showed that the EtOAc and BuOH fractions were active (**Fig. 36**). The EtOAc and BuOH combined fraction (120 g) was loaded onto a silica-gel column (20 × 80 cm, silica-gel 63-200 mesh), and eluted with gradient of n-hexane: acetone (from n-hexane: acetone 2:1 to 1:8). The eluates (500 mL in each flask) were combined into 7 fractions (F.1 to F.7) according to their TLC profiles. The seven fractions (F.1 to F.7) were tested in vitro 2-NBDG assay, along with checking the ^{1}H NMR to find out the active materials. Among these, fractions 4 (F.4), 5 (F.5), and 6 (F.6) exhibited strong insulin-sensitizing activity (**Fig. 37**). Fraction 4 (F.4) was further

chromatographed over LH-20 column (6.0 × 40 cm; 150 μm particle size), and eluted with gradient of MeOH to afford five subfractions (F.4.1 - F.4.5) according to their TLC profiles. These five subfractions were tested 2-NBDG assay for their insulin-sensitizing activity. The results showed that the fraction F.4.3 was active. Further purification of subfraction F.4.3 (600 mg) by semi-preparative Gilson HPLC systems [using RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size); mobile phase MeOH/H_2O containing 0.1% formic acid for 42 min (0–26 min: 48% MeOH, 26–28 min: 48–100%MeOH, 28–38 min: 100%MeOH, 38–40 min: 100–48% MeOH, 40–42 min: 48%MeOH); flow rate 2mL/min, UV detections at 205 and 254 nm] to yield compounds **6** (F.4.3.1, 13.1 mg, t_R = 18 min) and **7** (F.4.3.2, 85 mg, t_R = 22 min). Fraction 5 (F.5) was loaded onto a silica-gel column (10 × 50 cm, 63~200 μm particle size), and eluted with gradient of n-hexane-EtOAc-MeOH (from n-hexane: EtOAc 1:1 to EtOAc: MeOH 10:1) to yield six subfractions (F.5.1 - F.5.6). Further purification of subfraction F.5.4 (87 mg) by semi-preparative Gilson HPLC systems [using RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); mobile phase MeOH/H_2O containing 0.1% formic acid for 36 min (0–20 min: 29% MeOH, 20–22 min: 29–100% MeOH, 22–32 min: 100% MeOH, 32–34 min: 100–29% MeOH, 34–36 min: 29% MeOH); flow rate 2mL/min, UV detections at 205 and 254 nm] afforded compounds **8** (F.5.4.1, 15.1 mg, t_R = 15 min) and **9** (F.5.4.2, 10 mg, t_R = 18 min). Subfraction F.5.5 (185 mg) was also purified by preparative Gilson HPLC using an isocratic solvent system of 18% MeOH in H_2O containing 0.1% formic acid, over 28 min then increase to 100% MeOH for 10 min [RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); UV detections at 205 and 254 nm] to obtain compound **10** (F.5.5.1, 9 mg, t_R = 18 min). Subfraction F.5.6 (100 mg) was purified by preparative Gilson HPLC using an isocratic solvent system of 26% MeOH in H_2O containing 0.1% formic acid, over 30 min then increase to 100% MeOH for 10 min [RS Tech Optima

Pak C18 column (10 × 250 mm, 10 μm particle size); UV detections at 205 and 254 nm] to obtain compound **5** (F.5.6.1, 7.5 mg, t_R = 28 min). Subfraction F.5.2 (90 mg) was purified by preparative Gilson HPLC using an isocratic solvent system of 40% MeOH in H_2O containing 0.1% formic acid, over 30 min then increase to 100% MeOH for 10 min [RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); UV detections at 205 and 254 nm] to obtain compounds **12** (F.5.2.1, 7 mg, t_R = 25 min) and **13** (F.5.2.2, 8.5 mg, t_R = 29 min). Subfraction F.5.3 (45 mg) was purified by preparative Gilson HPLC using an isocratic solvent system of 34% MeOH in H_2O containing 0.1% formic acid, over 32 min then increase to 100% MeOH for 10 min [RS Tech Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size); UV detections at 205 and 254 nm] to obtain compound **11** (F.5.3.1, 6.8 mg, t_R = 30 min). Fraction F.6 (175 mg) was purified by preparative Gilson HPLC using an isocratic solvent system of 35% MeOH in H_2O containing 0.1% formic acid, over 36 min then increase to 100% MeOH for 10 min [RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); UV detections at 205 and 254 nm] to obtain compounds **2** (F.6.1, 7.8 mg, t_R = 21.1 min), **3** (F.6.2, 7.5 mg, t_R = 26.8 min), and **1** (F.6.3, 14.2 mg, t_R = 34.6 min). Further purification of fraction F.7 (177 mg) by preparative Gilson HPLC using an isocratic solvent system of 20% MeOH in H_2O containing 0.1% formic acid, over 22 min then increase to 100% MeOH for 10 min [RS Tech Optima Pak C18 column (10 × 250 mm, 10 μm particle size); UV detections at 205 and 254 nm] to obtain compound **4** (F.7.1, 19.8 mg, t_R = 18.2 min), and see **Scheme 1.**



A: OP-CC (6×60), SiO_2 (63~200 µm), Mobile phase: Hx:EA:MeOH

B: Gilson HPLC System, RP-C18 (10 × 250 mm, 10µm, MeOH / H_2O + 0.1%Formic acid)

C: (E : M: 5:1, 1:1, 1:10)

Scheme 1. Isolation of compounds (**1-13**) from EtOAc and BuOH fractions of *Tilia amurensis*

Compound 1, **(Z)-3-(4-Hydroxyphenyl) acrylaldehyde:**

It was yellow needles (MeOH), EIMS m/z (%): 178 ([M]+,100), 177 (29), 163 (11), 161 (20), 135 (25), 107 (18). ^{1}H NMR (600 MHz, acetone-d_6) δ: 9.63 (1H, d, J = 7.8 Hz, H-9), 8.41 (1H, s, 4-OH), 7.57 (1H, d, J = 15.6 Hz, H-7), 7.39 (1H, br, s, H-2), 7.21 (1H, br, d, J = 7.8 Hz, H-6), 6.91 (1H, br, d, J = 7.8 Hz, H-5), 6.66 (1H, dd, J = 7.8, 15.6 Hz, H-8), 3.89 (3H, s, 3-OCH_3); ^{13}C NMR (150 MHz, acetone-d_6) δ: 194.0 (CHO), 154.1 (C-7), 150.9 (C-3), 148.9 (C-4), 127.5 (C-1), 127.1 (C-8), 116.2 (C-5), 111.5 (C-2), 56.4 (3-OCH_3).

Compound 2, Benzoic acid:

It was a white, crystalline organic compound belonging to the family of carboxylic acids; ^{1}H NMR (600 MHz, CD_3OD) δ: 8.02 (2H, dd, J = 9.0 Hz, H-2 and H-6), 7.59 (1H, m, H-4), 7.48 (2H, m, H-3 and H-5); ^{13}C NMR (150 MHz, CD_3OD) δ: 173.0 (COOH), 130.9 (C-2 and C-6), 129.6 (C-3 and C-5), 134.1 (C-4).

Compound 3, Vanillin:

It was white colorless needles; mp 81°C; $C_8H_8O_3$; Mol. wt. 152.1; FTIR (KBr, cm^{-1}): 3539 (OH), 2832 (CH), 1680 (C=O), 1509 (C=C), 1159 (CH); MS m/z (rel. int., %): 152.1(M+, 6.70), 123.1 (5.12), 109.1 (15.45), 92.1 (2.14), 81.0 (63.90), 62.0 (30.33), 51.0 (100); ^{1}H NMR (600 MHz, CD_3OD) δ: 9.75 (1H, s, CHO), 7.44 (1H, d, J = 1.2 Hz, H-2), 7.43 (1H, d, J = 7.8 Hz, H-5), 6.94 (1H, dd, J = 1.2, 7.8 Hz, H-6), 3.92 (3H, s, 3-OCH_3); ^{13}C NMR (150 MHz, CD_3OD) δ: 193.0 (CHO), 154.8 (C-3), 149.8 (C-4), 130.8 (C-1), 128.1 (C-6), 116.4 (C-5), 111.3 (C-2), 56.5 (3-OCH_3).

Compound 4, Gallic acid:

It was yellow-green solid; UV λ_{max} (ethanol) nm: 220, 271; IR (KBr) ν cm^{-1}: 3491, 3377, 1703, 1617, 1539, 1453, 1254 cm^{-1}; HRMS (ESI) m/z 169.0137 $[M-H]^-$ (calcd.For $C_7H_5O_5$, 169.0137). ^1H NMR (600 MHz, CD_3OD) δ: 7.05 (2H, s, H-2 and H-6); ^{13}C NMR (150 MHz, CD_3OD) δ: 170.5 (COOH), 146.5 (C-3 and C-5), 139.5 (C-4), 122.0 (C-1), 110.2 (C-2 and C-6).

Compound 5, Fraxin:

It was colorless needles; mp: 198 ^oC; UV λ_{max} (MeOH) nm ($\log_\varepsilon$): 230 (3.2), 256 (2), 261 (1.5), 348 (2.0); EIMS *m/z*: 370 $[M]^+$; ^1H NMR (300 MHz, CD_3OD) δ: 7.88 (1H, d, *J* = 9.3 Hz, H-4), 6.99 (1H, s, H-5), 6.25 (1H, d, *J* = 9.3 Hz, H-3), 3.90 (3H, s, 6-OCH3), 5.00 (1H, d, *J* = 7.8 Hz, H-1′), 3.70 (1H, dd, H-2′), 3.45 (1H, dd, H-3′), 3.34 (1H, dd, H-4′), 3.56 (1H, ddd, H-5′), (2H, 3,79, dd, 3.47, dd, H-6′); ^{13}C NMR (75 MHz, CD_3OD) δ: 163.7 (C-2), 133.7 (C-7), 146.6 (C-4), 147.6 (C-6), 144.5 (C-8), 135.9 (C-9), 113.1 (C-3), 112.3 (C-10), 106.2 (C-5), 106.1 (C-1′), 75.6 (C-3′), 78.6 (C-5′), 77.9 (C-2′), 71.0 (C-4′), 62.3 (C-6′), 57.0 (6-OCH_3).

Compound 6, ((+)-Catechin):

It was obtained as a amorphous brown powder; mp: 230-232 ^oC; $[\alpha]_D^{25}$ -55.4^o (c 1.24, MeOH); EIMS m/z 290 $[M]^+$, 139, 138, 110, 152, 151 and 123; IR spectra (KBr): 3400 (broad), 1620, 1520, 1470, 1380, 1280, 1240, 1150, 1120, 1080, 1020, 820 cm^{-1}; UV (MeOH) spectra λ_{max} nm: 277, and 220. ^1H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 2** and **3**.

Compound 7, ((-)-Epicatechin):

It was obtained as a amorphous brown powder; mp: 230-232^{o}C; $[\alpha]_D^{25}$ -55.4^{o} (c 1.241, MeOH); EIMS m/z 291 $[M+H]^+$; ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 2** and **3**.

Compound 8, (+)-Gallocatechin:

It was obtained as a white amorphous solid; ESI-MS, m/z (relative intensity): 611 $[M+M-H]^-$ (9), 341 $[M+Cl]^-$ (23), 305 $[M-H]^-$ (100); MS-MS of 305: 287 $[M-H-H_2O]^-$ (4), 179 $[M-H-C_6H_6O_3]^-$ (100), 137 $[M-H-C_8H_8O_4]^-$ (31); ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 2** and **3**.

Compound 9, (-)-Epigallocatechin:

It was obtained as a white amorphous solid; ESI-MS, m/z (relative intensity): 611 $[M+M-H]^-$ (11), 341 $[M+Cl]^-$ (24), 305 $[M-H]^-$ (100); MS-MS of 305: 287 $[M-H-H_2O]^-$ (4), 179 $[M-H-C_6H_6O_3\ ]^-$ (100), 137 $[M-H-C_8H_8O_4]^-$ (33); ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 2** and **3**.

Compound 10, (+)-Catechin 3-O-β-D-glucopyranoside:

It was yellowish amorphous solid; UV (MeOH) λ_{max} (log ε): 280.0 (3.78), 210.0 (4.66); IR (KBr) ν_{max} cm^{-1}: 3385, 2924, 1623, 1503, 1462, 1365, 1282, 1199, 1142, 1073, 896, 825; ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 2** and **3**.

Compound 11, **(-)-Epigallocatechin-3-O-gallate (EGCG):**

It was off-white amorphous powder, mp 255–257 °C; $[\alpha]^{25}_{D}$ –41.5° (c 0.07, C_2H_5OH); IR (KBr) ν_{max}: 3427, 1693, 1608, 1520, 1454, 1383, 1234, 1142, 1038, 820, 768 cm^{-1}; EI-MS m/z: 458; UV (MeOH) λ_{max}: 269 nm. ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 4**.

Compound 12, (+)-gallocatechin-3-O-gallate (GCG):

It was white powder, $[\alpha]_D^{23.8}$ –24 (c 0.1, CH_3OH). UV (CH_3OH) λmax nm: 212 (log ε 4.52), 276 (log ε 3.71); IR (KBr) ν_{max}: 3426, 1704, 1631, 1531, 1450 cm^{-1}; ESI-MS m/z: 481 $[M+Na]^+$, 457 $[M-H]^-$; ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 4**.

Compound 13, (-)-Epicatechin-3-O-gallate (ECG):

Yellow powder, mp 255–257 °C; $[\alpha]^{20}_{D}$ –71.6° (c 0.07, C_2H_5OH); IR (KBr) ν_{max}: 3427, 1693, 1608, 1520, 1454, 1383, 1234, 1142, 1038, 820, 768 cm^{-1}; FAB-MS m/z: 443, 423, 391, 338, 185, 137; ^{1}H (300 MHz) and ^{13}C (75 MHz, CD_3OD) NMR data, and see **Table 4**.

3. Results and Discussions

3.1. Structure determination of isolated compounds from *Tilia amurensis*

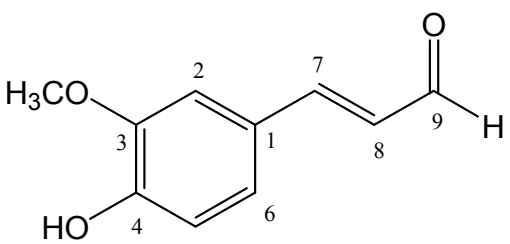
3.1.1. Structure determination of compounds 1-4

Compounds **1**-**4** were isolated as white amorphous powders, their ^{1}H and ^{13}C NMR spectra were similar with signals assignable for an aromatic benzen ring, and the differences between each compound were the substitution of the different functional groups. Compound **1** displayed a methoxy group at C-3 (δ_H 3.93, δ_C 56.9), an aldehyde moiety at δ_H 9.63 (1H, d, J = 7.8) and δ_C 193.9, and a pair of proton signals which can be assigned for H-7 at δ_H 7.57 (1H, d, J = 15.6) and H-8 at δ_H 6.66 (1H, dd, J = 15.6, 7.8) with corresponding carbon at 154.1 and 127.1, respectively. Two oxygenated quaternary carbons (δ_C 150.9 and 148.9) and one quaternary carbon (δ_C 127.5) were found. Thus structure of compound **1** was characterized as shown with the name (*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylaldehyde. [44]

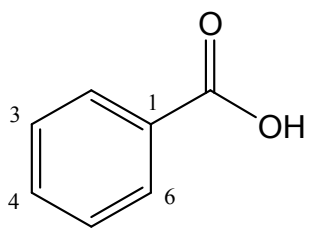
Compound **2** was a benzoic acid with a carboxylic carbon at δ_C 173.0, and the signals for benzene ring were appeared at δ_H 8.02 (2H, br, d, J = 9.0, H-2/H-6), 7.47 (2H, m, H-3/H-5), and 7.59 (1H, m, H-4).[45]

The ^{1}H and ^{13}C NMR spectra of compound **3** were similar to those of compound **1** with an aldehyde group at δ_H 9.75 (1H, s) and δ_C 193.0, a methoxyl group at C-3 (δ_H 3.92, δ_C 56.5), and an ABX aromatic proton spin system (δ_H 6.94, 7.43, and 7.44) were also found. However, two proton signals which can be assigned for H-7 at δ_H 7.57 (1H, d, J = 15.6) and H-8 at δ_H 6.66 (1H, dd, J = 15.6, 7.8) with corresponding carbon at 154.1 and 127.1 in compound **1** were not appeared in compound **3**. Therefore, compound **3** was thus determined to be 4-hydroxy-3-methoxybenzaldehyde.[46]

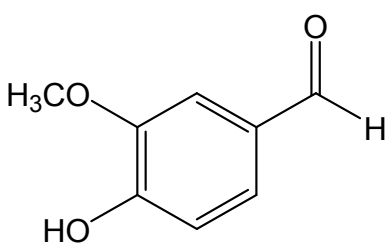
Compound **4** was an acid derivative of compound **2** with an carboxylic group at δ_C 174.4. However, 1H NMR spectrum displayed only one aromatic proton signal at δ_H 7.05 (2H, s) with corresponding carbon at δ_C 110.2, which was assigned for H-2 and H-6. The ^{13}C NMR spectrum of compound **4** displayed three oxygenated quaternary carbons at δ_C 146.5 (2 x C) and 139.5, and a quaternary carbon at δ_C 122.0. This observation revealed a 1,3,4,5-tetrasubstituted ring. Thus, compound **4** was elucidated as 3,4,5-trihydroxybenzoic acid [47] as shown in the figure below.



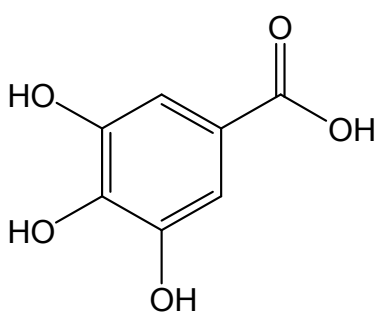
Compound 1



Compound 2



Compound 3



Compound 4

Chemical structure of isolated compounds **1-4**

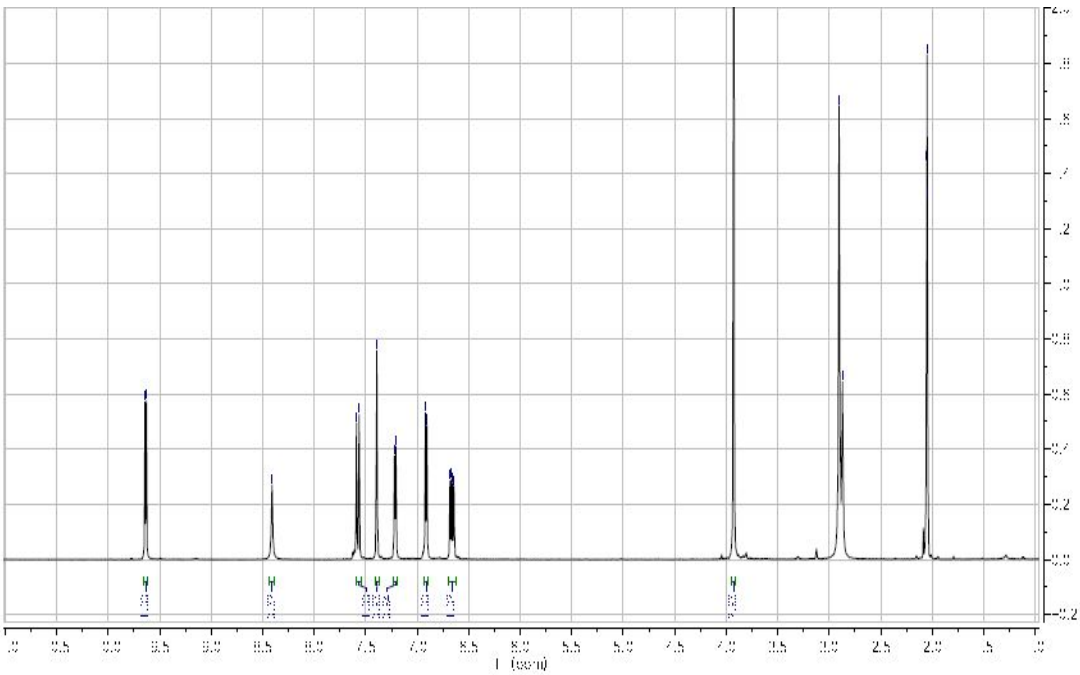


Fig. 8. ^{1}H-NMR spectrum of compound **1** (600 MHz, acetone-d_6)

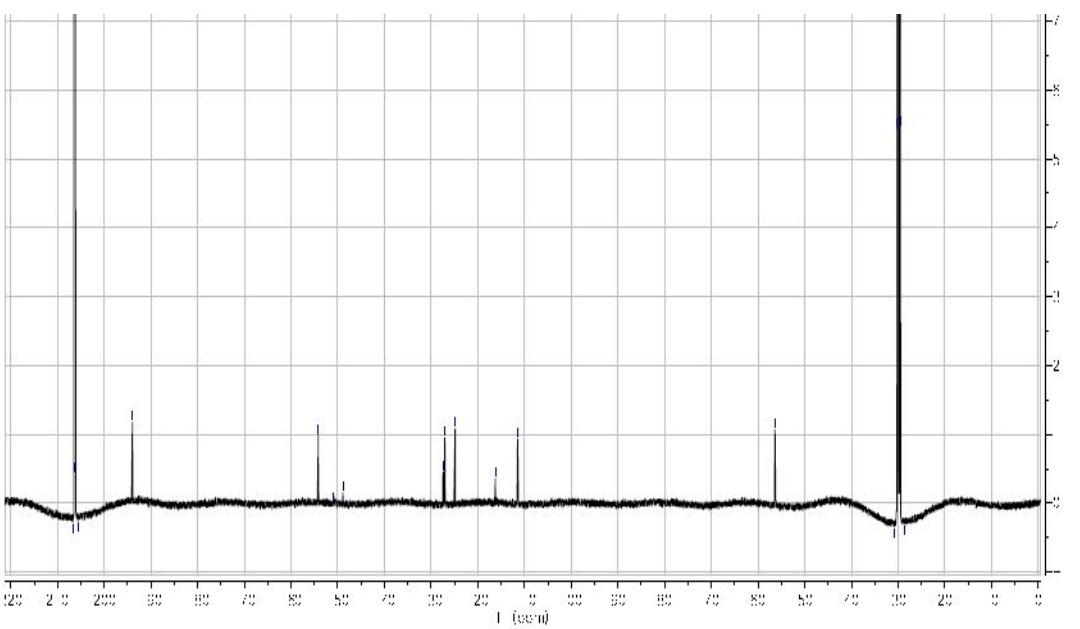


Fig. 9. ^{13}C-NMR spectrum of compound **1** (150 MHz, acetone-d_6)

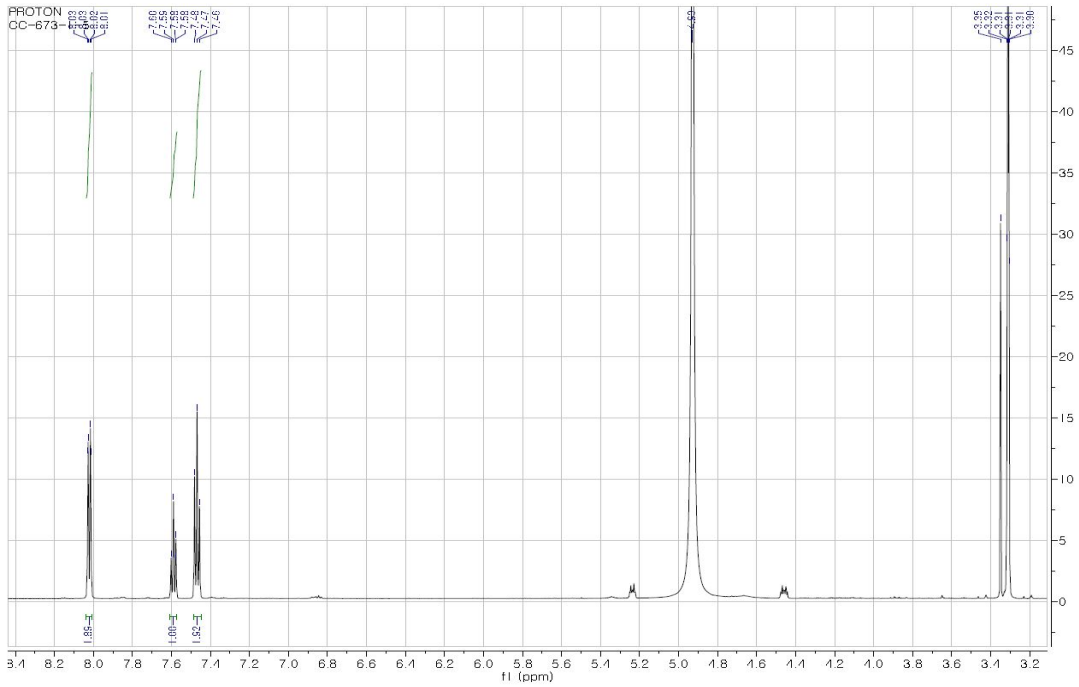


Fig. 10. ^{1}H-NMR spectrum of compound **2** (600 MHz, CD_3OD)

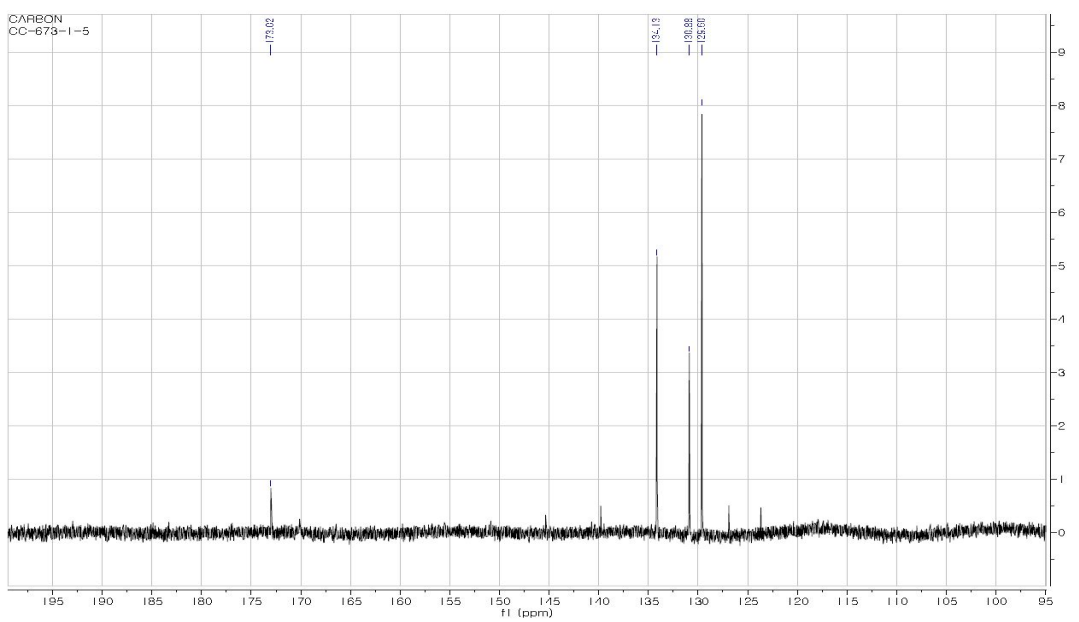


Fig. 11. ^{1}H-NMR spectrum of compound **2** (150 MHz, CD_3OD)

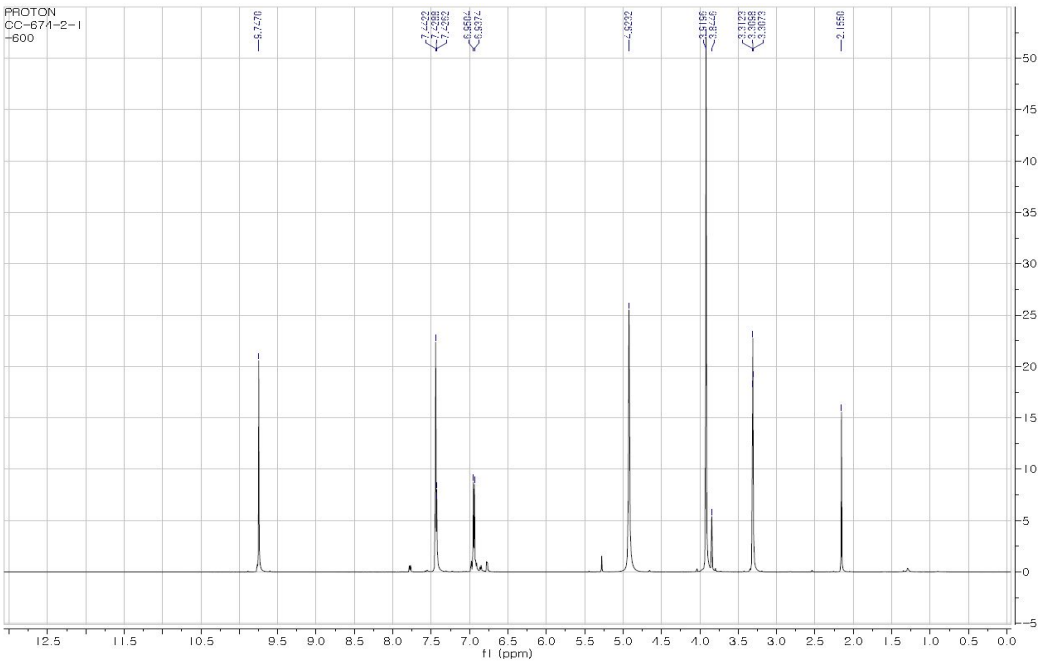


Fig. 12. ^{1}H-NMR spectrum of compound **3** (600 MHz, CD_3OD)

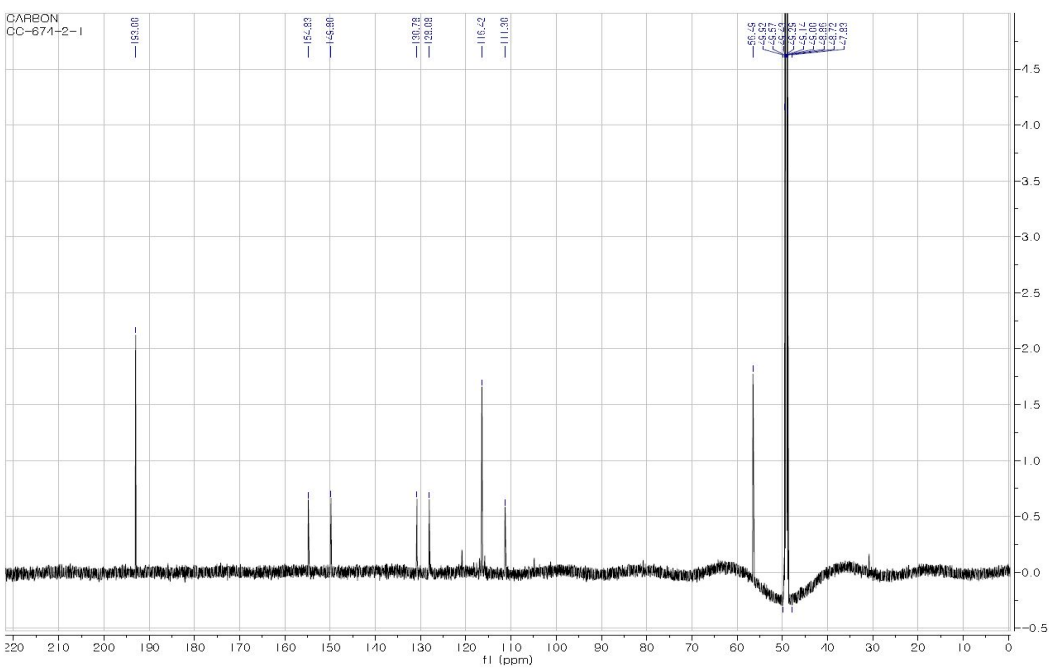


Fig. 13. ^{1}H-NMR spectrum of compound **3** (150 MHz, CD_3OD)

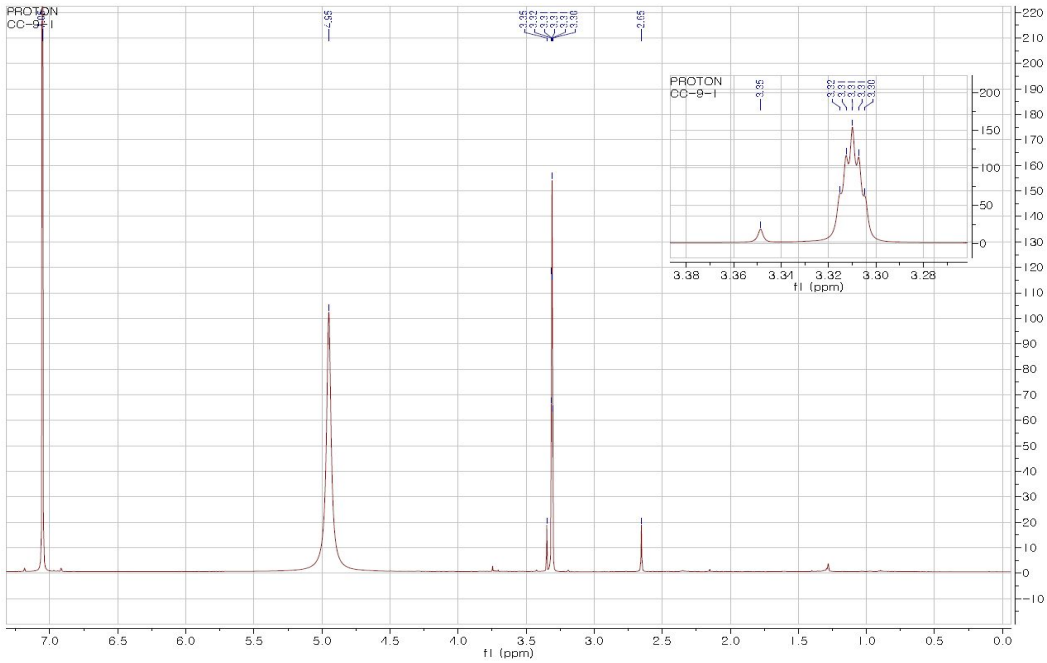


Fig. 14. ^{1}H-NMR spectrum of compound **4** (600 MHz, CD_3OD)

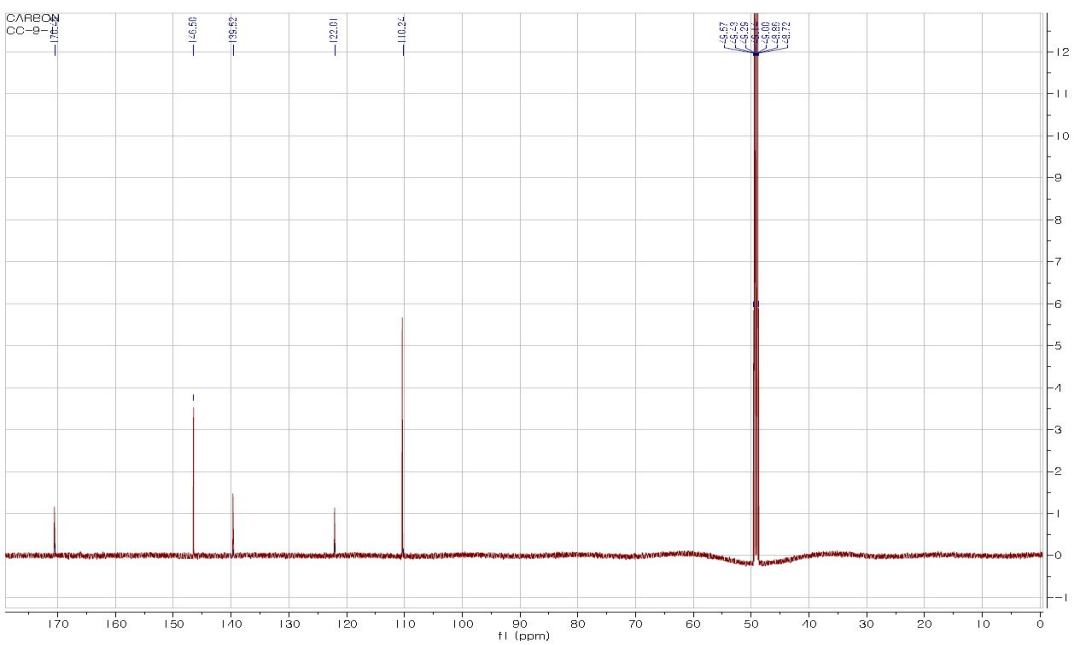
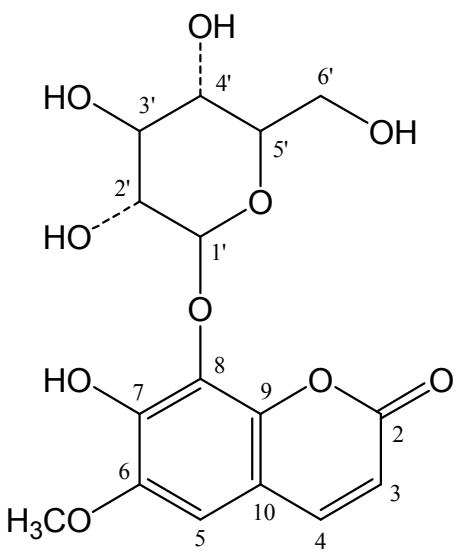


Fig. 15. ^{1}H-NMR spectrum of compound **4** (150 MHz, CD_3OD)

3.1.2. Structure determination of compound 5

Compound **5** was obtained as a white amorphous powder with molecular formula $C_{16}H_{18}O_{10}$ as determined by FAB-MS with m/z = 393 $[M+Na]^+$. Its UV (MeOH) spectrum displayed maxima at 230, 256, 261, and 348 nm. The ^1H and ^{13}C NMR spectra showed a characteristic of a coumarin derivative with typical aromatic proton signals at δ_H 6.25 (1H, d, *J* = 9.3, H-3) and 7.88 (1H, d, *J* = 9.3, H-4) with corresponding carbons at δ_C 113.1 (C-3) and 146.6 (C-4), and the carboxylic carbon at δ_C 163.7 (C-2). In addition, a methoxy moiety was observed at δ_H 3.90 (3H, s) and δ_C 57.0. The remaining one aromatic proton singlet at 6.99 ppm with the corresponding carbon at 106.2, and four oxygenated quaternary carbons at δ_C 147.6 (C-6), 133.7 (C-7), 144.5 (C-8), and 135.9 (C-9) indicated that ring A was 6, 7, and 8 substitution by oxygenated functional group (OH and or OCH_3). In addition, the ^1H and ^{13}C NMR spectra of **5** revealed a glucose moiety (The ^1H NMR multiplet signals appeared in the 3.2- to 4-ppm region, the ^{13}C NMR signals appeared from 62.3 to 78.6 ppm), with an anomeric proton doublet at δ_H 5.00 (1H, d, *J* = 7.8, H-1') and the corresponding carbon at 106.1 ppm further evidenced this conclusion. Comparison of its ^1H and ^{13}C NMR spectroscopic data of compound **5** with those published in literature led to the identification of compound **5** to be fraxin, a 7-hydroxy-6-methoxy-8-O-β-D-glucocoumarin. [17, 18]



Chemical structure of compound **5** fraxin

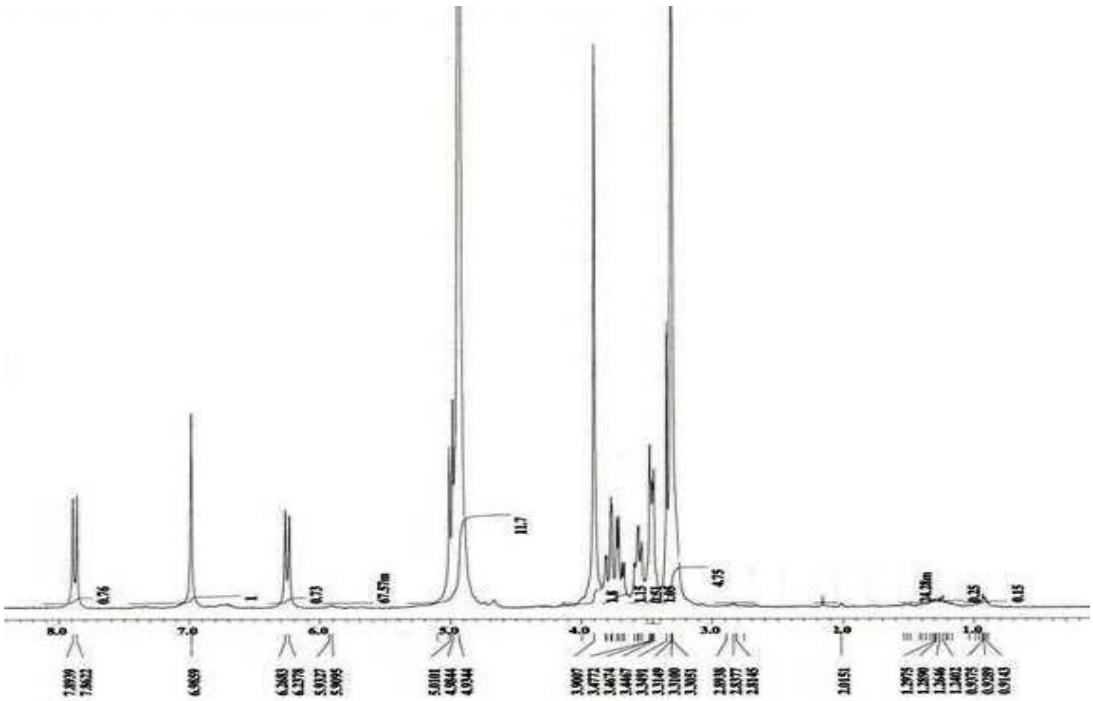


Fig. 16. ^{1}H-NMR spectrum of compound **5** (300 MHz, CD_3OD)

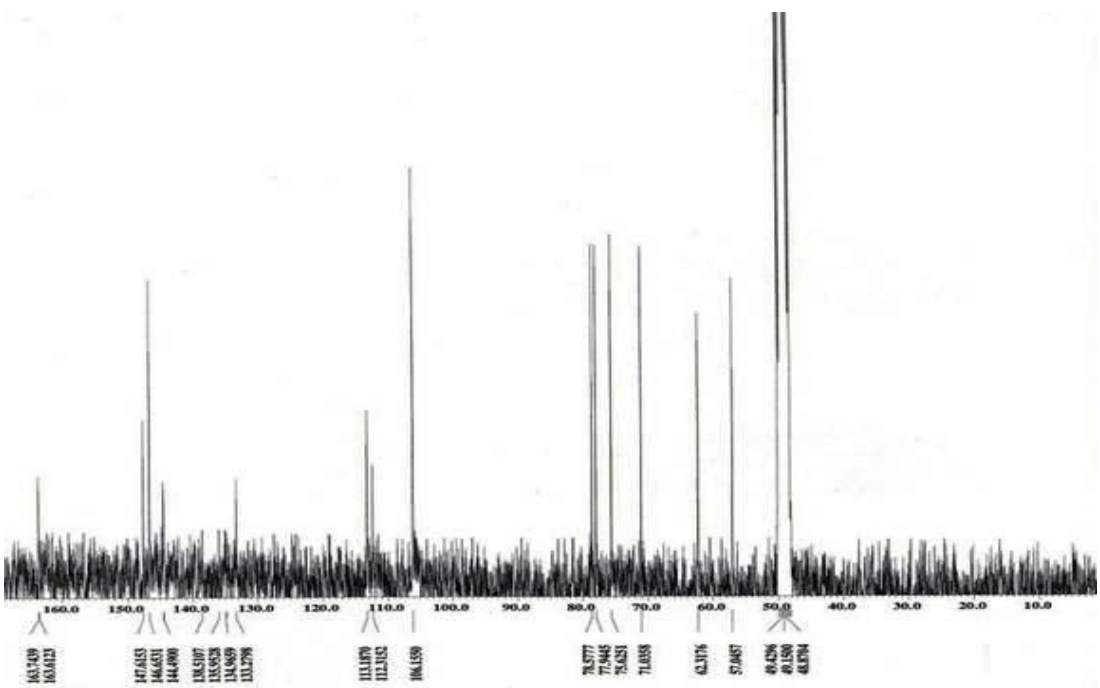
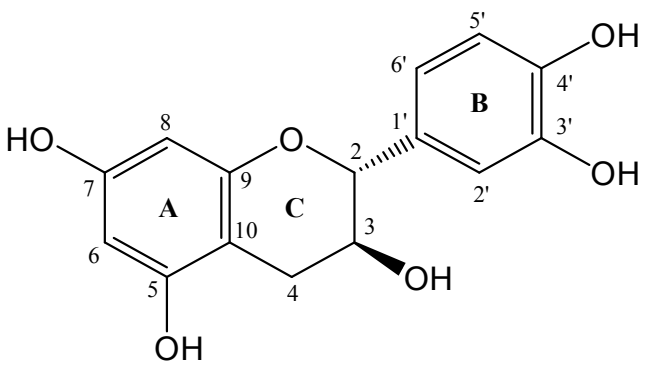


Fig. 17. ^{13}C-NMR spectrum of compound **5** (75 MHz, CD_3OD)

3.1.3. Structure determination of compound 6

Compound **6** was obtained as amorphous brown powder with molecular formula of $C_{15}H_{14}O_6$. Its IR spectra (KBr) showed band at 3400 (OH), 1620, 1520, 1470, 1380, 1280, 1240, 1150, 1120, 1080, 1020, and 820 cm^{-1}. The mass spectra showed maximum at 290 and minimum at 55. The other fragments were seen at 139, 138, 110, 152, 151 and 123. The UV spectra (MeOH) showed absorption maxima at 277 and 220 nm. The ^1H NMR gave a group of typical ABX coupling proton signals [δ_H 6.97 (1H, d, J = 1.8 Hz), 6.75 (1H, d, J = 8.1 Hz), and 6.80 (1H, dd, J = 8.0, 1.8 Hz)], which could be assignable for ring B, and a group of *meta*-aromatic proton signals [δ_H 5.93 (1H, d, J = 2.1 Hz, H-5), and 5.92 (1H, d, J = 2.1 Hz, H-6)], ring A. Its ^{13}C NMR spectrum showed 15 carbon signals including one methylene (δ 29.4) and two oxymethines at δ 67.6 (C-3) and 80.0 (C-2), respectively. The ^1H and ^{13}C NMR spectra together with the UV data of **1** revealed a 5, 7, 3', 4'-substituented flavan-3-ol skeleton. The coupling pattern of H-2 at δ 4.56 (1H, d, J = 7.0 Hz) and the chemical shifts of C-2 (δ 80.0) and C-3 (δ 67.6) indicated that H-2 and H-3 of **6** were in the trans-form. Comparison of our data for compound **6** with those reported in literature led us to conclude that compound **6** was (+)-catechin.[13, 14]



Chemical structure of compound **6** (+)-catechin

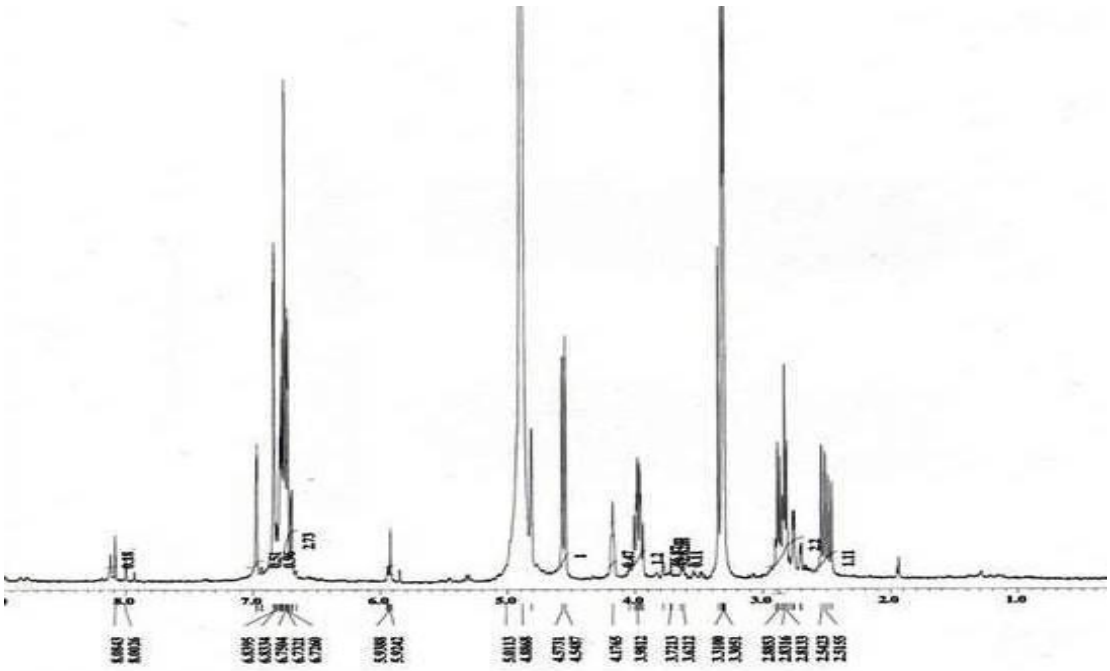


Fig. 18. ^{1}H-NMR spectrum of compound **6** (300 MHz, CD_3OD)

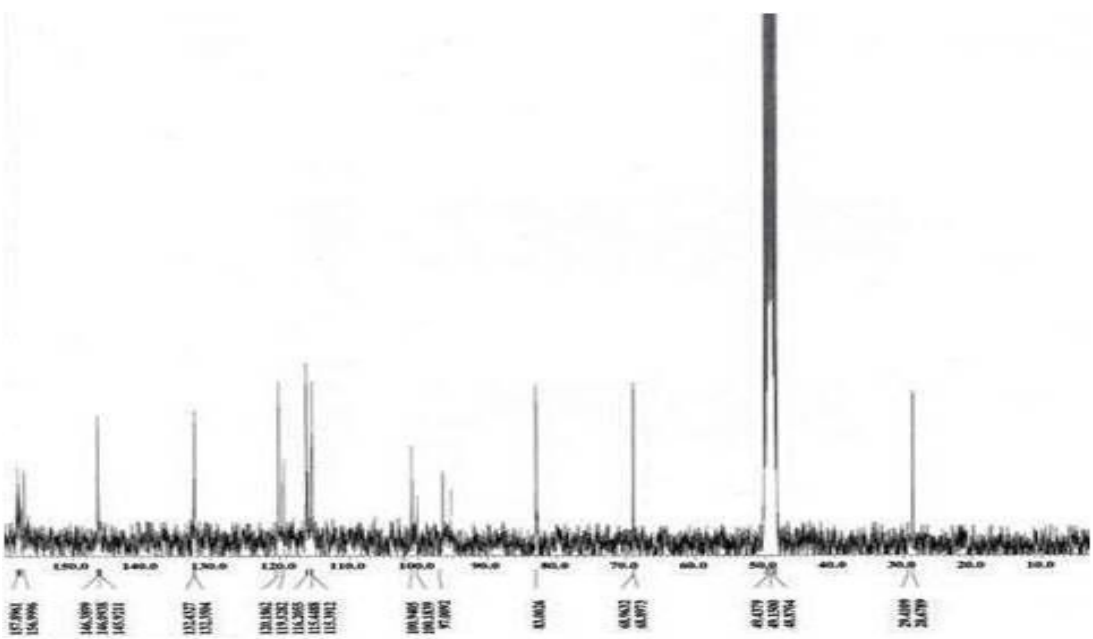
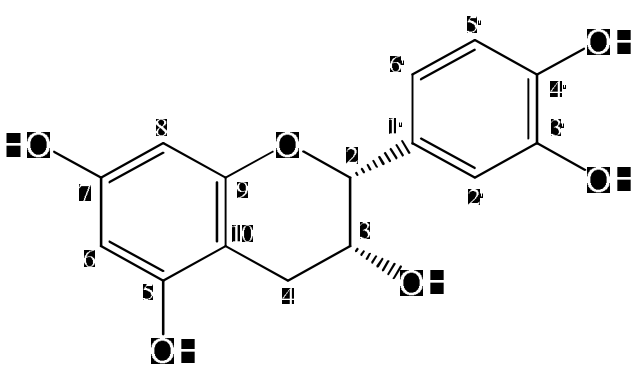


Fig. 19. ^{13}C-NMR spectrum of compound **6** (75 MHz, CD_3OD)

3.1.4. Structure determination of compound 7

Compound **7** was an white amorphous solid with molecular formula of $C_{15}H_{14}O_6$ from positive ES-MS: m/z 313 $[M + Na]^+$, 391 $[M + H]^+$. The ^1H and ^{13}C NMR spectra of compound **7** closely resembled those of (+)-catechin (**6**). The ^1H NMR also gave a group of typical ABX coupling proton signals [δ_H 6.97 (1H, d, J = 1.8 Hz), 6.69 (1H, d, J = 8.1 Hz), and 6.81 (1H, dd, J = 8.1, 1.8 Hz)], which were belong to the B ring, two oxygenated quaternary carbons at 146.1 (C-3') and 146.2 (C-4') further supported for this observation. A group of *meta*-aromatic proton signals at δ_H 5.95 (1H, s, H-6), and 5.95 (1H, s, H-8) and the remaining three oxygenated quaternary carbons at 158.0 (C-5), 157.9 (C-7), and 157.4 (C-9) were fully indicated for ring A. In addition, its ^{13}C NMR spectrum showed carbon signals assignable for one methylene (δ 27.0, C-4) and two oxymethines δ_C 70.1 (C-3) and 78.8 (C-2), (see **Table 2** and **3**). All of the physicochemical data and the 1D NMR spectroscopic data of **7** suggested a 5, 7, 3', 4'-substituented flavan-3-ol skeleton. However, the coupling pattern of H-2 at δ 5.03 (1H, br, s) and the chemical shifts of C-2 (δ 78.8) and C-3 (δ 70.1) indicated that H-2 and H-3 of **7** were in the *cis*-form. Therefore, compound **7** was elucidated to be (-)-epicatechin.[15]



Chemical structure of compound **7** (-)-epicatechin

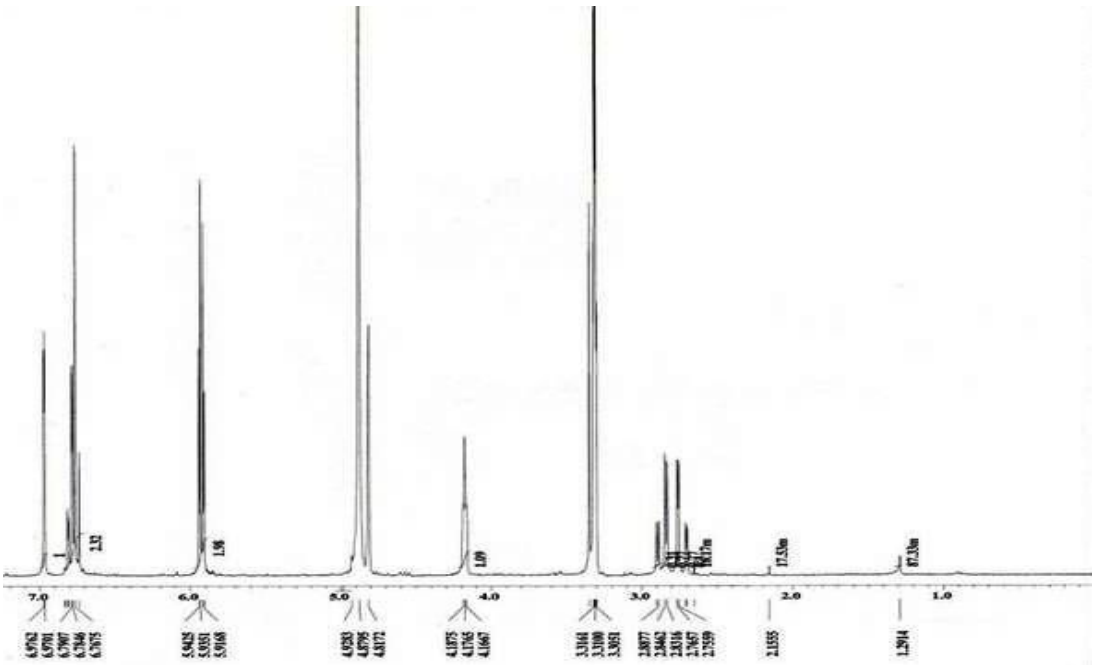


Fig. 20. ^{1}H-NMR spectrum of compound **7** (300 MHz, CD_3OD)

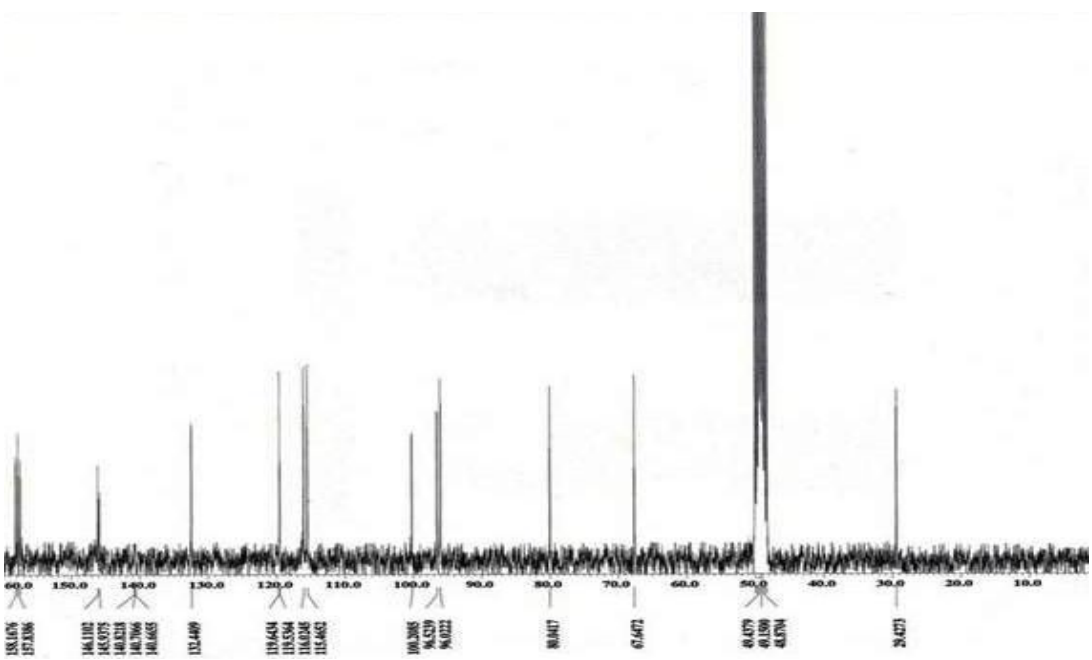
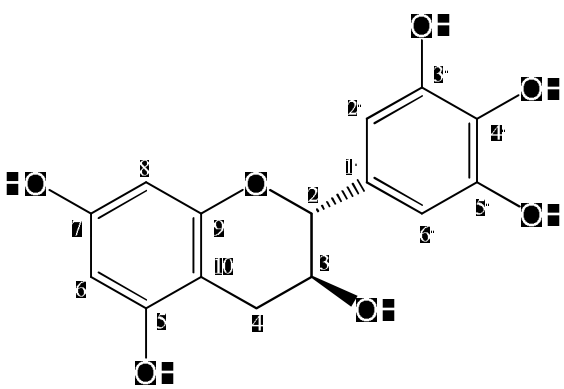


Fig. 21. ^{13}C-NMR spectrum of compound **7** (75 MHz, CD_3OD)

3.1.5. Structure determination of compound 8

Compound **8** was obtained as a white amorphous solid with molecular formula of $C_{15}H_{14}O_7$. The Electro Spray Mass Spectrum of compound **8** gave quasi-molecular ions at 307 m/z $[M+H]^+$ and 329 m/z $[M+Na]^+$ in good agreement with the calculated mass for a molecular formula of $C_{15}H_{14}O_7$. The ^1H NMR spectrum of compound **8** displayed a doublet appearing at δ_H 4.52 (1H, d, J = 6.9 Hz, H-2) and two double doublets at δ_H 2.50 (1H, J = 17.3, 8.1 Hz, H-4α) and δ_H 2.81 (1H, J= 17.2, 5.1 Hz, H-4β) which are characteristic signals of ring C from (+)-catechin nucleus. Two doublets at δ_H 5.91 and 5.92 ppm (J = 2.4 Hz) are assigned to H-6 and H-8 protons respectively. The observation of an additional singlet at δ_H 6.41 ppm (2H, br, s, H-2'/H-6') suggested the presence of C-3', C-4' and C-5' trihydroxy group substitutions in ring B. Comparison of ^1H and ^{13}C NMR data with those of the literature, suggested that compound **8** has a catechin skeletal pattern. Three oxygenated aromatic quaternary carbons at 158.0, 157.8, and 157.0 were assigned for C-5, C-7, and C-9 in ring A, respectively. The remaining three other ones were assigned for C-3' (147.0), C-4' (131.8), and C-5' (145.3) in ring C. This observation suggested that C aromatic ring is trisubstituted by three hydroxy groups in positions 3', 4' and 5', respectively In conclusion, analysis of **8** and 1D NMR spectra compared with the literature values were allowed to propose for compound **8** as the structure of (+)-gallocatechin.[16]



Chemical structure of compound **8** (+)-gallocatechin

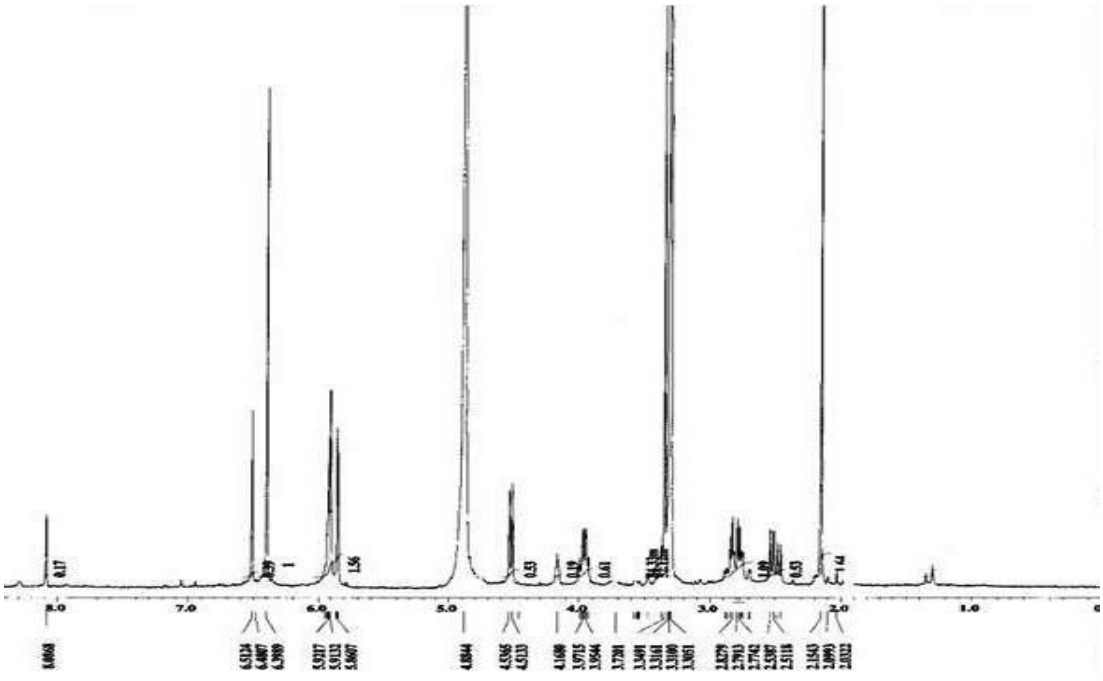


Fig. 22. ^{1}H-NMR spectrum of compound **8** (300 MHz, CD_3OD)

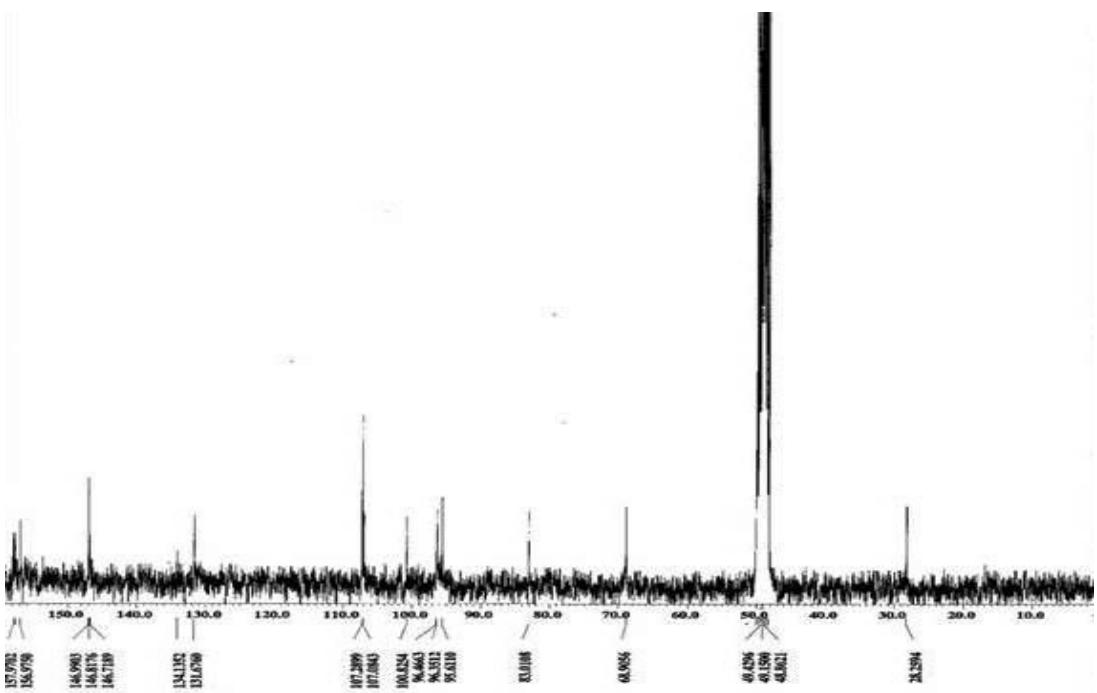
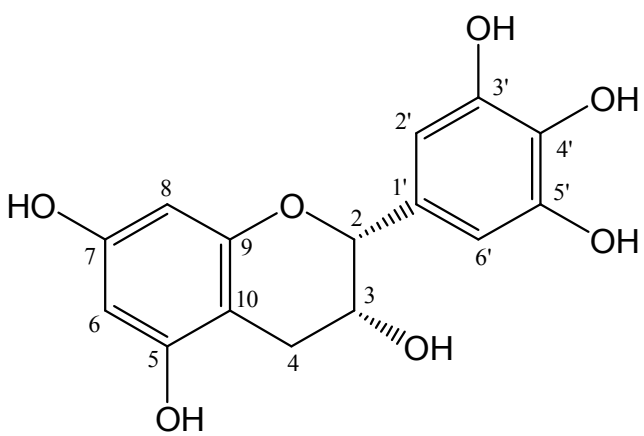


Fig. 23. ^{13}C-NMR spectrum of compound **8** (75 MHz, CD_3OD)

3.1.6. Structure determination of compound 9

Compound **9** was obtained as a white amorphous solid with molecular formula $C_{15}H_{14}O_7$. The Electro Spray Mass Spectrum of compound **9** gave quasi-molecular ions at 307 m/z $[M+H]^+$ and 329 m/z $[M+Na]^+$ in good agreement with the calculated mass for a molecular formula of $C_{15}H_{14}O_7$. When the ^1H and ^{13}C NMR spectra of compound **9** were compared with those of compound **9** suggested that they were identical. The ^{13}C NMR spectra gave 15 carbons, of which 6 were of oxygenated quaternary carbons (ring A, and ring B), and the others 6 carbons were of aromatic methines and 2 quaternary carbons. The ^1H NMR spectrum also gave two set of meta-coupled aromatic protons for ring A [5.91 (1H, d, *J* = 2.1, H-6), 5.93 (1H, d, *J* = 2.1, H-8), and 6.51 (2H, br, s, H-2'/H-6'). However, the coupling patterns of H-2 at δ 4.75 and H-3 (4.17) were appeared as broad singlet suggested that H-2 and H-3 of **9** were in the *cis*-form. Thus, compound **9** was determined to be (-)-epigallocatechin.[16]



Chemical structure of compound **9** (-)-epigallocatechin

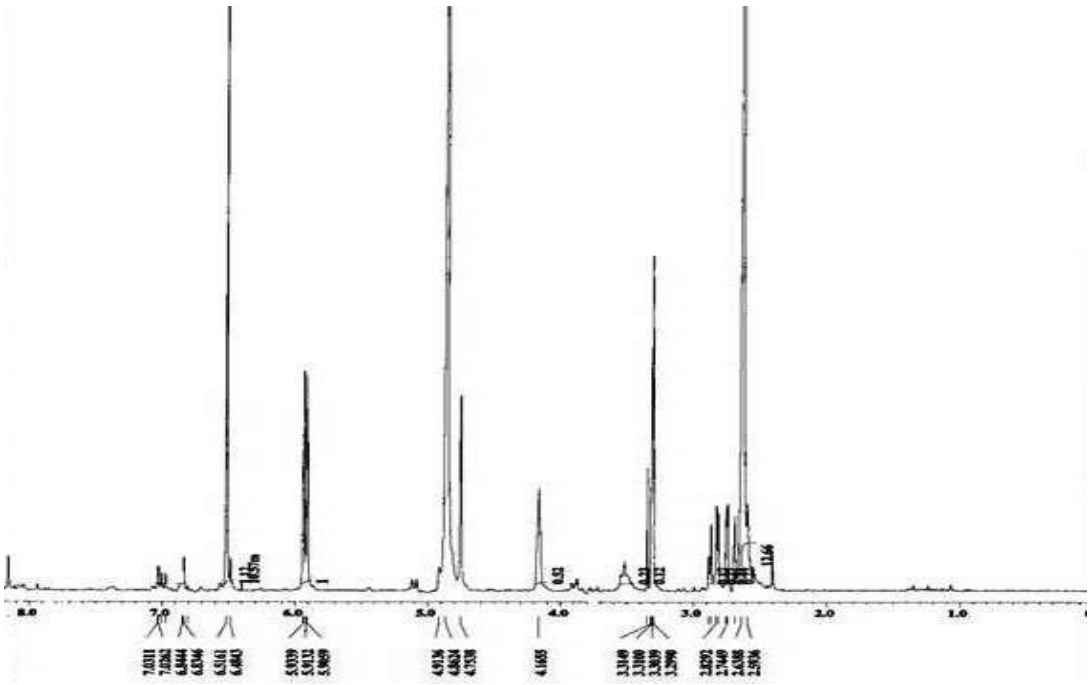


Fig. 24. ^{1}H-NMR spectrum of compound **9** (300 MHz, CD_3OD)

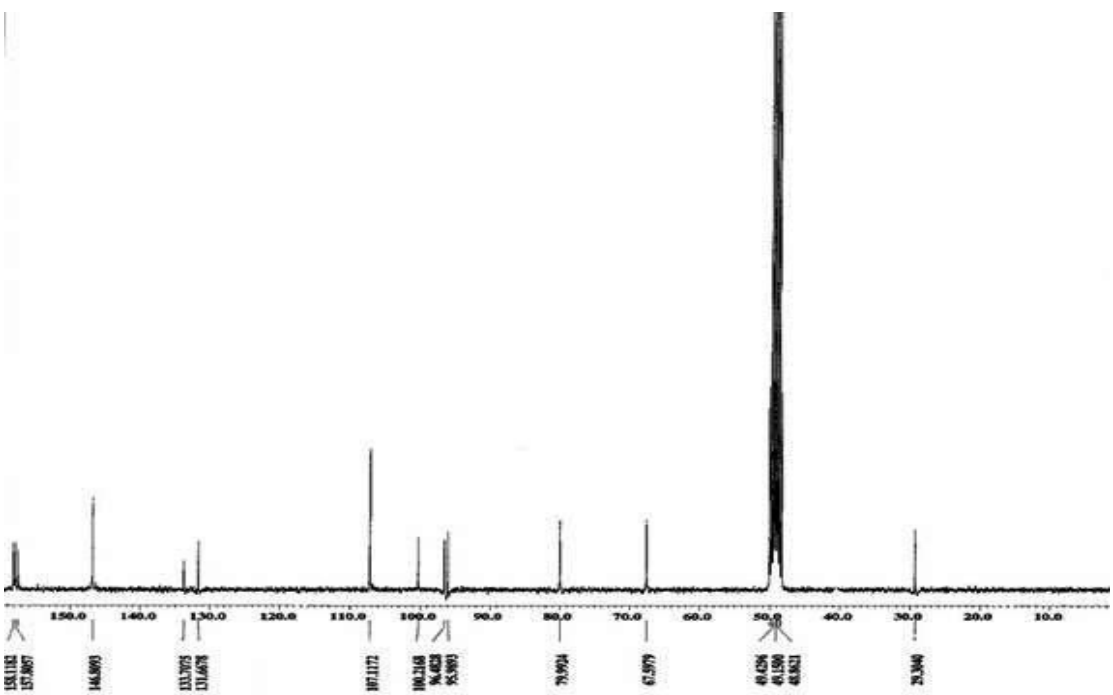
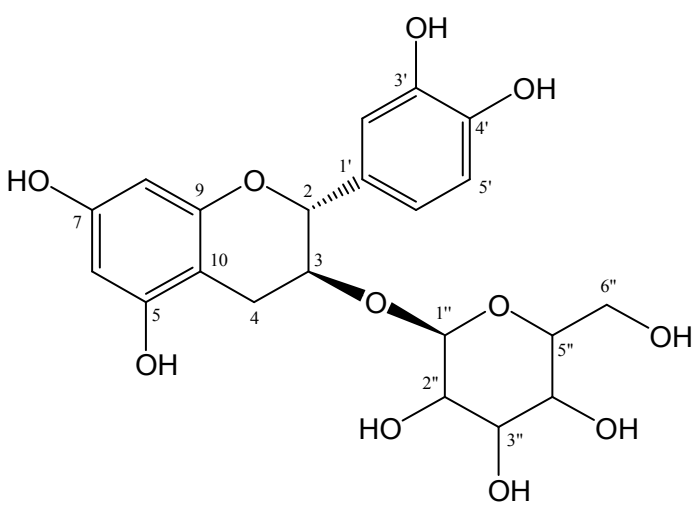


Fig. 25. ^{13}C-NMR spectrum of compound **9** (75 MHz, CD_3OD)

3.1.7. Structure determination of compound 10

Compound **10** was obtained as colorless needles (H_2O) with molecular formula $C_{21}H_{24}O_{11}$. The ^{1}H and ^{13}C NMR spectra of compound **10** closely resembled those of (+)-catechin (**6**), except for the presence of additional signals in the oxymethine regions. The appearance of an anomeric proton signal at δ_H 4.59 (1H, d, J = 7.2 Hz) with corresponding carbon at δ_C 102.5 (C-1"), as well as five aliphatic carbon signals at 78.2 (C-5"), 78.1 (C-2"), 75.0 (C-3"), 71.5 (C-4"), and 62.6 (C-6") with corresponding protons from 3.40 to 3.90 ppm. These proton and carbon chemical shifts are clearly revealed those of methyl β-D- glucoside, this observation fully suggested that compound **10** is a glucoside of (+)-catechin (**6**). Furthermore, the fairly downfield shift at δ_C 68.7 of the C-3 signals, as compared with that of compound **6** (δ 67.6), clearly indicated the location of the glucose moiety was at the C-3 position. Therefore, the structure of compound **10** was thus determined to be (+)-catechin-3-O-glucopyranoside .[14]



Chemical structure of compound **10** (+)-catechin-3-O-glucopyranoside

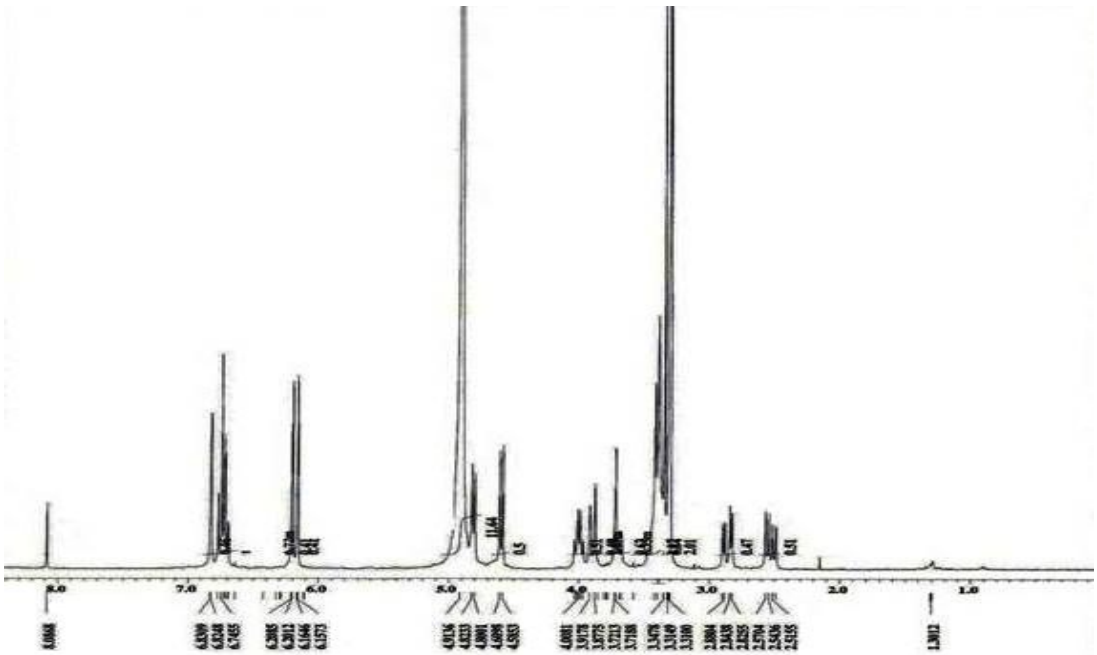


Fig. 26. ^{1}H-NMR spectrum of compound **10** (300 MHz, CD_3OD)

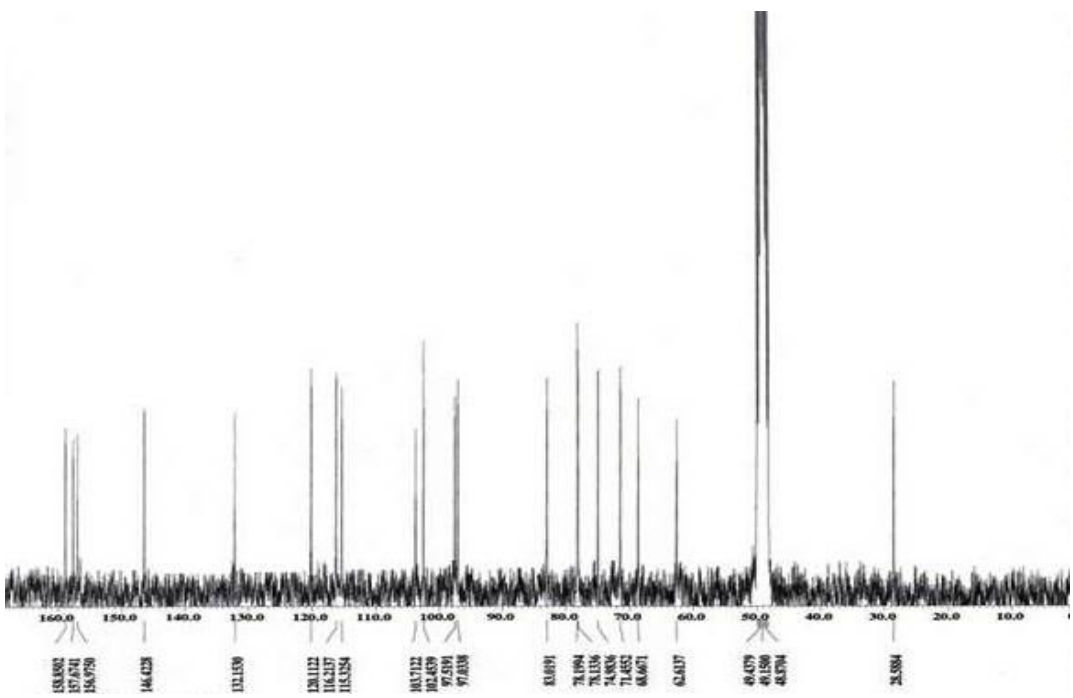
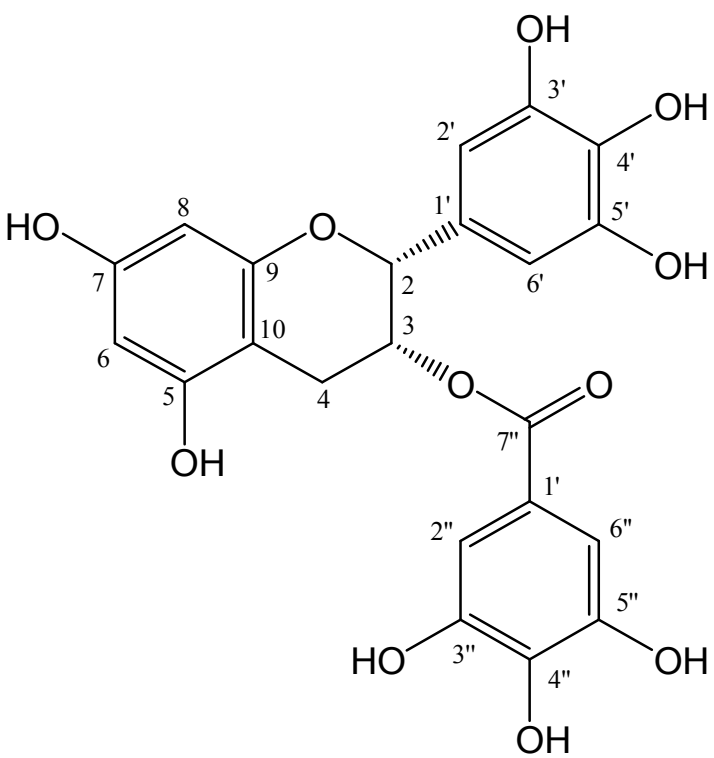


Fig. 27. ^{13}C-NMR spectrum of compound **10** (75 MHz, CD_3OD)

3.1.8. Structure determination of compound 11

Compound **11** was obtained as amorphous powder with a molecular formula of $C_{22}H_{18}O_{11}$. Except for an additional gallate moiety (Table 4), all other signals in the ^{1}H and ^{13}C NMR spectra of compound **11** were identical with those of (-)-epigallocatechin (**9**). Two *singlet* two proton signals at δ_H 5.95 (2H, s, H-6 and H-8) and 6.50 (2H, s H-2' and H-6') revealing that the B ring was 1,3,4,5-tetrasubstitution which demonstrated an gallocatechin-type structure. The proton pattern of H-2 and H-3 were appeared as *singlet* (H-2, 4.98) and broad singlet (H-3, 5.52) in its 1H NMR spectrum indicating that the configuration at C-2 and C-3 was a *cis*-form. Therefore, compound **11** was an *epi*-type skeleton of catechin. Thus, chemical structure of compound **11** was drown as shown and characterized as (-)-epigallocatechin-3-O-gallate. [49]



Chemical structure of compound **11** (-)-epigallocatechin-3-O-gallate

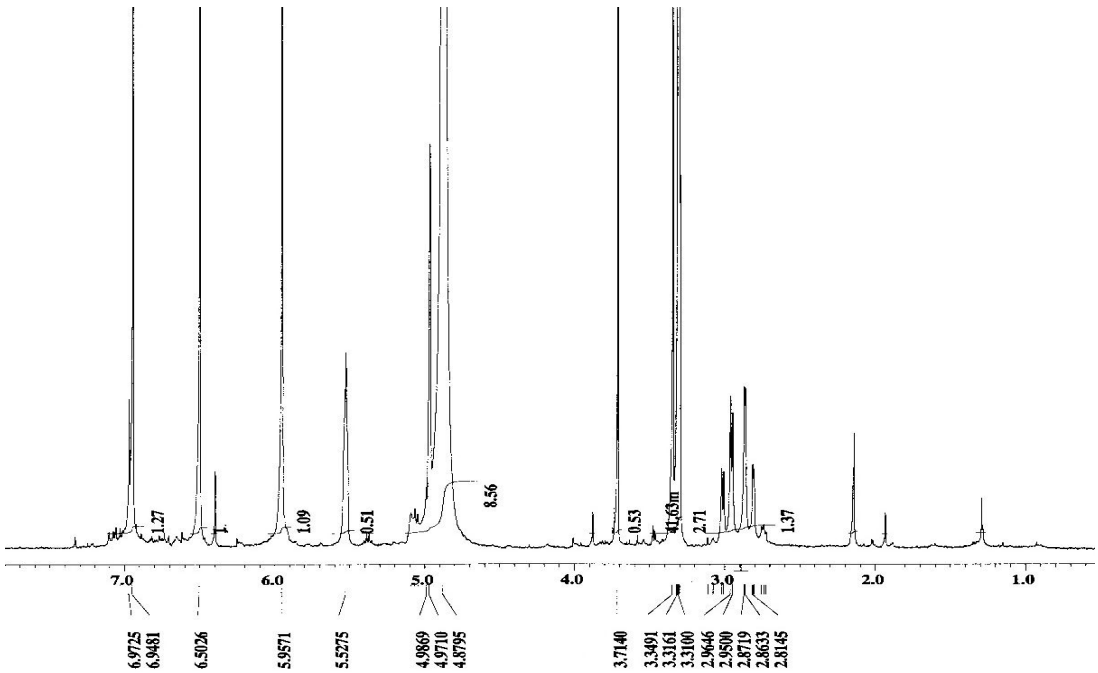


Fig. 28. ^{1}H-NMR spectrum of compound **11** (300 MHz, CD_3OD)

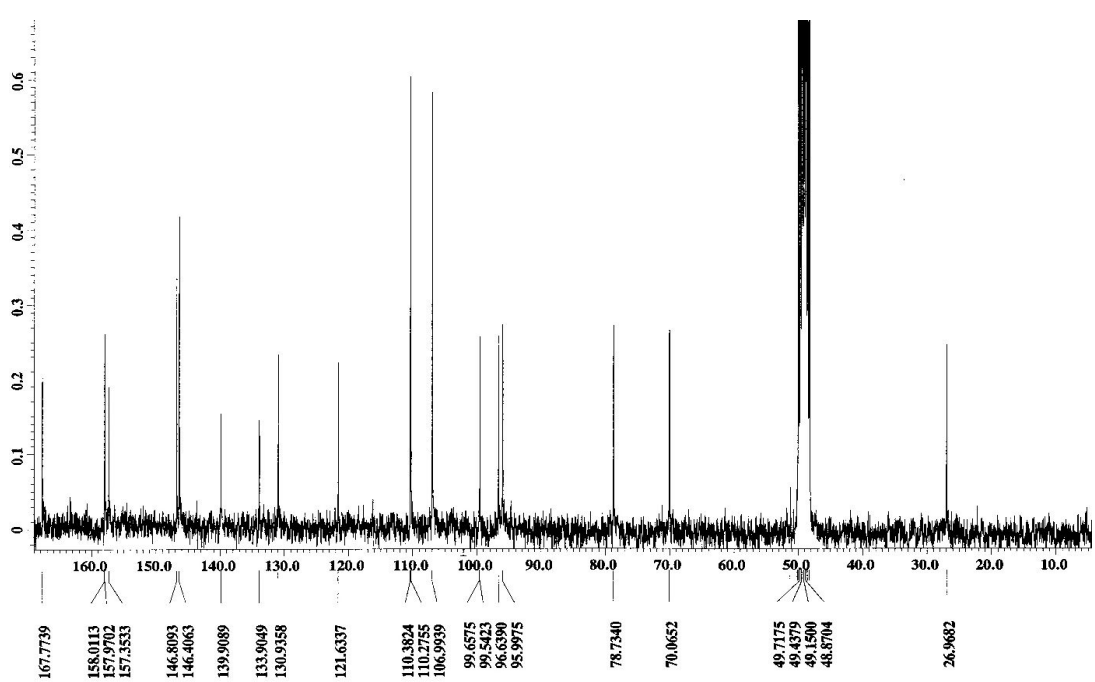
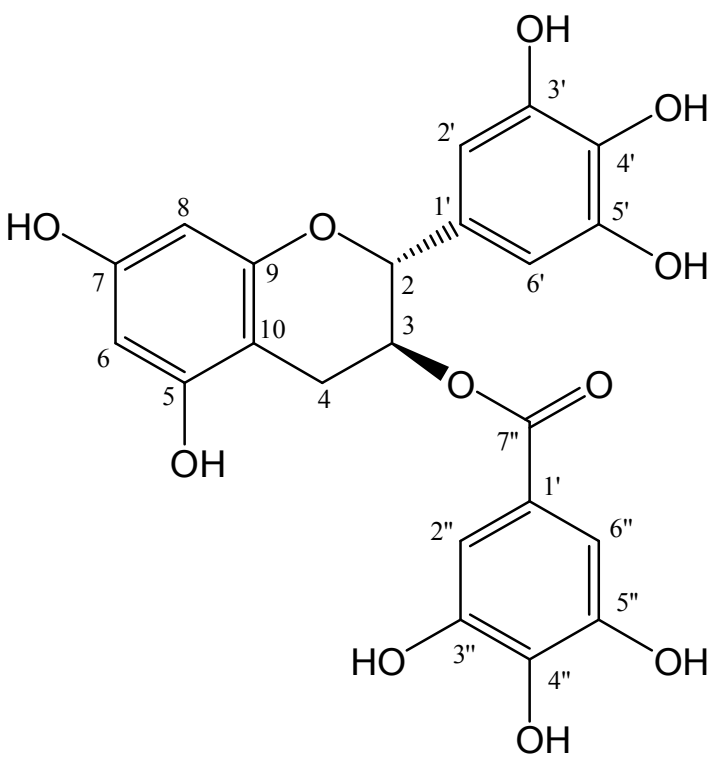


Fig. 29. ^{13}C-NMR spectrum of compound **11** (75 MHz, CD_3OD)

3.1.9. Structure determination of compound 12

Compound **12** was also obtained as amorphous powder with molecular formula of $C_{22}H_{18}O_{11}$. The ^{1}H and ^{13}C NMR spectra of compound **12** closely resembled those of (-)-epigallocatechin-3-O-gallate (**11**) with one gallate moiety [δ_H 6.95 (2H, br, s, H-2'' and H-6'') with corresponding carbon at δ_C 110.3, three oxygenated quaternary carbons at δ_C 146.5 (C-3'' and C-5'') and 139.9 (C-4'')]. Two broad singlet peaks which could be assigned for H-6 and H-8 (δ_H 5.95), and H-2' and H-6' (δ_H 6.39). However, the proton signals which assignable for H-2 and H-3 were appeared as doublet (δ_H 4.98, d, J = 5.1 Hz) and double of doublet (δ_H 5.37, dd, 8.1, 10.2). This observation indicated that the configuration at C-2 and C-3 of compound **12** was *trans*-form which demonstrated that the chemical structure of compound **12** was to be (+)-gallocatechin-3-O-gallate.



Chemical structure of compound **12** (+)-gallocatechin-3-O-gallate

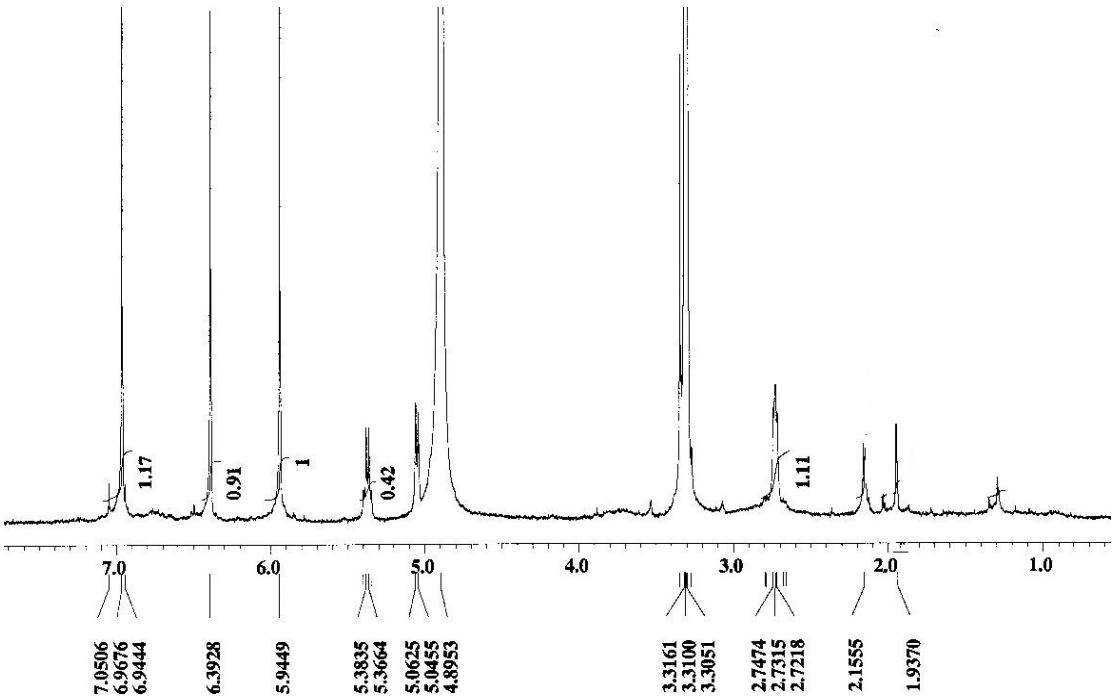


Fig. 30. ^{1}H-NMR spectrum of compound **12** (300 MHz, CD_3OD)

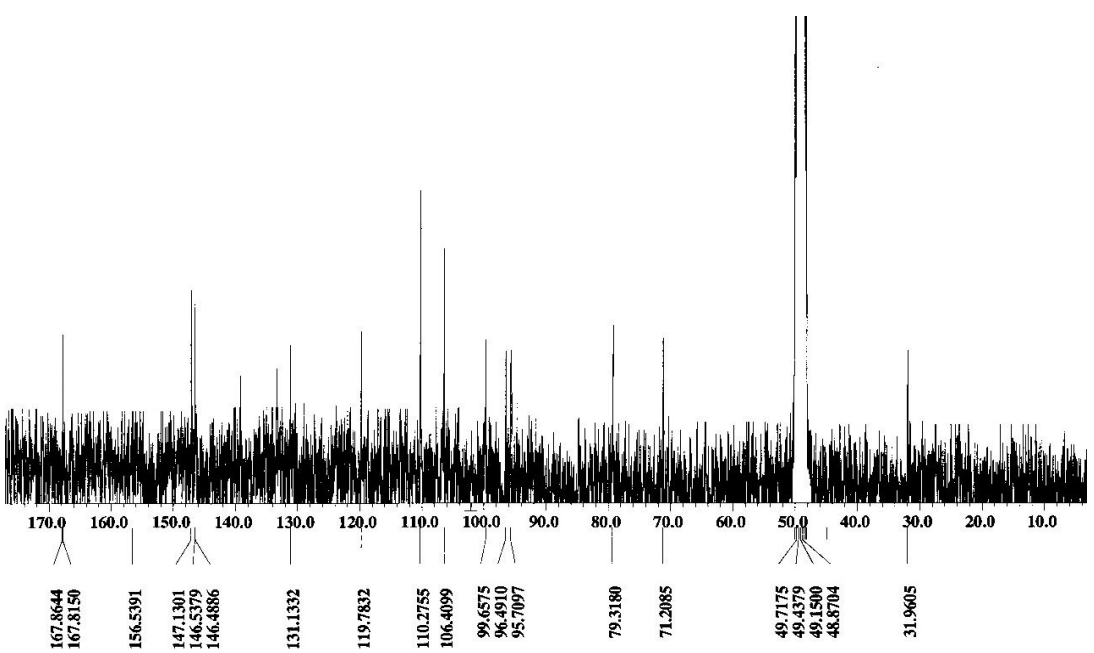
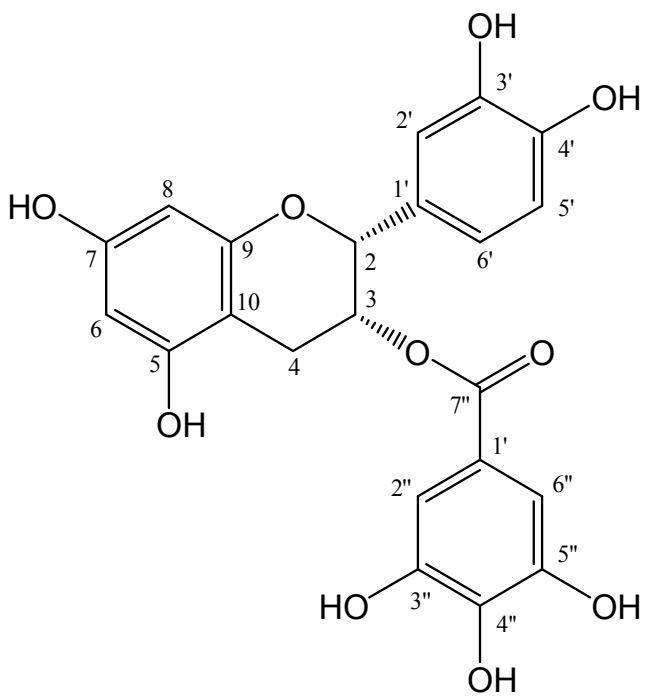


Fig. 31. ^{13}C-NMR spectrum of compound **12** (75 MHz, CD_3OD)

3.1.10. Structure determination of compound 13

Compound **13** was obtained as yellowish amorphous powder, its molecular formula was determined as $C_{21}H_{24}O_{11}$ by EIMS with m/z = 442.0902. Comparison of its ^{1}H and ^{13}C NMR spectra with those of (-)-epigallocatechin-3-O-gallate (**11**) indicated that they are identical. However, mass data showed the loss of one oxygen and an ABX aromatic spin system was observed in the ^{1}H and ^{13}C NMR spectra of compound **13** [δ_H 6.92 (1H, d, J =1.8 Hz, H-2'), 6.81 (1H, dd, J = 1.8, 8.1 Hz, H-6'), and 6.69 (1H, d, J = 8.1 Hz, H-5'), with corresponding carbon at δ_C 115.3 (C-2'), 119.5 (C-6'), and 116.1 (C-5')]. This observation revealed a 1,3,4-trisubstituted B ring indicating that compound **13** was an catechin-type skeleton. The proton pattern of H-2 and H-3 was appeared as broad *singlet* indicating that the configuration at C-2 and C-3 was *cis*-form. Therefore, compound **13** was elucidated to be (-)-epicatechin-3-O-gallate as shown in the figure below.



Chemical structure of compound **13** (-)-epicatechin-3-O-gallate

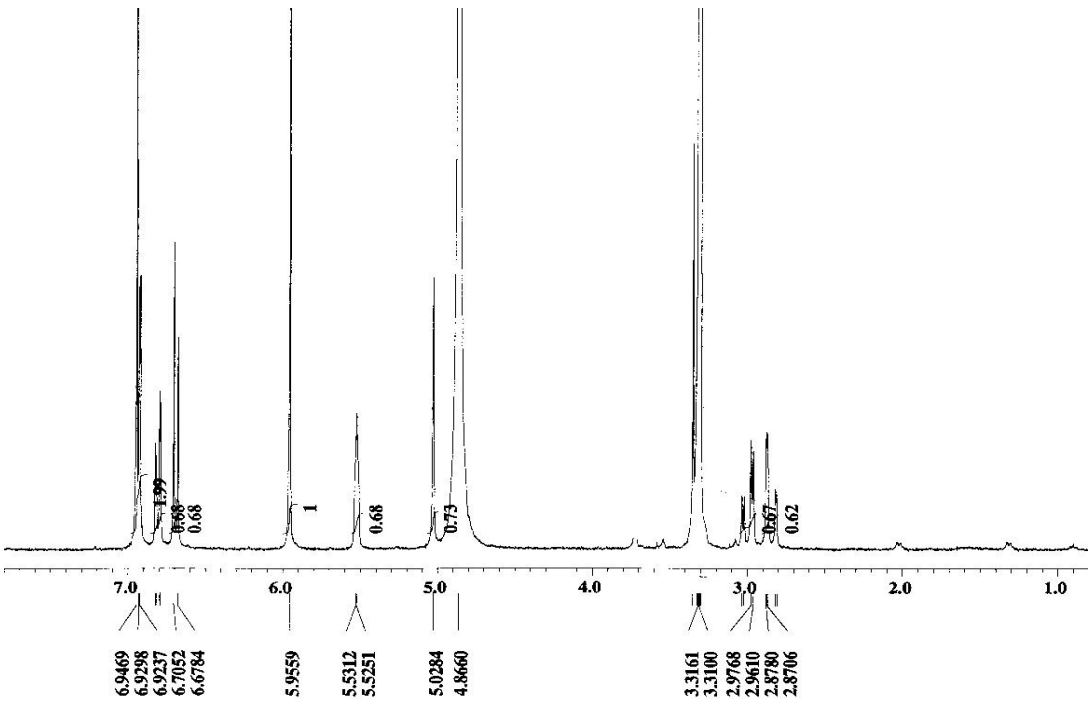
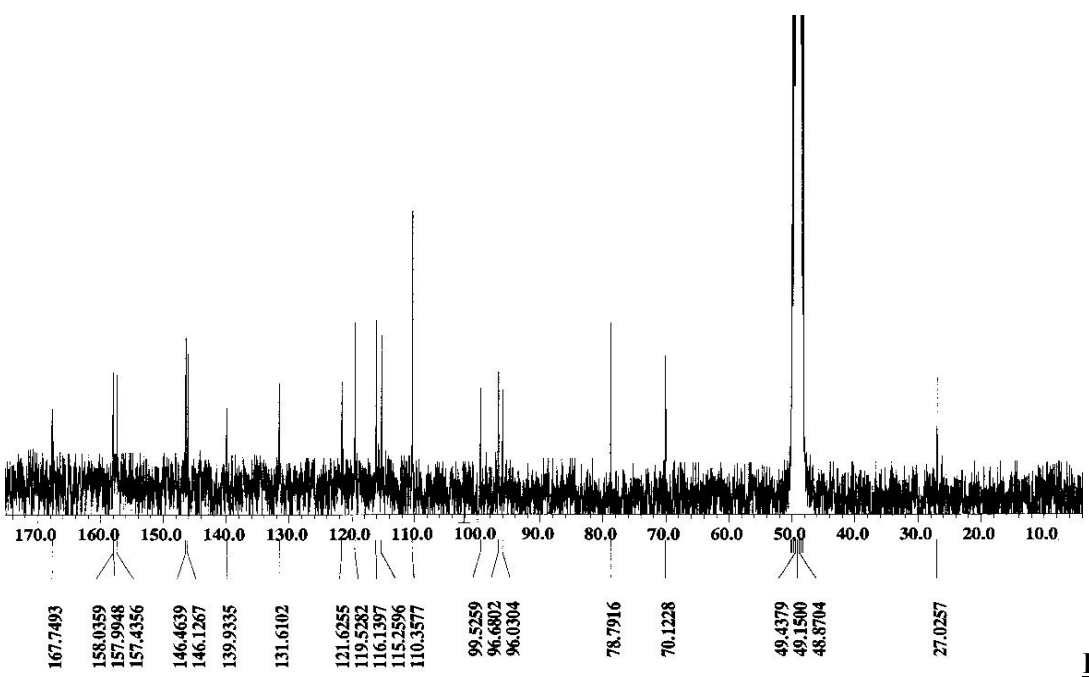
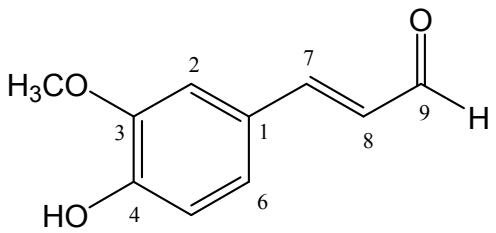


Fig. 32. ^{1}H-NMR spectrum of compound **13** (300 MHz, CD_3OD)

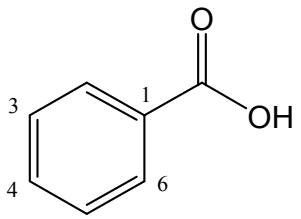


F

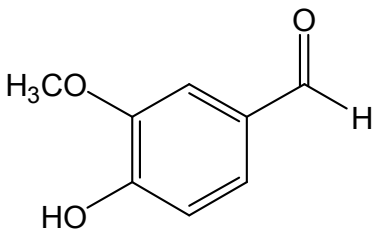
ig.33. ^{13}C-NMR spectrum of compound **13** (75 MHz, CD_3OD)



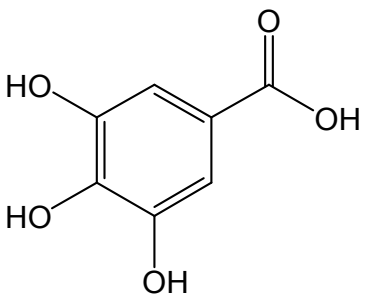
Compound **1**
(*E*)-3-(4-hydroxy-3-methoxyphenyl)acrylaldehyde



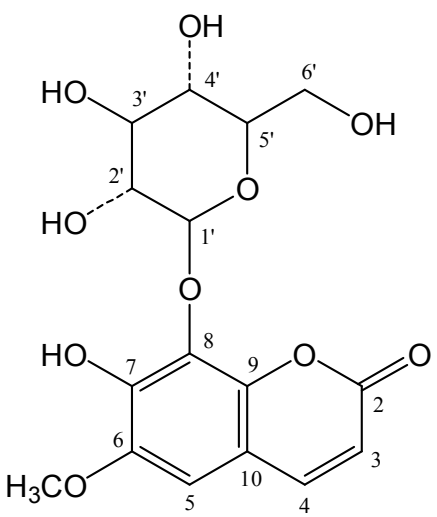
Compound **2**
benzoic acid



Compound **3**
4-hydroxy-3-methoxybenzaldehyde

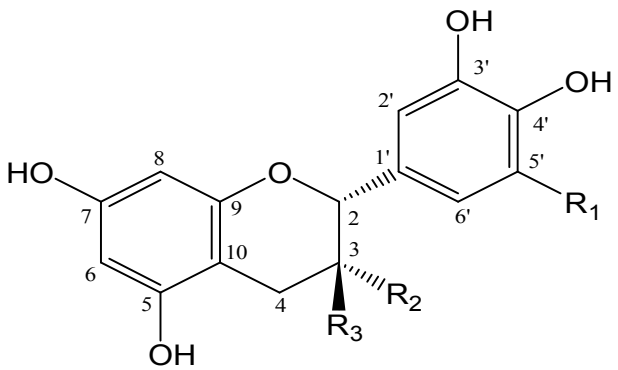


Compound **4**
3,4,5-trihydroxybenzoic acid

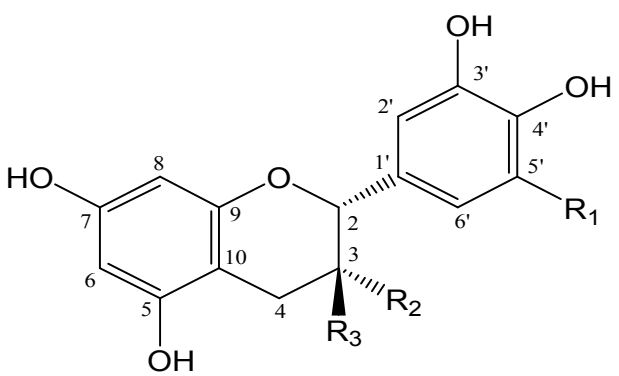


Compound **5**
Fraxin

Fig. 34. Chemical structures of isolated compounds **1-5** from *Tilia amurensis*



Flava-3-ol	R1	R2	R3
(+)-Catechin (**6**)	H	H	OH
(-)-Epicatechin (**7**)	H	OH	H
(+)-Gallocatechin (**8**)	OH	H	OH
(-)-Epigallocatechin (**9**)	OH	OH	H
(+)-Catechin-3-O-beta-D-glucopyranoside (**10**)	H	H	O-glucose



Flava-3-ol	R1	R2	R3
(-)-epigallocatechin-3-O-gallate (**11**)	OH	Gal	H
(+)-gallocatechin-3-O-gallate (**12**)	OH	H	Gal
(-)-epicatechin-3-O-gallate (**13**)	H	Gal	H

Gal =

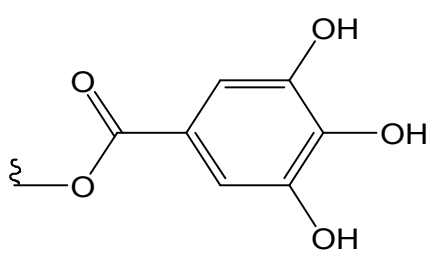


Fig. 35. Chemical structures of isolated compounds **6-13** from *Tilia amurensis*

Table 2. ^{1}H (300 MHz, CD_3OD) NMR data of isolated compounds **6-10** from *Tilia amurensis*

Pos.	**6**	**7**	**8**	**9**	**10**
	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	4.56, d, 7.0	5.03, s, br	4.52, d, 6.9	4.75, s	4.81, d, 6.9
3	3.99, ddd	5.52, m	3.96, m	4.17, br, s	4.00, ddd
4	2.73, dd, 8.1, 17.2 2.86, dd, 5.1, 17.2	2.70, dd, 5.4, 17.4 2.84, dd, 2.5, 17.4	2.50, dd, 8.1, 17.2 2.81, dd, 5.1, 17.2	2.50, dd, 8.1, 17.2 2.81, dd, 5.1, 17.2	2.86, dd, 5.1, 16.2 2.54, dd, 2.5, 16.2
5					
6	5.92, d, 2.1	5.95, s	5.91, d, 2.4	5.91, d, 2.1	6.16, d, 2.1
7					
8	5.93, d, 2.1	5.95, s	5.92, d, 2.4	5.93, d, 2.1	6.20 ,d, 2.1
9					
10					
1'					
2'	6.97, d, 1.8	6.97, d, 8.1	6.40, s	6.51, s	6.83, d, 2.1
3'					
4'					
5'	6.75, d, 8.1	6.69, d, 8.1			
6'	6.80, dd, 8.0, 1.8	6.81, dd, 1.8, 8.1	6.41, s	6.51, s	6.71, dd, 1.8, 8.1
1"					4.59, d, 7.2
2"					3.72, m
3"					3.41, m
4"					3.42, m
5"					3.44, m
6"					3.90, dd, 11.5, 5.0 3.70, dd, 11.5, 5.0

Table 3. ^{13}C (75 MHz, CD_3OD) NMR data of isolated compounds **6-10** from *Tilia amurensis*

Position	**6**	**7**	**8**	**9**	**10**
	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)	δ_C (ppm)
1					
2	80.0	78.8	83.0	80.0	83.0
3	67.6	70.1	68.9	67.7	68.7
4	29.4	27.0	28.3	29.3	28.6
5	158.1	158.0	158.0	158.1	158.8
6	96.0	96.7	96.3	96.0	97.0
7	157.8	157.9	157.8	157.8	157.7
8	96.5	96.0	95.6	96.5	97.5
9	157.5	157.4	157.0	157.5	157.0
10	100.2	99.5	100.3	100.2	103.7
1’	132.4	131.6	134.1	133.7	132.1
2’	116.0	115.3	107.3	107.1	115.3
3’	145.1	146.1	147.0	146.8	146.4
4’	146.1	146.2	138.1	131.7	146.4
5’	115.4	116.1	145.3	145.3	116.2
6’	119.5	119.5	105.6	105.6	120.1
1”					102.5
2”					78.1
3”					75.0
4”					71.5
5”					78.2
6”					62.6

Table 4. ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data of compounds **11-13** from *Tilia amurensis*

No.	**11**		**12**		**13**	
	δ_H (J in Hz)	δ_C (ppm)	δ_H (J in Hz)	δ_C (ppm)	δ_H (J in Hz)	δ_C (ppm)
1						
2	4.98, s	78.8	4.98, d, 5.1	79.3	5.03, s	78.8
3	5.52, br, s	70.1	5.37, dd, 8.1, 10.2	71.2	5.52, m	70.1
4	2.96, dd, 4.5, 17.1 2.84, dd, 1.5, 17.1	27.0	2.73, br, dd,	32.0	3.00, dd, 5.4, 17.4 2.84, dd, 2.5, 17.4	27.0
5		158.0		158.0		158.0
6	5.95, s	96.6	5.95, br, s	96.5	5.95, s	96.7
7		157.9		157.9		157.9
8	5.95, s	96.0	5.95, br, s	95.7	5.95, s	96.0
9		157.4		157.4		157.4
10		99.5		99.7		99.5
1’		130.9		131.1		131.6
2’	6.50, br, s	117.0	6.39, br, s	106.4	6.92, d, 1.8	115.3
3’		146.8		147.1		146.1
4’		133.9		133.9		146.2
5’		146.8		147.1	6.69, d, 8.1	116.1
6’	6.50, br, s	117.0	6.39, br, s	106.4	6.81, dd, 1.8, 8.1	119.5
1”		121.6		119.8		121.6
2”	6.95, br, s	110.4	6.95, br, s	110.3	6.95, br, s	110.4
3”		146.4		146.5		146.5
4”		139.9		139.9		139.9
5”		146.4		146.5		146.5
6”	6.95, br, s	110.4	6.95, br, s	110.3	6.95, br, s	110.4
7’’		167.8		167.8		167.7

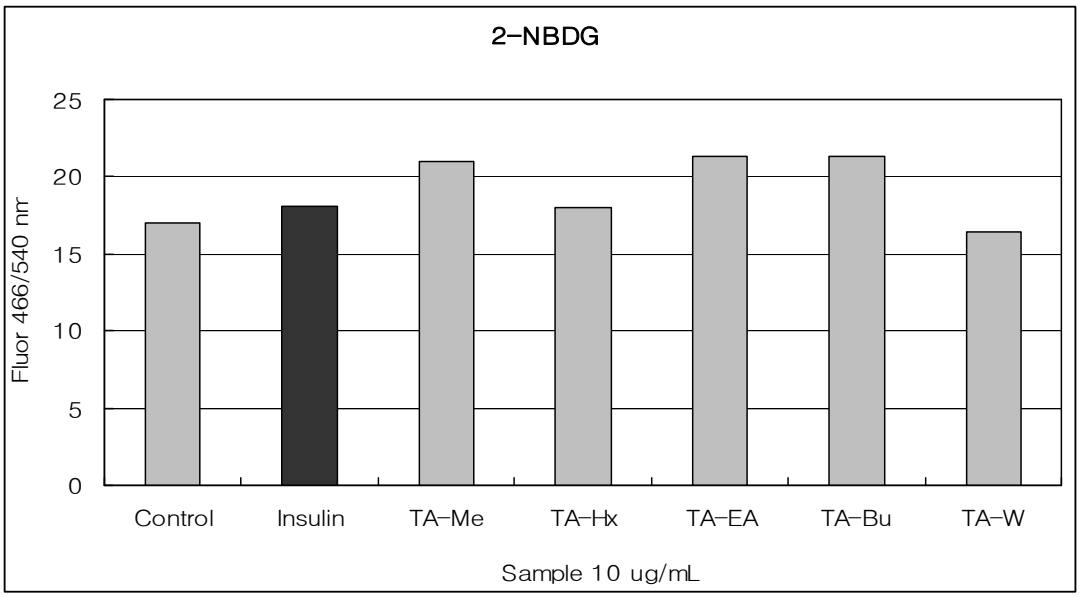


Fig. 36: Insulin-sensitizing effects of the MeOH extract, Hexan fraction, EtOAc fraction, and BuOH fraction from *Tilia amurensis*

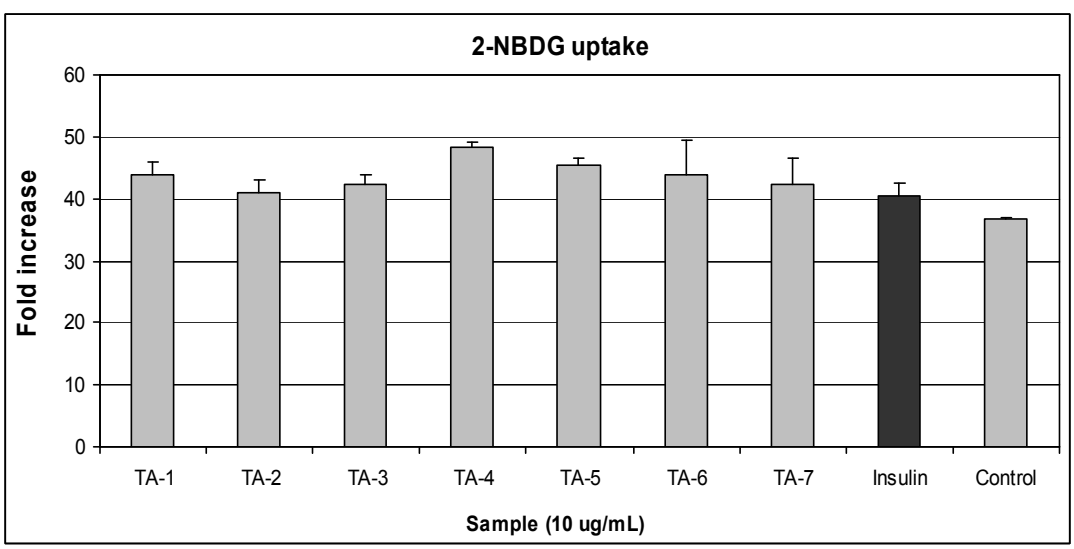


Fig. 37: Insulin-sensitizing effects of fractions TA.1 – TA.7 from *Tilia amurensis*

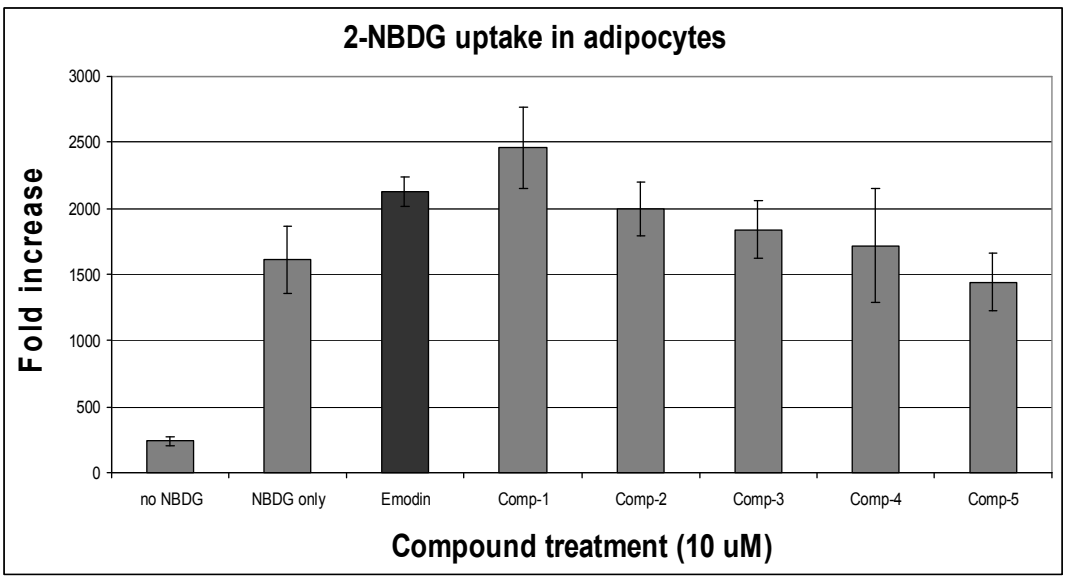


Fig. 38: Insulin-sensitizing effects of isolated compounds **1** – **5** from *Tilia amurensis*

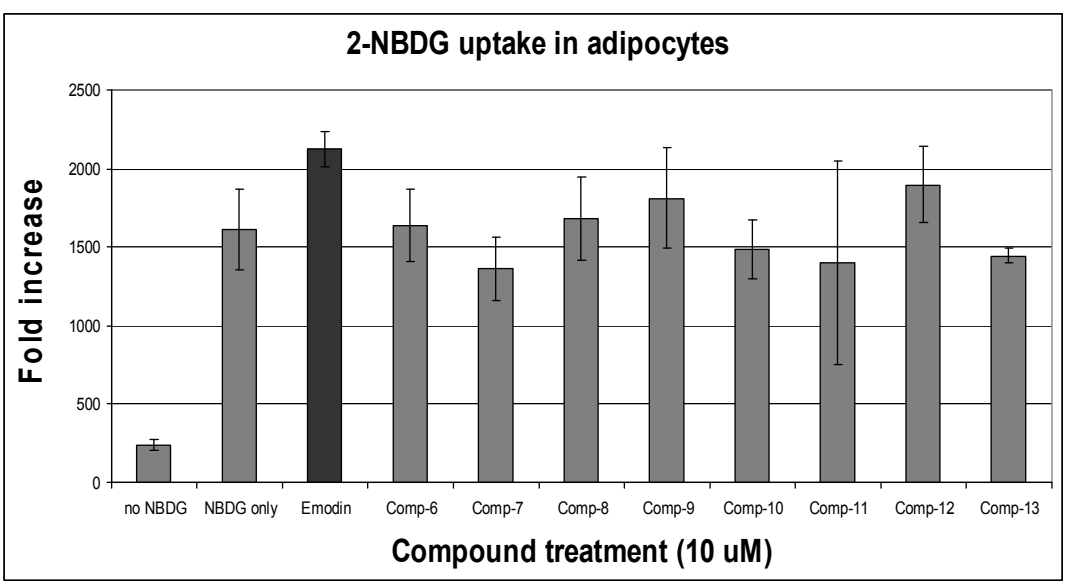


Fig. 39: Insulin-sensitizing effects of isolated compounds **6** – **13** from *Tilia amurensis*

3.2. Discussions

A diabetes epidemic is underway. An estimated 30 million people world-wide had diabetes in 1985. By 1995, this number had shot up to 135 million. The latest WHO (World Health Organization) estimate (for the number of people with diabetes, world-wide, in 2011) is 366 million. This will increase to at least 522 million by 2030. Diabetes caused 4.6 million deaths and caused at least USD 465 billion dollars in healthcare expenditures in 2011. Therefore, there is a need to search for new anti-diabetic agents that can act as insulin mimics and insulin sensitizers for diabetes treatment by stimulating glucose uptake in cells.

2-NBDG was initially developed as fluorescent probes to monitor glucose uptake in live cells and the study of GLUT1 receptor kinetics, respectively. The use of NBDG by the research community to monitor glucose uptake has steadily increased. A PubMed search (U.S. National Library of Medicine National Institutes of Health) retrieved 54 hits for NBDG since 1985, with 10 hits for the years 2009/2010. However, recent research using NBDG has focused more on the monitoring of glycolysis in cancer cells, rather than diabetes related research.

In this study, six compounds were isolated from activity-guide isolation of the EtOAc and BuOH combined fraction of the MeOH-soluble extract of the bark of *T. amurensis*. After structure determination, isolated compounds were tested using an *in vitro* assay on 2-NBDG using 3T3-L1 adipocyte cells to investigate their glucose uptake stimulation activity in cells for the first time. The result showed that compounds and its enriched extract from *T. amurensis* have the possibility for development of agents for the treatment of type II diabetes.

4. Conclusions

Insulin is the only agent developed for the treatment of either type-1 diabetes or severe type-2 diabetes. In this study, to search for new anti-diabetic agents for effectively treating diabetes by mimicking the action of insulin (insulin mimetics), we found that the combined EtOAc and BuOH fractions of the MeOH-soluble extract of *T. amurensis* stimulated glucose uptake in 3T3-L1 adipocyte cells. As the active constituents, four gallic acid derivatives (**1-4**), eight flavan-3-ols, (+)-catechin (**6**), (-)-epicatechin (**7**), (+)-gallocatechin (**8**), (-)-epigallocatechin (**9**), (+)-catechin-3-O-β-D-glucopyranoside **(10)**, (-)-epigallocatechin-3-O-gallate (**11**), gallocatechin-3-O-gallate (**12**), epicatechin-3-O-gallate (**13**), and one coumarin glycoside, fraxin (**5**) were isolated from this plant through activity-guided isolation. When the effects of the isolates (**1-13**) were tested on glucose uptake using 2-NBDG assay, compound **1** showed significant stimulation on insulin-stimulated glucose uptake in 3T3-L1 adipocyte cells (**Fig. 38**). The 3T3-L1 adipocytes cell, which has been widely used in studies of insulin-stimulated glucose uptake, was used to identify glucose uptake enhancers for insulin-mimetic and/or insulin-sensitizing activity. Among them, compound **1** as an acrylaldehyde showed the most potent effect comparable with emodin, a natural compound used as positive control in this assay, followed by (+)-gallocatechin-3-O-gallate **12**, gallic acid derivatives (**2-4**), and (-)-epigallocatechin (**9**). Compounds **5**, a coumarin glycoside, compound **7**, **11**, and **13** which are epicatechin-type derivative showed weak or no activity on this assay. This data revealed that attachment of sugar and/or galloyl moiety at 3-O in the structure of flavonol and at C-8 of the courmarin may responsible for the decrease of activity, and also the epicatechin-type compounds possessed weaker activity than the catechin ones. In the garlic acid derivatives, compounds **1** and **3** with an aldehyde group and a methoxyl

moiety in the structure possessed potential activity, while compounds **2** and **4** with carboxylic group and no methoxy moiety presented in the structure showed weaker activity (**Fig. 38, 39**). The observation of this study suggest that the isolated compounds, especially, the flavan-3-ols might be act as insulin mimics and/or insulin sensitizers to improve the effects of glucose uptake in 3T3-L1 adipocyte cells. And that the active constituents of *Tilia amurensis* and its enrich-extract could be considered as nontoxic source for development of agents against diabetes.

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(50)

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South Korea, June, 2012

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논문제목	한글: 피나무로부터 인슐린 작용을 증가시키는 화합물의 분리 정제 영문: Isolation and identification of increasing materials on insulin sensitivity from *Tilia amurensis*				

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

- 다 음 -

1. 저작물의 DB 구축 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 5 년으로 하고, 기간종료 3 개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.

5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1 개월 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.

2012 년 8 월 24 일

동의 여부: 동의 (O) 조건부 동의( ) 반대 ( )

저작자: 도 뚜안 펑 (서명 또는 인)

조선대학교 총장 귀하