약학석사 학위논문

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## 단삼에서 분리된 화합물과 LPS 유도 염증반응 억제 활성

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이 논문을 약학석사 학위논문으로 제출함

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정관영의 약학석사 학위논문을 인준함

2023 년 2 월

위 원 장 _____ (인)

부위원장 _____ (인)

위 원 _____ (인)

# Abstract

*Salvia miltiorrhiza* Bunge is a perennial plant belonging to the family Lamiaceae, genus Salvia. It is native to China and nowadays it is cultivated in Korea. The root of *Salvia miltiorrhiza* has traditionally been used for various cerebrovascular and cardiovascular diseases. To date, more than 70 kinds of compounds have been reported, including lipophilic diterpenoid represented by Tanshinone IIA and hydrophilic phenolic acids represented by Salvianolic acid B. Tanshinone IIA has reported by its anti-inflammatory and antioxidant activity. Salvianolic acid B has reported by its cardiovascular protection activity. The domestic use of danshen depended entirely on imports from China until 2010. Domestic cultivation began in 2010, and a variety called “Dasan” was developed in 2016 and “Gosan” in 2018. This study constitutes a chemical library with compounds separated from danshen and confirms the inhibitory activity of LPS-induced inflammatory response in RAW264.7 macrophages, which is intended to help in the development of more varieties of danshen or the determination of its post-development application in the future. Two types of danshen originated China and Korea extracted. Fifteen kinds of compounds were isolated using various chromatography techniques from the ethyl acetate fraction of Chinese danshen and the butanol fraction of Korean danshen. All 15 types of compounds were identified as base compounds through 1D NMR and HR-ESIMS analysis. compounds **3,4,8,11,12,13** showed similar or stronger activity to positive control Quercetin.


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**Keyword**: *Salvia miltiorrhiza*, Lamiaceae, Diterpenoids, Phenolic acids, anti-inflammatorry activites.



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# List of Abbreviations

*n*-BuOH: *n*-butanol

ACN: acetonitrile

d: doublet

dd: doublet of doublet

ddd: doublet of doublet of doublet

EtOAc, EA: Ethyl acetate

Hex: *n*-hexane

HPLC: high performance liquid chromatography

HRESIMS: high-resolution electrospray ionization mass spectrometry

Hz: hertz

m: multiplet

MeOH: methanol

MHz: mega hertz

MPLC: middle pressure liquid chromatography

*m/z*: mass to charge ratio

NMR: nuclear magnetic resonance

NP: normal-phase (silica gel)

RP-$C_{18}$: $C_{18}$-reversed phase silica gel

s: singlet

t: triplet

$t_R$: retention time

TLC: thin layer chromatography

# I. Introduction

1. *Salvia miltiorrhiza*

*Salvia* is genus of plants in the family of Lamiaceae. *Salvia miltiorrhiza* is a plant used as a traditional Chinese medicine.[1] *Salvia miltiorrhiza* has been used in China whether alone or with other Chinese medicinal plants. Moreover, it has been used as a treatment for various cerebrovascular and cardiovascular diseases in other countries. Recently, it has accepted as a health product in the western countries because of its notable and dependable biological activities, especially for the therapy of cardiovascular deseases.[2] Nowadays, more than 70 compounds have been isolated and confirmed its structures from *Salvia miltiorrhiza* with diverse concentrations[3]. The major compounds reported from *Salvia miltiorrhiza* are the diterpenoids which is lipophilic and hydrophilic depsides derivatives. Salvianolic acids, are very rich in *Salvia miltiorrhiza*, which can be considered as the condensation derivatives of caffeic acid in different linkage forms and numbers[4]. In Addition, Chemical compounds isolated from Salvia miltiorrhiza include 15,16-dihydrotanshinone,[5] tanshinone I, and tanshinone IIA.[6,7,8] Tanshinone IIA is also one of the most abundant constituents of the Danshen.[6]

2. Purpose of research

The roots of *Salvia miltiorrhiza*, Danshen is used for many purpose, such as ingredients for cosmetics, resource for nutritional supplements and other more. Even though Before, 2010 Danshen usage of Korea relied entirely on imports from China. Danshen started to cultivated in Korea in 2010. Two varients called ‘Dasan’ and ‘Gosan’ has been developed, which compound composition is differ with previous Danshen. Since, demands on nutritional supplements are increasing, there may be a possibility of Danshen to new variant would be developed. The purpose of this study is to isolate various compounds from roots of *Salvia miltiorrhiza.* then see anti-inflammation bioactivity of isolated compounds. Results of this research can offer a direction or possibility of usage of new variant.

**Figure 1**. Morphological difference of roots of Chines and Korean *Salvia miltiorrhiza*. *Salvia miltiorrhiza* from Korea (Left) was thinner than Chinese one, and showed more rootlets. Roots of Salvia *miltiorrhiza* from China (Right) was thicker than Korean one and did not showed rootlets.

3. Diterpenoids

Diterpenoids are a secondary metabolite which is rich in *Salvia miltiorrhiza* and sharing a core structure made of 20 carbons. This Diterpenoids have shown major bioactivity such as anti-bacterial, anti-inflammatory, anti-oxidative and anti-cancer. Until these days, more than 81 diterpenoids from *Salvia miltiorrhiza* have been reported. According to research so far, two kinds of diterpenoids in *Salvia miltiorrhiza*. One is tanshinones which are the richiest diterpenoids in *Salvia miltiorrhiza.* Tanshinones share their ortho-naphtoquinone structure. The other kind of diterpenoid in *Salvia miltiorrhiza* is royleanones. They are abietane diterpenes sharing para-naphthoquinone chromophore. These two kinds of compounds are found especially in *Salvia* genus[20]. Moreover, according to our experience, roots of *Salvia miltiorrhiza* from China turned out to contain more amount of diterpenoids then Korean ones.

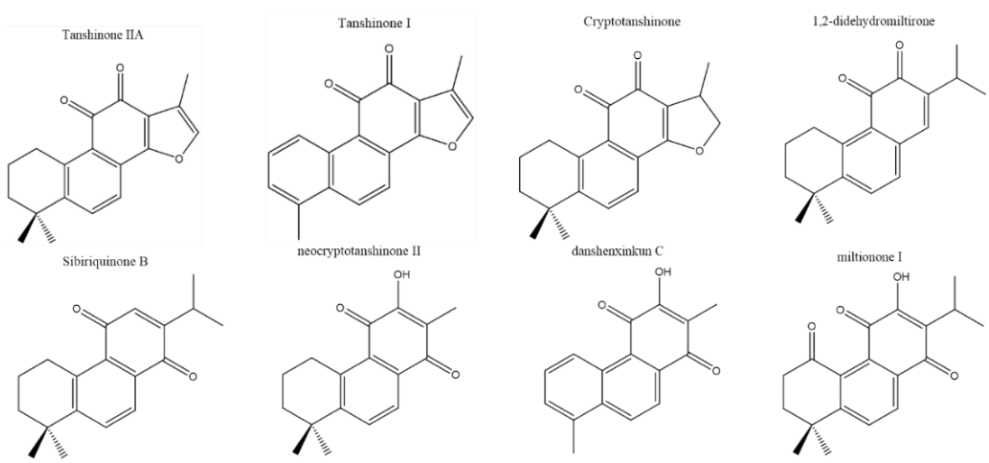



**Figure 2**. Reported diterpenoids that isolated from *S. miltiorrhiza*

4. Phenolic acids

Hydrophilic phenolic acids have been explained for the bioactivity of roots of *Salvia miltiorrhiza.* Several researchers from China and Japan put their efforts to those components[21,22,23]. These phenolic acids from roots of *Salvia miltiorrhiza* have shown diverse biological activities, such as anti-inflammation, anti-oxidative and anti-coagulation[24,25,26]. Phenolic acids from roots of *Salvia miltiorrhiza* were can divided into two groups. First group is single phenolic acids which mainly containing a skeleton of phenylpropanoid, for example, caffeic acid, danshensu, protocatechuic aldehyde and more. Second group is polyphenolic acids. Compounds of these group are mainly conjugate of Danshensu and derivatives or dimer of caffeic acid. Also these group contains depsides such as Salvianolic acid A-E, lithospermic acid and rosmarinic acid.

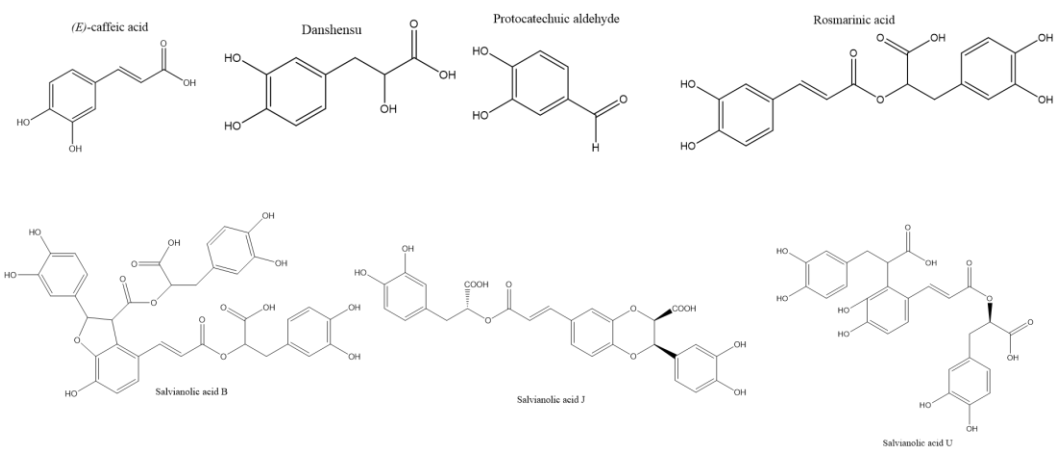



**Figure 3**. Isolated Phenolic acids from roots of *Salvia miltiorrhiza*

5. NO assay

Oxidative responses are mainly mediated by nitric oxide (-NO) and superoxide (-$O_2^-$) that are produced from a number of activated cells such as endothelial cells, Kupffer cells, neutrophils, and macrophages.[15] Especially, NO is a notable reactive component generated from L-arginine by at least three different isoforms of NO synthase (NOS).[16] Even though NO plays important physiological roles in many different cellular processes, excessive production of NO mediated by inducible NOS (iNOS) in inflammatory cells such as macrophages has been reported to mediate acute and chronic inflammatory diseases, such as rheumatoid arthritis, sepsis and pancreatic cancer.[17] Hence, protection against excessive NO production is attributed to important tool in controlling such relevant diseases.

6. MTT assay

MTT assay is a colorimetric analysis for evaluating cellular metabolic activity. NAD(P)H-dependent cellular redox enzymes may reflect the number of surviving cells present under defined conditions. This enzyme can reduce the tetrazolium dye MTT 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide to a purple insoluble formazan. Other closely related tetrazolium dyes, including XTT, MTS, and WST, are used in conjunction with 1-methoxy phenazine methosulfate (PMS), an intermediate electron acceptor. Cellular impermeability, WST-1, is reduced outside the cell via

protoplasmic membrane electron transfer. This existing explanation now comes because evidence has been found that MTT is reduced to formazans in lipid cell structures without the apparent involvement of oxidoreductase.

## 7.Traditional Usage of Danshen

Danshen, known as diverse bioactivity, has been used in traditional Korean and Chinese medicine for approximately two millennia. Activity of Danshen is explained traditionally as promoting blood circulation to get rid of blood stasis, nourishing blood to clear the mind, cooling blood to remove carbuncles. It is used to treat various pains of chest and abdomen caused by blood stasis, pyogenic infection and carbuncle of the skin, insomnia and heart palpitation. Danshen was originally recorded in Shin-Nong-Bon-Cho-Kyung (the first Chinese Materia Medica). Furthermore, it was used for external treatment as well. Myung-Ui-Byeol-Rok (Han Dynasty, 219 AD) has also mentioned that the danshen can be used for stiffness along the vertebral column and numbness of feet. Moreover, clear and well-explained record of the morphology of danshen was kept in Chock-Bon-Cho (Five Dynasties and Ten States periods, 935 AD−960 AD). As a matter of a fact, its functions and many other aspects were recorded in a specific and comprehensive way in Bon-Cho-Gang-Mok (Ming Dynasty, 1578 AD). Nowadays, the activities of Danshen, promoting blood circulation to remove blood stasis, facilitating menstrual discharge to reduce menalgia, removing heart heat to reduce anxiety and cooling blood to scatter carbuncles, have been written in officially, the

Chinese Pharmacopoeia (2019). The therapeutic uses of Danshen spread abroad, therefore, usage of danshen recorded in YI Xin-Fang (Japan, 982 AD) and Dong-Ui-Bo-Gam (Korea, 1611AD). Products containing Danshen are sold commercially for facilitate circulation and alleviating blood stasis in Korea, China and Japan. Similar products are also available in American and European countries. Danshen is one of the earliest and most commonly used herbal medicines in traditional treatment, which are mainly formulated as infusion. For example, Danshen infusion could be used for treat fever of children. Nowadays, the preparations of Danshen have been widely used in clinic for various diseases in many contries. For example, Fufang Danshen tablet is used to treat chest pain caused by angina pectoris, Guanxin Danshen capsule and Fufang Danshen Dripping pill have therapeutic effects on treating chest impediment syndrome due to blood-stasis

## 8. Anti-inflammation activity of Danshen

Inflammatory reactions caused by cytokines and chemokines can be reason of diverse inflammatory vascular illness. Therefore, anti-inflammation is a very consequential therapeutic strategy. Some compounds isolated from Danshen have been studied for its anti-inflammatory activity[32]. Recent studies have showed that salvianolic acid B and an aqueous ethanol extract from Danshen had anti-inflammatory activity. They restrained tumor necrosis factor-α-induced nuclear factor-κB activation in human aortic endothelial cells.[33] Another study has showed that a mechanism for the anti-

inflammatory activity of tanshinone IIA may related with down-regulation of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 through blockage of alpha phosphorylation by the inhibition of IκB kinase-α and IκB kinase-β pathway in endothelial progenitor cells[34], TNF-α-induced NF-κB activation and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor,. Moreover, cryptotanshinone also showed anti-inflammatory activity through diverse mechanisms, including inhibition of the NF-κB and mitogen-activated protein kinase signaling pathways.[35]

9. Anti-oxidative activity of Danshen

Oxidative stress is possible reasons for lots of pathological illness. Antioxidants may show protective activities against these illnesses. Phenolic acids and tanshinones have antioxidative efficacy, though the effectiveness of the Phenolic acids are more strong. Salvianolic acids, especially salvianolic acid A and salvianolic acid B, have shown cardiovascular protective activity due to their anti-oxidative effectiveness.[36] Moreover, it has studied that tanshinone IIA showed activity to the inhibition of myocardial ischemia reperfusion injury, myocardial remodeling, tanshinone IIA also showed neuroprotection from experimental ischemic stroke, and activity of preventing cirrhosis by maintaining antioxidant effect[37]. Additionally, because of its antioxidative activity, tanshinone I could show mitochondrial protection against $H_2O_2$ and protection activity against pyramidal neurons of the gerbil hippocampal CA1 region by ischemic

damage induced by transient cerebral ischemia[38].

10. Effects on heart of Danshen

A recent study showed that Danshen can be important herbal medicine in treatment for patients with coronary heart disease. It may reduce the risk of coronary heart disease, and biomarker of patients seemed better.[39] It has showed that Fufang Danshen Dripping pill with is much more effective than therapy with only aspirin for coronary heart disease[40]. Myocardial ischemia-reperfusion injury (MIRI) is inevitable while open-heart surgery and cardioplegic arrest. Danshen can solve problem against MIRI through diverse mechanisms. For instance, tanshinone IIA can protect against MIRI by initiate the PI3K/protein kinase B (Akt)/mTOR signaling pathway, decreasing oxidative stress, reducing HMGB1 expression, and inflammatory response[41] Dihydrotanshinone I showed cardio-protective activity against MIRI through inhibiting arachidonic acid hydroxylase.[42]

11. Side effects of Danshen

Danshen has been used in the therapy of pregnancy-induced hypertension by its inhibiting effects of angiotensin-converting enzyme. Anyway, many studies have showed that the use of angiotensin-converting enzyme inhibitors can be reason to fetal toxicity, even stillbirths, which is caused by the chemical compounds from Danshen, in

the second and third trimesters of pregnant women[43]. It has demonstrated that depside salt injection made from Danshen had some side drug effects, such as facial blushing, headache, skin itching, thrombocytopenia, dizziness and liver malfunction, which may be activated by rapid infusion rate and other more reasons. Long-term toxicity tests on beagles demonstrated the safety dose of depside salt injection of Danshen, was lower than 80 $mg \cdot kg^{-1}$, and a dose at 320 $mg \cdot kg^{-1}$ becomes toxicity[44]. One research has showed that high-dose (5.76 $g \cdot kg^{-1} \cdot day^{-1}$) Danshen injection could result in peripheral vascular dysfunction, for example, the increase of serum nitrite and endothelin-1, the raise of vascular leakage, and the apoptosis of vascular endothelial cells[45]. Additionally, another study has demonstrated the safety of Danshen and Fufang Danshen injection with 2715 patients from 35 randomized control. From this research, five trials showed some minor side drug effects, such as stomachache, local pain and itchy skin, which were showed to be not severe[46].

# II. Materials and Methods

## 1. Plant materials

The roots of Chinese *S.miltiorrhiza* were purchased from Dukhyundang dispensary of oriental medicine, Seoul, Korea in May 2022. Roots of Korean *S.miltiorrhiza* were collected at the Medicinal Plant Garden (Seoul National University, Siheung, Republic of Korea). The sample was identified by S.I. Han (The medicinal Plant Garden, College of Pharmacy, Seoul National University) and P.G. Min (The medicinal Plant Garden, College of Pharmacy, Seoul National University).

## 2. Chemicals, reagents and chromatography

- Industrial-grade Methanol, distilled water, and ethyl acetate were used for extraction and purification. All solvents were purchased from Daejung Chemical (Siheung, Korea).
- Silica gel 60 $F_{254}$ and RP-18 $F_{254}$S TLC plates were obtained from Merck (Darmstadt, Germany).
- NP silica gel (63-200 μm) from ZEOchem AG (Switzerland).
- RP silica gel Cosmosil 75$C_{18}$-PREP was purchased from Nacalai Tesque (Japan)
- Sephadex LH-20 (18-111 μm) was purchased from GE Healthcare (USA).

- 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (Thiazolyl Blue Tetrazolium Bromide) (Sigma-Aldrich Co., St. Louis, MO, USA)
- Dimethyl sulfoxide (DMSO) (Junsei Chemical Co Ltd., Japan)
- Formic acid (Sigma-Aldrich Co., St. Louis, MO, USA)
- DMEM without phenol red (WelGene, Korea)

### 3. Experimental instruments

- Evaporator : KSB-202 from EYELA (Japan).
- HRESIMS spectrometer : Waters Xevo G2 QTOF mass spectrometer (Waters MS Technologies, Manchester, UK)
- Sonicator : Powersonic 420, Hwa Shin Tech. (Korea).
- UV lamp : VL-4. LC from Vilber Lourmat (France).
- MPLC column : Watchers flash catridges Silica C-18 66 g (USA)
- MPLC system : Biotage Isolera one system (USA)
- HPLC column : YMC $C_{18}$ column (10 × 250 mm, 5 $\mu$m particle size; YMC, Korea), Optima Pak $C_{18}$ column (particles: 5μm, 10 × 250 mm, 5 $\mu$m particle size, RS Tech, Korea)
- HPLC system : 321 pump and Gilson UV/VIS 155 detector from Gilson (USA).
- NMR spectrometers

AVANCE digital 400 spectrometer from Bruker (Germany)

JNM-ECA-600 spectrometer from JEOL (USA).

- Autoclave : LAC-5080S from LAB Tech. (Korea)
- Centrifuge (for filteration) : AVANTI J2 from Beckman Coulter (USA).

  (for bioassay) : FLETA S from Hanil Science (Korea)
- Clean Bench : Class II Biological Safety Cabinet from ESCO (Korea)
- $CO_2$ Incubator : Forma Series II water jacketed $CO_2$ incubator from Thermo (USA)
- ELISA Microplate reader : VersaMax from Molecular Devices (USA)

## 4. Extraction and isolation schemes

4-1. Extraction and fractionation of *S.miltiorrhiza* roots

The roots of *S.miltiorrhiza* (3.0 kg) were extracted with 10% MeOH (3 × 5 L, for 2 h each) then 90% MeOH ( 3 × 5 L, for 2 h) each in a sonicator at 35 °C. The combined extract was concentrated by an evaporator to yield a dry residue (808.1 g). The dried residue was suspended in $H_2O$, partitioned successively with EtOAc (3 × 5 L).

The roots of *S.miltiorrhiza* from China (600 g) were extracted with 75% MeOH (3 × 1 L, for 2 h each) The extract was concentrated by an evaporator to yield a dry residue (125.2 g). The dried residue was suspended in $H_2O$, partitioned successively with EtOAc (3 × 1.5 L).

The roots of *S.miltiorrhiza* from China (1.8 kg) were extracted with 100% MeOH (3

× 1 L, for 2 h each) with avoidance of light and heat. The extract was concentrated by an evaporator to yield a dry residue (33.3 g). The dried residue was suspended in $H_2O$, partitioned successively with EtOAc (3 × 1.5 L).

4-2 Isolation scheme of fraction

The *n*-BuOH fraction was of Korean *salvia miltiorrhiza* earned 41.7 g. 13.8 g of *n*-BuOH fraction was subjected to MPLC with gradient solvent system of MeOH/$H_2O$ (0.1% formic acid) (v/v, 10/90 →70/30 → 100/0), to obtain seven sub-fractions (F1-F7). Fractionation was monitored by TLC. Fraction 1 was subjected to MPLC (Medium pressure liquid chromatography) with gradient solvent system of MeOH/$H_2O$ (0.1% formic acid) (v/v, 10/90 →30/70 → 100/0), to obtain nine subfractions (F1_1-F1_9). Fractionation was monitored by TLC. Fraction 1_6 was identified as a compound **1**. Fraction 2 was subjected to MPLC with gradient solvent system of MeOH/$H_2O$ (0.1% formic acid) (v/v, 10/90 →70/30 → 100/0) again to obtain eleven sub-fractions (F2_1-F2_11). Subfraction 2_8 was identified as compound **9**. Subfraction 2_6 was subjected to HPLC with isocratic solvent system of ACN/$H_2O$ (0.1% formic acid) (v/v, 20/80), to obtain compound **10**. Fraction 15 was subjected to MPLC with gradient solvent system of MeOH/$H_2O$ (0.1% formic acid) (v/v, 10/90 →70/30 → 100/0), to obtain seven subfractions (F15_1-F15_7). Fractionation was monitored by TLC. Fraction 15_4 was subjected to Sephadex LH-20 with 100% MeOH, resulted with six subfractions (F15_4_1-F15_4_6). Fraction

15_4_3 and Fraction 15_4_5 were identified as Compound **6** and compound **7**

The EtOAc fraction from Chinese *Salvia miltiorrhiza* (3.9 g) was subjected to silica gel column chromatography (10 × 60 cm; 40-68 μm particle size) using gradient solvents systems of *n*-hexane/EtOAc/MeOH mixtures (1:0:0 → 19:1:0 → 8:2:0 → 7:3:0 → 6:3.5:0.5 → 5:4:1→4:5:1→0:0:1) to afford 14 fractions. Fraction 4 and Fraction 6 were washed with *n*-hexane, then identified as Compound **2** and **3**. Fraction 8 was identified as Compound **4**. From fraction 10, powder was naturally made during evaporating procedure. Powder was collected and identified as Compound **5.** Fraction 2 was subjected to HPLC with isocratic solvent system of ACN/$H_2O$ (0.1% formic acid) (v/v, 80/20), resulted in compound **8**

The EtOAc fraction from light and heat avoided Chinese *Salvia miltiorrhiza* (16.6 g) was subjected to subjected to MPLC with gradient solvent system of MeOH/$H_2O$ (0.1% formic acid) (v/v, 20/80 →90/10 → 100/0), to obtain six subfractions (F1-F6). Fraction 4 was subjected to MPLC with gradient solvent system of MeOH/$H_2O$ (0.1% formic acid) (v/v, 60/40 →80/20 → 90/10 → 100/0), to obtain eight subfractions (F4_1-F4_8). Fractionation was monitored by TLC. Fraction 4_3 was subjected to Sephadex LH-20 with 100% MeOH, resulted with compound **11**. Fraction 4_4 was subjected to HPLC with isocratic solvent system of ACN/$H_2O$ (0.1% formic acid) (v/v,

52/48), resulted in compound **12,13,14** and compound **15.**

5. Chemical and spectral properties of isolated compounds

*(E)*-Caffeic acid (**1**)

Brown, amorphous solid

$C_9H_8O_4$

ESIMS: m/z 179.0339 $[M-H]^-$

$^1H$ NMR Data (400 MHz, MeOD-*d4*), $\delta_H$ 7.47 (d, *J*=15.9 Hz, 1H), 6.99 (d, *J*=2.1 Hz, 1H), 6.89 (dd, *J*=8.2, 2.1 Hz, 1H), 6.73 (d, *J*=8.2,1 Hz, 1H), 6.18 (d, *J*=15.9, 1H)

$^{13}C$ NMR Data (100 MHz, MeOD-*d4*), 127.9 (C-1), 127.8 (C-2), 116.5 (C-3), 149.4 (C-4), 146.8 (C-5), 115.9 (C-6), 146.8 (C-7), 115.0 (C-8), 171.4 (C-9)

Tanshinone IIA (**2**)

Red Powder

$C_{18}H_{12}O_3$

ESIMS: m/z 295.1317 $[M+H]^+$

$^1H$ NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.63 (d, *J*=9.1 Hz, 1H), 7.55 (d, *J*=9.1 Hz, 1H), 7.22 (q, *J*=1.2 Hz, 1H), 3.18 (t, *J*=6.4 Hz, 2H), 2.26 (d, *J*=1.3 Hz, 3H), 1.80 (m, 2H), 1.65 (m, 2H), 1.31 (s, 6H)

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 30.1 (C-1), 19.3 (C-2), 38.0 (C-3), 34.8 (C-4), 144.7 (C-5), 133.6 (C-6), 120.4 (C-7), 127.6 (C-8), 126.6 (C-9), 150.3 (C-10), 183.8 (C-11), 175.9 (C-12), 120.0 (C-13), 161.9 (C-14), 141.4 (C-15), 121.3 (C-16), 9.0 (C-17), 32.0 (C-18,19)

Tanshinone I (**3**)

Brown Powder

$C_{18}H_{12}O_3$

ESIMS: m/z 277.0822 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 9.24 (d, *J*=9.0 Hz, 1H), 8.29 (d, *J*=8.7 Hz, 1H), 7.80 (d, *J*=8.7 Hz, 1H), 7.54 (dd, *J*=8.9, 7.0 Hz, 1H), 7.34 (d, *J*=7.0 Hz, 1H), 7.29 (q, *J*=1.3 Hz 1H), 2.68 (s, 3H), 2.29 (d, *J*=1.3 Hz 3H)

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 118.9 (C-1), 133.8 (C-2), 129.8 (C-3), 135.4 (C-4), 124.9 (C-5), 133.1 (C-6), 124.9 (C-7), 130.8 (C-8), 128.5 (C-9), 133.8 (C-10), 183.6 (C-11), 175.8 (C-12), 121.9 (C-13), 161.4 (C-14), 142.2 (C-15), 120.6 (C-16), 9.0 (C-17), 20.0 (C-18).

Cryptotanshinone (**4**)

Orange Powder

$C_{19}H_{20}O_3$

ESIMS: m/z 297.1478 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.63 (d, *J*=8.1 Hz, 1H), 7.49 (d, *J*=8.1 Hz, 1H), 4.89 (t, *J*=9.5 Hz, 1H), 4.36 (dd, *J*=9.3, 5.9 Hz, 1H), 3.60 (m, 1H), 3.21 (t, *J*=6.4 Hz 2H), 1.79 (m, 2H), 1.66 (m, 2H), 1.35 (d, *J*=6.7 Hz, 3H), 1.31 (s, 6H).

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 29.8 (C-1), 19.2 (C-2), 37.9 (C-3), 35.0 (C-4), 143.9 (C-5), 132.7 (C-6), 122.7 (C-7), 128.5 (C-8), 126.4 (C-9), 152.5 (C-10), 184.4 (C-11), 175.9 (C-12), 118.4 (C-13), 171.0 (C-14), 81.6 (C-15), 34.7 (C-16), 19.0 (C-17), 32.1 (C-18,19).

15,16-dihydrotanshinone (**5**)

Oragne Powder

$C_{18}H_{14}O_3$

ESIMS: m/z 279.1010 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 9.29 (d, *J*=8.9 Hz, 1H), 8.30 (dd, *J*=8.7, 1.1 Hz, 1H), 7.75 (dd, *J*=8.7, 1.1 Hz, 1H), 7.57 (dd, *J*=8.9, 7.0 Hz, 1H), 7.40 (d, *J*= 6.9 Hz, 1H), 4.97 (t, *J*=9.6 Hz 1H), 4.44 (dd, *J*=9.4, 6.2 Hz, 1H), 3.66 (m, 1H), 2.70 (s, 3H), 1.41 (d, *J*= 6.8 Hz 3H).

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 125.2 (C-1), 130.6 (C-2), 129.0 (C-3), 135.1 (C-

4), 132.3 (C-5), 132.1 (C-6), 120.5 (C-7), 128.4 (C-8), 126.2 (C-9), 134.9 (C-10), 184.5 (C-11), 175.9 (C-12), 118.5 (C-13), 170.8 (C-14), 81.8 (C-15), 34.8 (C-16), 19.0 (C-17), 20.0 (C-18)

Rosmarinic acid (**6**)

Colorless gum

$C_{18}H_{16}O_8$

ESIMS: m/z 359.0671 $[M-H]^-$

$^1$H NMR Data (400 MHz, MeOD-*d4*), $\delta_H$ 7.52 (d, *J*=15.9 Hz, 1H), 7.01 (d, *J*= 2.1 Hz, 1H), 6.92 (dd, *J*=8.2, 2.1 Hz, 1H), 6.75 (d, *J*=8.1 Hz, 1H), 6.72 (d, *J*= 2.1 Hz, 1H), 6.66 (d, *J*=8.0 Hz 1H), 6.58 (dd, *J*=8.1, 2.1 Hz, 1H), 6.24 (d, *J*=15.9 Hz, 1H), 5.15 (dd, *J*=8.3, 4.3 Hz, 1H), 3.07 (dd, *J*=14.3, 4.4 Hz, 1H), 2.97 (dd, *J*= 14.3, 8.4 Hz 1H).

$^{13}$C NMR Data (100 MHz, MeOD-*d4*), 127.6 (C-1), 115.2 (C-2), 146.8 (C-3), 149.8 (C-4), 116.3 (C-5), 123.2 (C-6), 147.7 (C-7), 114.4 (C-8), 168.5 (C-9), 134.9 (C-1′), 117.6 (C-2′), 146.2 (C-3′), 145.3 (C-4′), 116.5 (C-5′), 121.8 (C-6′), 37.9 (C-7′), 74.7 (C-8′), 173.6 (C-9′).

Salvianolic acid B (**7**)

Yellowish gum

$C_{36}H_{30}O_{16}$

ESIMS: m/z 717.1447 $[M-H]^-$

$^1$H NMR Data (400 MHz, MeOD-*d4*), $\delta_H$ 7.52 (d, *J*=15.9 Hz, 1H), 7.16 (d, *J*= 8.5 Hz, 1H), 6.83 (d, *J*=8.4, 1H), 6.76 (d, *J*=2.1 Hz, 1H), 6.75 (d, *J*= 2.1 Hz, 1H), 6.74 (d, *J*=2.2 Hz 1H), 6.70 (d, *J*=8.0 Hz, 1H), 6.65 (dd, *J*=8.2, 2.1 Hz, 1H), 6.62 (dd, *J*=8.1, 2.0 Hz, 1H), 6.54 (d, *J*=8.1 Hz, 1H), 6.52 (d, *J*= 2.1 Hz 1H), 6.31 (dd, *J*=8.1, 2.2 Hz, 1H), 6.20 (d, *J*= 15.9 Hz, 1H), 5.86 (d, *J*= 4.8 Hz, 1H), 5.17 (m, 2H), 4.35 (d, *J*= 4.9 Hz, 1H), 3.07 (dd, *J*=14.3, 4.4 Hz, 1H), 3.02 (m, 1H), 2.83 (dd, *J*=14.3, 9.6 Hz, 1H).

$^{13}$C NMR Data (100 MHz, MeOD-*d4*), 124.6 (C-1), 126.4 (C-2), 149.0 (C-3), 145.1 (C-4), 118.3 (C-5), 122.2 (C-6), 143.5 (C-7), 116.5 (C-8), 168.0 (C-9), 128.9 (C-1′), 117.3 (C-2′), 145.9 (C-3′), 145.1 (C-4′), 116.4 (C-5′), 121.7 (C-6′), 37.9 (C-7′), 75.6 (C-8′), 172.6 (C-9′), 129.2 (C-1″), 117.5 (C-2″), 146.1 (C-3″), 145.2 (C-4″), 116.4 (C-5″), 122.1 (C-6″), 37.5 (C-7″), 74.7 (C-8″), 173.7 (C-9″), 133.6 (C-1‴), 113.3(C-2‴), 146.6 (C-3‴), 146.7 (C-4‴), 116.5 (C-5‴), 118.4 (C-6‴), 88.3 (C-7‴), 57.9 (C-8‴), 172.3 (C-9‴).

Sibiriquinone B (**8**)

Red Powder

$C_{19}H_{22}O_2$

ESIMS: m/z 283.1694 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.59 (d, *J*=8.0 Hz, 1H), 7.10 (d, *J*=8.0 Hz, 1H), 7.07 (s, 1H), 3.17 (t, *J*=6.4 Hz, 2H), 3.02 (sept, *J*= 6.9 Hz, 1H), 1.79 (m, 1H), 1.65 (m, 2H), 1.29 (s, 6H), 1.16 (d, *J*=6.9 Hz, 1H s, 6H)

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 29.7 (C-1), 19.0 (C-2), 37.8 (C-3), 34.5 (C-4), 149.6 (C-5), 134.5 (C-6), 127.9 (C-7), 133.9 (C-8), 139.8 (C-9), 144.5 (C-10), 182.4 (C-11), 139.0 (C-12), 145.0 (C-13), 181.5 (C-14), 26.9 (C-15), 21.5 (C-16), 21.5 (C-17), 31.7 (C-18), 31.7 (C-19).

Isolithospermic acid (**9**)

Yellowish gum

$C_{27}H_{22}O_{12}$

ESIMS: m/z 537.1033 $[M-H]^-$

$^1$H NMR Data (400 MHz, MeOD-*d4*), $\delta_H$ 7.50 (d, *J*=15.9 Hz, 1H), 7.07 (d, *J*= 8.5 Hz, 1H), 6.76 (d, *J*=8.4, 1H), 6.68 (s, 1H), 6.66 (d, *J*= 7.4 Hz, 1H), 6.56 (dd, *J*=8.2, 2.2 Hz 1H), 6.53 (s, 1H), 6.52 (d, *J*=5.6 Hz, 1H), 6.30 (dd, *J*=8.1, 2.1 Hz, 1H), 6.11 (d, *J*=15.9 Hz, 1H), 5.77 (d, *J*= 4.6 Hz 1H), 5.06 (dd, *J*=6.4, 3.8 Hz, 1H), 4.25 (d, *J*= 4.6 Hz, 1H), 2.96 (dd, *J*= 14.3, 3.9 Hz, 1H), 2.83 (dd, *J*= 14.3, 4.9 Hz, 1H).

$^{13}$C NMR Data (100 MHz, MeOD-*d4*), 124.9 (C-1), 126.1 (C-2), 149.0 (C-3), 144.8

(C-4), 118.3 (C-5), 122.0 (C-6), 143.1 (C-7), 117.7 (C-8), 170.7 (C-9), 133.7 (C-1′), 113.2 (C-2′), 146.6 (C-3′), 146.7 (C-4′), 116.4 (C-5′), 118.3 (C-6′), 88.2 (C-7′), 57.8 (C-8′), 172.2 (C-9′), 129.0 (C-1″), 117.3 (C-2″), 146.0 (C-3″), 145.1 (C-4″), 116.4 (C-5″), 121.8 (C-6″), 37.5 (C-7″), 75.6 (C-8″), 172.6 (C-9″).

8-epibechnic acid (**10**)

colorless gum

$C_{18}H_{14}O_8$

ESIMS: m/z 357.0598 $[M-H]^-$

$^1$H NMR Data (400 MHz, MeOD-*d4*), $\delta_H$ 7.74 (d, *J*=15.9 Hz, 1H), 7.16 (d, *J*= 8.5 Hz, 1H), 6.78 (d, *J*=8.5 Hz, 1H), 6.73 (d *J*=8.1 Hz, 1H), 6.68 (dd, *J*=8.2, 2.0 Hz, 1H), 6.25 (d, *J*=15.9 Hz, 1H), 5.85 (d, *J*=4.9 Hz, 1H), 4.29 (d, *J*=4.9 Hz, 1H).

$^{13}$C NMR Data (100 MHz, MeOD-*d4*), 124.8 (C-1), 127.5 (C-2), 148.9 (C-3), 144.9 (C-4), 118.2 (C-5), 121.5 (C-6), 143.4 (C-7), 117.6 (C-8), 170.8 (C-9), 133.9 (C-1′), 113.4 (C-2′), 146.7 (C-3′), 146.6 (C-4′), 116.4 (C-5′), 118.2 (C-6′), 88.9 (C-7′), 57.8 (C-8′), 175.3 (C-9′).

R-(+)-grandifolia D **(11)**

Red Powder

$C_{19}H_{22}O_3$

ESIMS: m/z 299.1653 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.59 (d, *J*=7.9 Hz, 1H), 7.17 (s, 1H), 7.11 (d, *J*=7.9 Hz, 1H), 3.69 (m, 2H), 3.08 (m, 1H), 1.79 (m, 2H), 1.63 (m, 2H), 1.28 (d, *J*=2.5 Hz, 6H), 1.21 (d, *J*=7.0 Hz, 1H s, 3H)

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 30.0 (C-1), 19.0 (C-2), 37.9 (C-3), 34.6 (C-4), 150.3 (C-5), 134.0 (C-6), 128.3 (C-7), 134.2 (C-8), 128.3 (C-9), 144.5 (C-10), 182.1 (C-11), 181.9 (C-12), 140.6 (C-13), 142.9 (C-14), 35.6 (C-15), 15.6 (C-16), 66.5 (C-17), 31.9 (C-18), 31.9 (C-19).

Miltipolone **(12)**

White Powder

$C_{19}H_{24}O_3$

ESIMS: m/z 301.1778 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.42 (s, 1H), 7.30 (s, 1H), 4.70 (dd, *J*=3.8, 1.8 Hz, 1H), 4.38 (d, *J*=9.0 Hz, 1H), 3.00 (dd, *J*= 9.1, 1.8 Hz, 1H), 2.46 (s, 3H), 2.12 (ddd, *J*=13.8, 6.2, 3.8 Hz, 1H), 1.95 (td, *J*=13.7, 4.3 Hz, 1H), 1.73 (m, 2H), 1.63 (m 2H), 1.23 (m 2H), 1.17 (s, 3H), 0.87 (s, 3H).

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 29.9 (C-1), 19.0 (C-2), 41.0 (C-3), 34.4 (C-4), 41.5

(C-5), 29.2 (C-6), 74.4 (C-7), 138.7 (C-8), 160.1 (C-9), 40.6 (C-10), 120.0 (C-11), 172.0 (C-12), 166.4 (C-13), 131.3 (C-14), 136.4 (C-15), 67.4 (C-16), 21.3 (C-17), 33.0 (C-18), 21.3 (C-19).

Trijuganone B **(13)**

Red Powder

$C_{18}H_{16}O_3$

ESIMS: m/z 281.1191 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.53 (d, *J*=7.9 Hz, 1H), 7.43 (d, *J*=7.9 Hz, 1H), 6.10 (td, *J*=4.6, 1.3 Hz, 1H), 4.91 (t, *J*=9.5 Hz, 1H), 4.37 (dd, *J*=9.3, 6.0 Hz, 1H), 3.62 (m, 1H), 3.38 (t, *J*=7.9 Hz, 2H), 2.26 (m, 2H), 2.07 (q, *J*=1.7 Hz, 3H), 1.38 (d, *J*=6.8 Hz, 3H)

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 24.6 (C-1), 22.5 (C-2), 123.2 (C-3), 140.8 (C-4), 116.7 (C-5), 129.7 (C-6), 127.2 (C-7), 131.0 (C-8), 126.1 (C-9), 143.4 (C-10), 184.7 (C-11), 176.0 (C-12), 111.2 (C-13), 170.6 (C-14), 81.6 (C-15), 34.6 (C-16), 20.0 (C-17), 18.9 (C-18)

Isograndifoliol **(14)**

Yellow Powder

$C_{19}H_{26}O_3$

ESIMS: m/z 303.1906 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 7.61 (s, 1H), 7.42 (s, 1H), 3.99 (d, *J*=11.4 Hz, 1H), 3.88 (d, *J*=11.4 Hz, 1H), 3.03 (ddd, *J*=18.3, 7.6, 2.6 Hz, 1H), 2.95 (dd, *J*=16.4, 8.8 Hz, 1H), 2.67 (dd, *J*=12.7, 3.0 Hz, 1H), 1.24 (m, 1H), 2.38 (s, 3H), 1.90 (m, 2H), 1.67 (m, 2H), 1.52 (m, 1H), 1.40 (m, 1H), 1.24 (m, 1H), 0.97 (s, 3H), 0.96 (s, 3H).

$^{13}$C NMR Data (100 MHz, $CDCl_3$), 34.2 (C-1), 19.2 (C-2), 41.0 (C-3), 33.9 (C-4), 48.8 (C-5), 18.6 (C-6), 34.4 (C-7), 137.6 (C-8), 151.4 (C-9), 45.3 (C-10), 118.7 (C-11), 160.3 (C-12), 174.3 (C-13), 138.8 (C-14), 143.1 (C-15), 64.7 (C-16), 33.2 (C-17), 22.4 (C-18), 22.3 (C-19).

Danshenxinkun A (**15)**

Red Powder

$C_{18}H_{16}O_4$

ESIMS: m/z 297.1498 $[M+H]^+$

$^1$H NMR Data (400 MHz, $CDCl_3$), $\delta_H$ 9.42 (d, *J*=8.8 Hz, 1H), 8.42 (d, *J*=8.8 Hz, 1H), 8.26 (d, *J*=8.8, Hz, 1H), 7.63 (dd, *J*=8.9, 7.0 Hz, 1H), 7.47 (d, *J*=6.8 Hz, 1H), 4.00 (dd, *J*=10.7, 7.8 Hz, 1H), 3.89 (dd, *J*=10.7, 5.3 Hz, 1H) 3.50 (m, 1H), 2.74 (s, 3H), 1.33 (d, *J*=7.1 Hz, 3H)

$^{13}C$ NMR Data (100 MHz, $CDCl_3$), 126.0 (C-1), 130.5 (C-2), 129.6 (C-3), 135.8 (C-4), 134.0 (C-5), 132.4 (C-6), 122.6 (C-7), 130.8 (C-8), 125.4 (C-9), 135.6 (C-10), 184.4 (C-11), 156.3 (C-12), 122.7 (C-13), 186.5 (C-14), 33.4 (C-15), 14.9 (C-16), 65.4 (C-17), 19.9 (C-18)

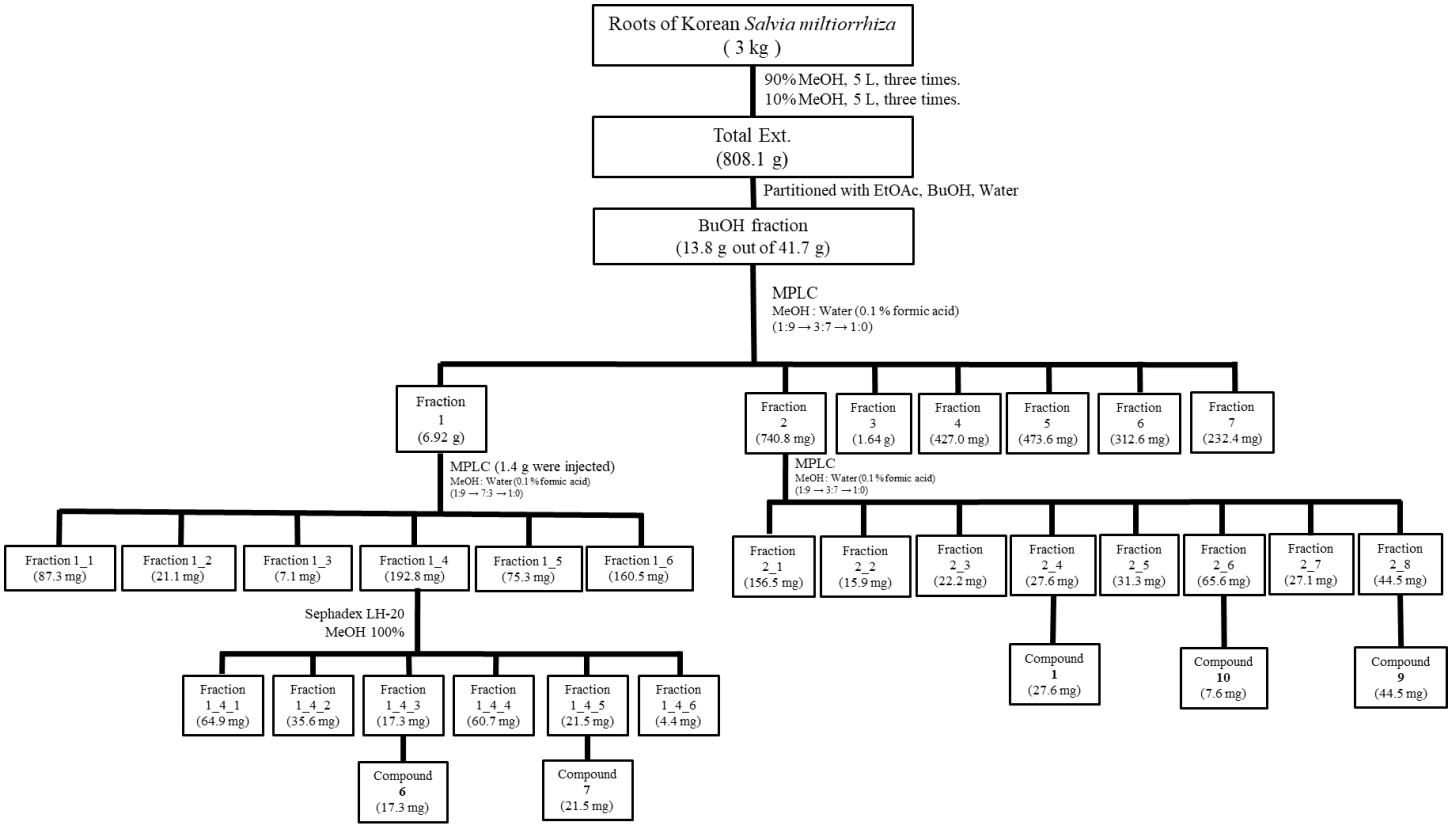
6. Bio assay procedure

6-1 NO assay

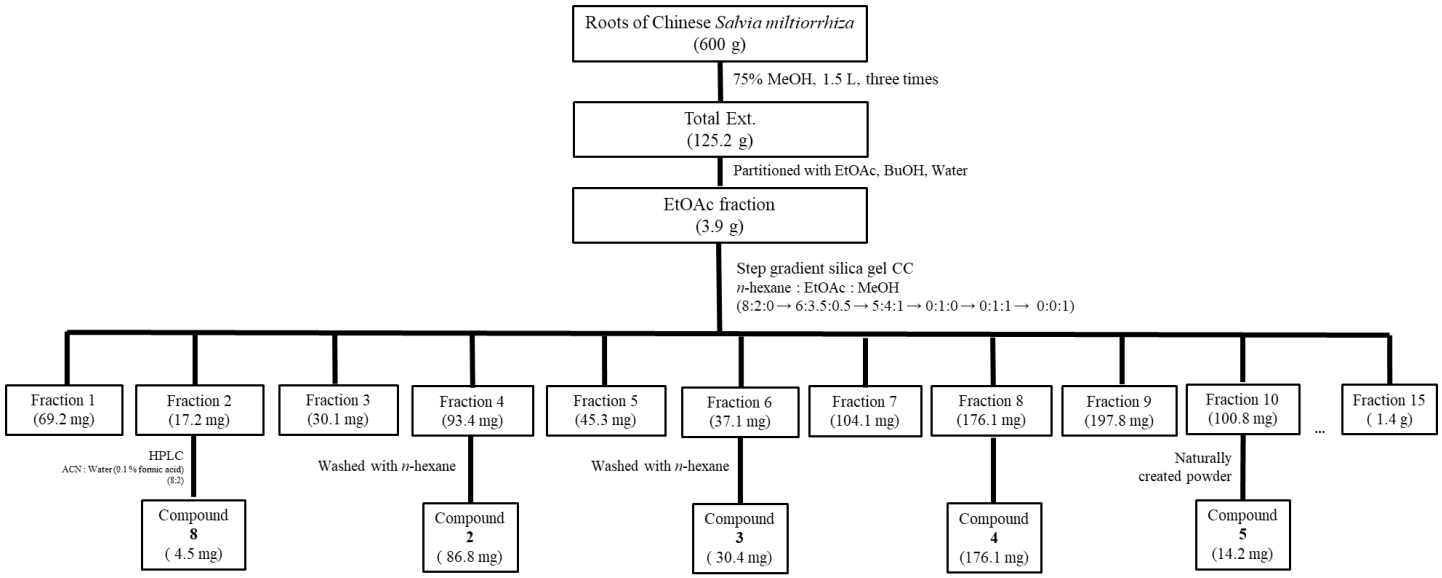
Dulbecco′s Modified Eagle′s Medium (WelGene, Korea) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin (Gibco) under 37 °C was used for the NO production assay. Briefly, RAW 264.7 cells were seeded into 96-well plates and incubated for 1 day at 37 ◦C under a 5% CO2 atmosphere. Nitric oxide production was evaluated by using the Griess reagent method, and the absorbance was measured at 540 nm18. For the NO assay stimulated by lipopolysaccharide (LPS), RAW 264.7 cells were pretreated with the test compounds for 1 h, and 1 ug/mL LPS was added to the cells. After 24 h of incubation, NO production was measured as described above. Also, Cell viability was tested by MTT reagent.

6-2 C2C12 MTT assay

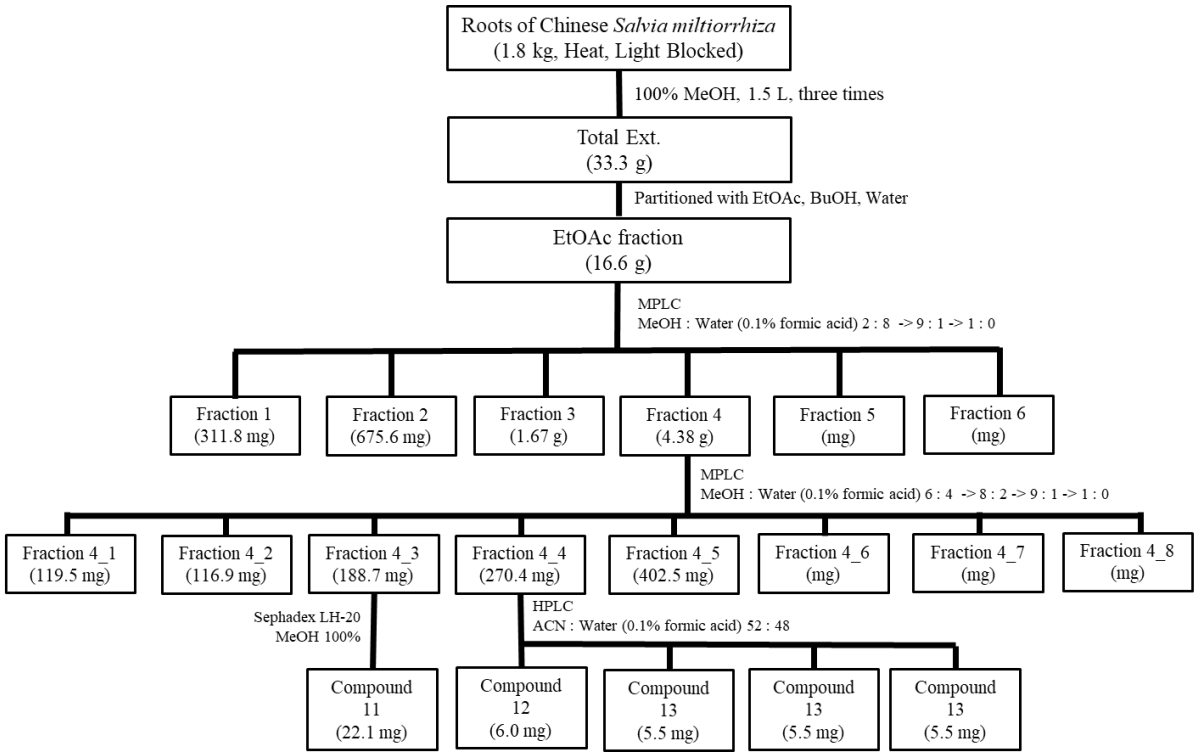
Dulbecco′s Modified Eagle′s Medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone), 1% penicillin-streptomycin (Gibco) was used for the C2C12 MTT assay. Myoblast cells from mouse (C2C12) were cultured in under 37 °C, 5% CO2 humidified atmosphere and used at passages 10−25. A 3-(4, 5-dimethyl-2-thuazolyl)-2,5-diprenyl-2H-tetrazolium bromide (MTT) assay was used to measure cytoxity. C2C12 cells were seeded in 96-well plates at density 5000 cell/well each sample treated triplicate. After overnight incubation, cells were treated 100 μL of sample containing serum free media for 24h. 20 μL of MTT solution (2mg/ mL) was directly added to each well. After four hours, purple MTT formazam precipitate was then dissolved in 100 μL of DMSO, and the absorbance at 540 nm was measured on a multiwall reader (VersaMax).




Scheme 1. Isolation scheme of BuOH fraction of Korean *S.miltiorrhiza* roots.




Scheme 2. Isolation scheme of EtOAc fraction of Chinese *S.miltiorrhiza* roots




Scheme 3. Isolation scheme of EtOAc fraction of Chinese *S.miltiorrhiza* roots

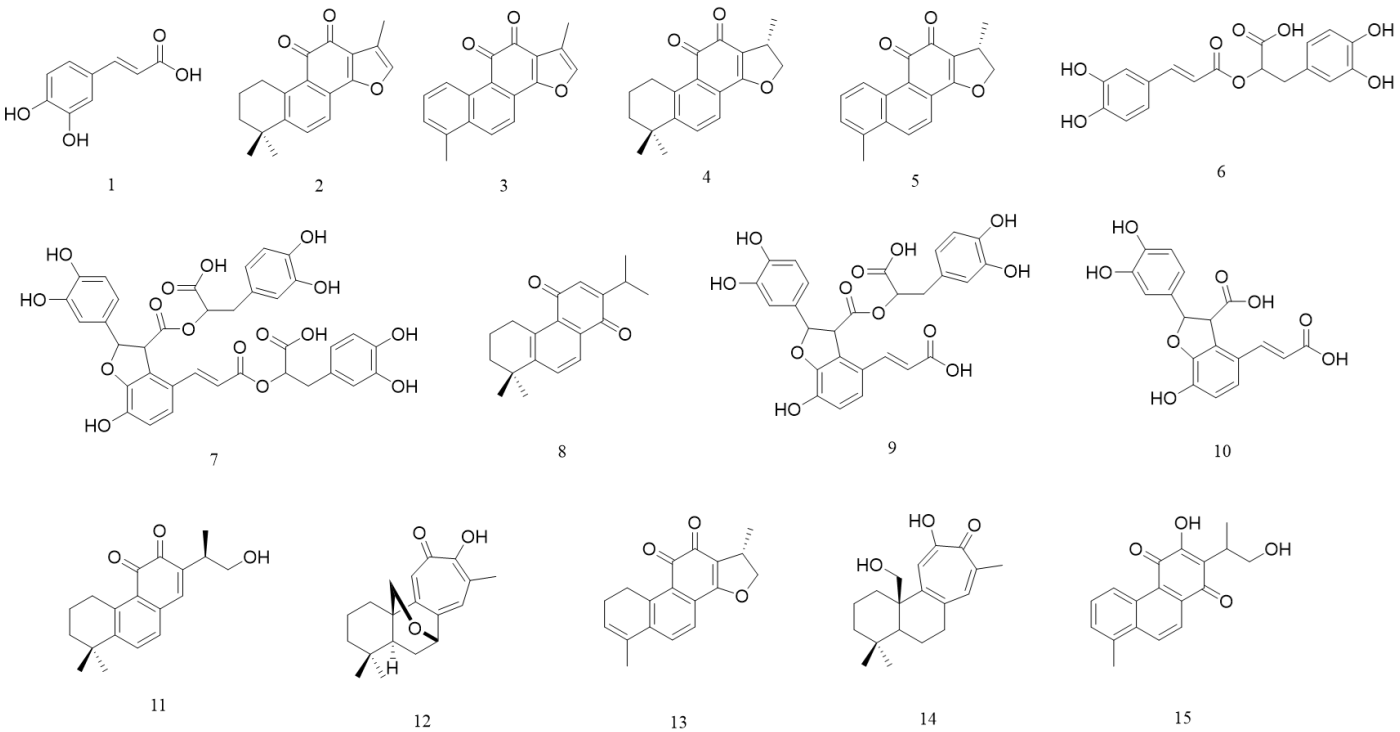



Figure 4. Isolated Compounds from roots of *Salvia miltiorrhiza*

# III. Results and Discussion

1. Elucidation of chemical structures of isolated compounds from the roots of *Salvia miltiorhiza*

## 1-1. Compound **1**

Compound **1** was obtained as a brown, and amorphous solid. Its molecular formula was established as $C_9H_7O_4$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 179.0339 $[M - H]^-$ (calcd for $C_9H_6O_4$, 179.0344) corresponding to six degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two singlets for olefinic protons which coupling constants indicated *trans* double bond. [($\delta_H$ 7.47, d, *J* = 15.9 Hz, 1H), ($\delta_H$ 6.18 d, *J* = 15.9 Hz, 1H)], three aromatic protons which showed splitting pattern and coupling constants of ABX spins system. [($\delta_H$ 6.99, d, *J*=2.1 Hz. 1H), ($\delta_H$ 6.89, dd, *J*=8.2, 2.1 Hz, 1H), ($\delta_H$ 6.73, d, *J*=8.2 Hz ,1H)], Based on an analysis of the spectra and reported data[1], active compound was identified as *(E)*-Caffeic acid.

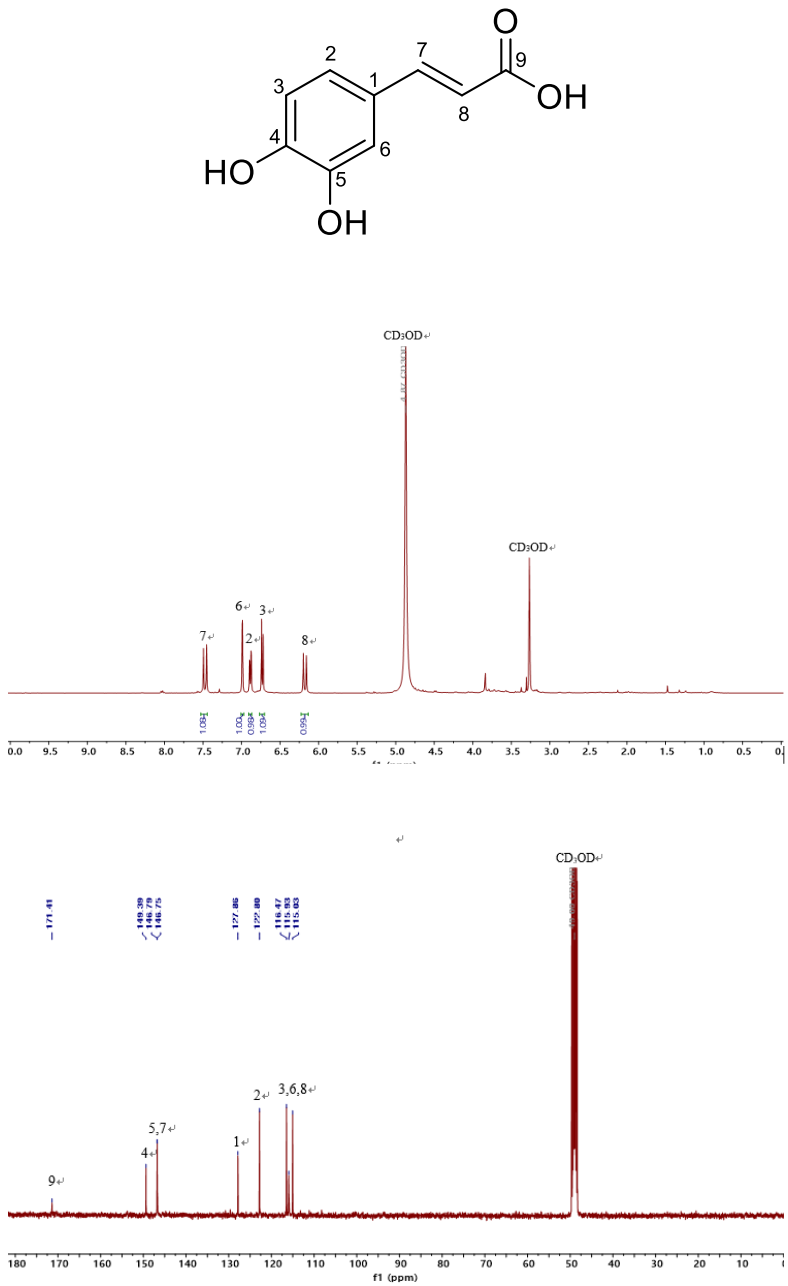



Figure 5.$^{1}$H, $^{13}$C NMR spectra of compound **1** in Methanol-*d4,* 400 MHz

## 1-2 Compound **2**

Compound 2 was obtained as a red powder. Its molecular formula was established as $C_{19}H_{18}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 295.1317 $[M + H]^+$ (calcd for $C_{19}H_{19}O_3$, 295.1334) corresponding to eleven degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two aromatic protons which showed splitting patterns of AB quaurtet. [($\delta_H$ 7.63, d, *J*=9.1 Hz. 1H), ($\delta_H$ 7.55, d, *J*=9.1 Hz, 1H)], One proton attached to oxygenated and double-bonded carbon ($\delta_H$ 7.22, q, *J*=1.2 Hz. 1H), Three methyl protons, [($\delta_H$ 2.26, s, 3H), ($\delta_H$ 1.31, s, 6H)], and six more protons attached to A ring of skeleton. [($\delta_H$ 1.65, m, 2H), ($\delta_H$ 1.80, m, 2H), ($\delta_H$ 3.18, t, *J*=6.4 Hz, 2H)] Based on an analysis of the spectra and reported data[9], active compound was identified as Tanshinone IIA.

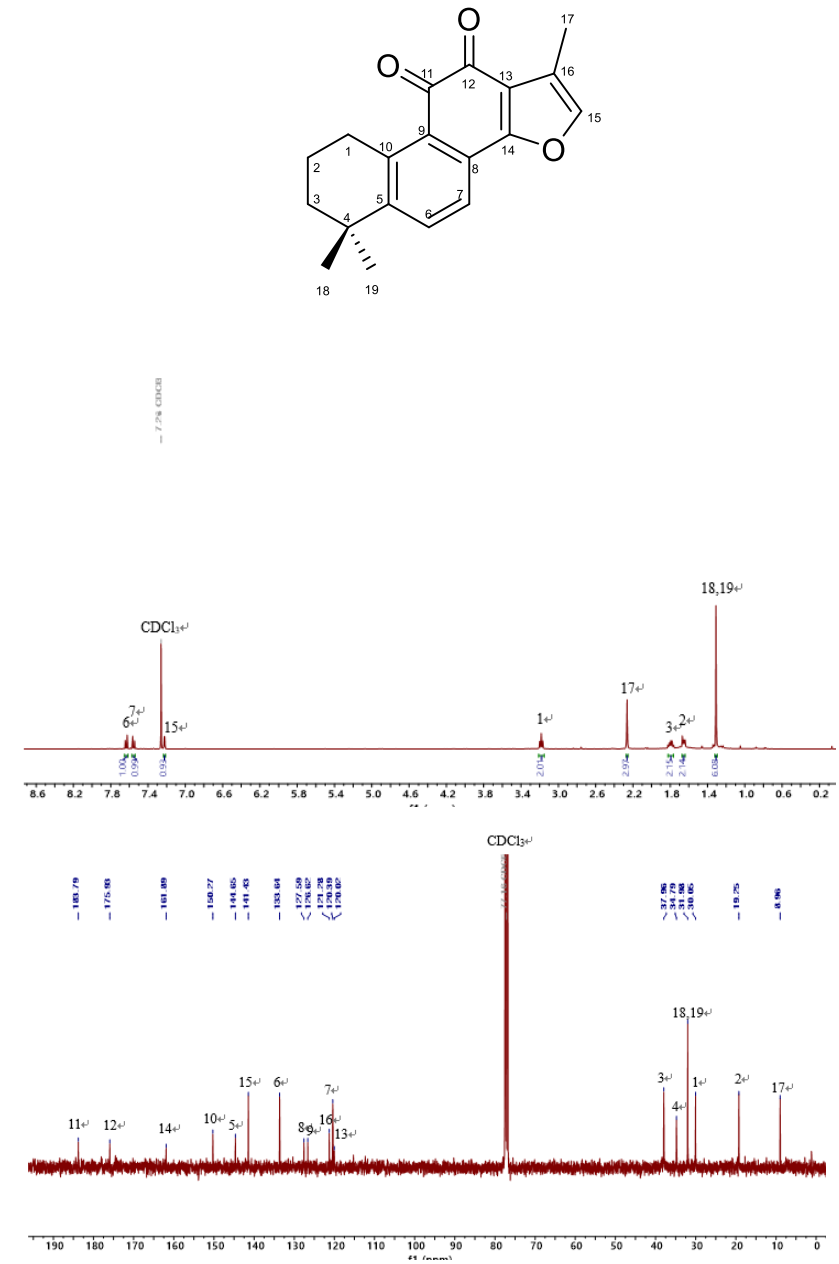
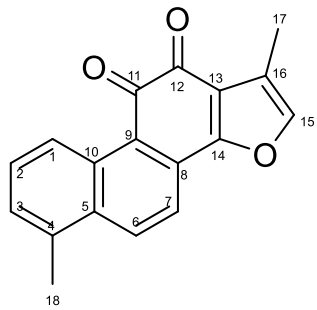



Figure 6.$^{1}$H, $^{13}$C NMR spectra of compound **2** in $CDCl_3$, 400 MHz

## 1-3 Compound **3**

Compound 3 was obtained as a brown powder. Its molecular formula was established as $C_{18}H_{12}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 277.0822 $[M + H]^+$ (calcd for $C_{18}H_{13}O_3$, 277.0864) corresponding to thirteen degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two aromatic protons which showed splitting patterns of AB quaurtet. [($\delta_H$ 7.80, d, *J*=8.7 Hz. 1H), ($\delta_H$ 8.29, d, *J*=8.7 Hz, 1H)], One proton attached to oxygenated and double-bonded carbon ($\delta_H$ 7.29, q, *J*=1.3 Hz. 1H), Two methyl protons, [($\delta_H$ 2.29, d, *J*=1.3 Hz, 3H), ($\delta_H$ 2.68, s, 3H)], and three more aromatic protons attached to A ring of skeleton. [($\delta_H$ 9.24, d, *J*=9.0 Hz 1H), ($\delta_H$ 7.54, dd, *J*=8.9, 7.0 Hz 1H), ($\delta_H$ 7.34, d, *J*=7.0 Hz, 1H)] Based on an analysis of the spectra and reported data[10], active compound was identified as Tanshinone I.



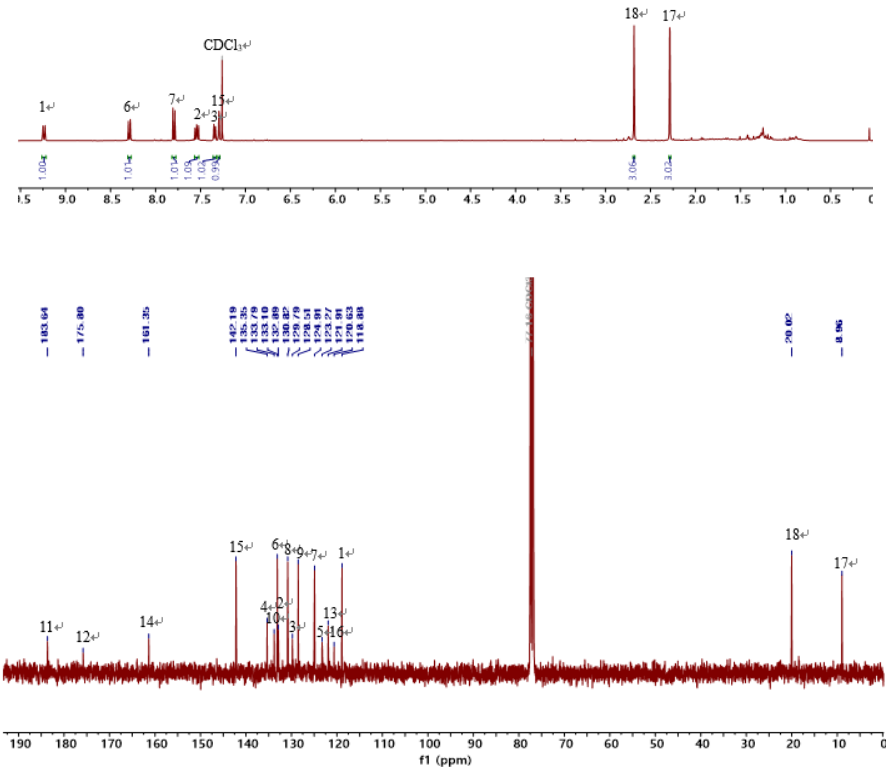




Figure 7.$^{1}$H, $^{13}$C NMR spectra of compound **3** in $CDCl_3$, 400 MHz

## 1-4 Compound **4**

Compound 4 was obtained as a orange powder. Its molecular formula was established as $C_{19}H_{20}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 297.1478 $[M + H]^+$ (calcd for $C_{19}H_{21}O_3$, 297.1491) corresponding to ten degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two aromatic protons which showed splitting patterns of AB quaurtet. [($\delta_H$ 7.63, d, *J*=8.1 Hz. 1H), ($\delta_H$ 7.49, d, *J*=8.1 Hz, 1H)], Methylene protons attached to oxygenated carbon [($\delta_H$ 4.89, t, *J*=9.5 Hz. 1H), ($\delta_H$ 3.60, m, 1H)], Three methyl protons, [($\delta_H$ 1.35, d, *J*=6.7 Hz, 3H), ($\delta_H$ 1.31, s, 6H)], six more protons attached to A ring of skeleton. [($\delta_H$ 3.21, t, *J*=6.4 Hz 2H), ($\delta_H$ 1.66, m, 2H), ($\delta_H$ 1.79, m, 2H)], and one proton from D ring of skeleton. ($\delta_H$ 4.36, dd, *J*=9.3, 5.9 Hz 1H). Based on an analysis of the spectra and reported data[10], active compound was identified as Cryptotanshinone.

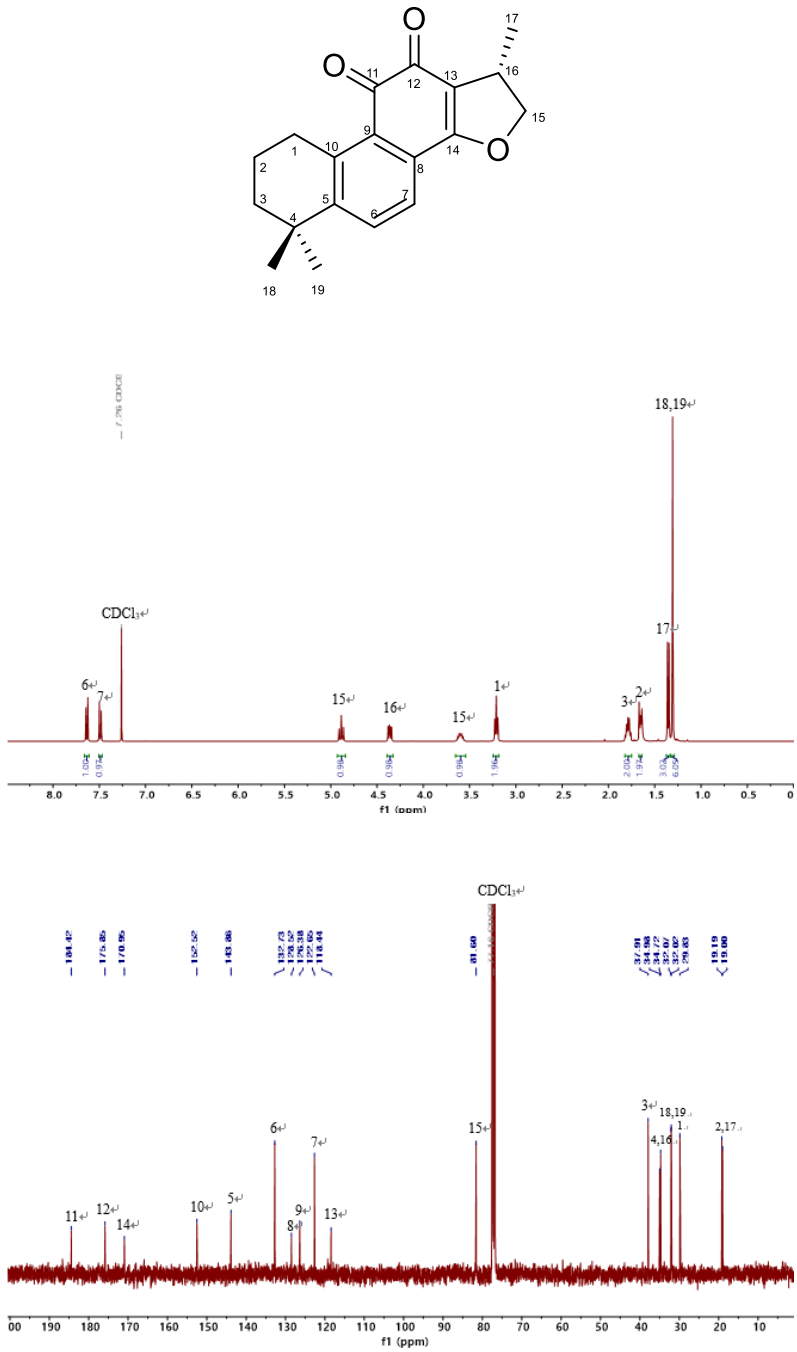



Figure 8.$^{1}$H, $^{13}$C NMR spectra of compound **4** in $CDCl_3$, 400 MHz

## 1-5 Compound **5**

Compound 5 was obtained as a red powder. Its molecular formula was established as $C_{18}H_{14}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 279.1010 $[M + H]^+$ (calcd for $C_{18}H_{15}O_3$, 279.1021) corresponding to thirteen degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed five aromatic protons. [($\delta_H$ 9.29, d, *J*=8.9 Hz. 1H), ($\delta_H$ 8.30, dd, *J*=8.7, 1.1 Hz, 1H), ($\delta_H$ 7.75, dd, *J*=8.7, 1.1 Hz. 1H), ($\delta_H$ 7.40, d, *J*=6.9 Hz. 1H)], Methylene protons attached to oxygenated carbon [($\delta_H$ 4.97, t, *J*=9.6 Hz. 1H), ($\delta_H$ 3.66, m, 1H)], Two methyl protons, [($\delta_H$ 1.41, d, *J*=6.8 Hz, 3H), ($\delta_H$ 2.70, s, and one proton from D ring of skeleton. ($\delta_H$ 4.34, dd, *J*=9.4, 6.2 Hz 1H). Based on an analysis of the spectra and reported data[10], active compound was identified as 15,16-dihydrotanshinone.

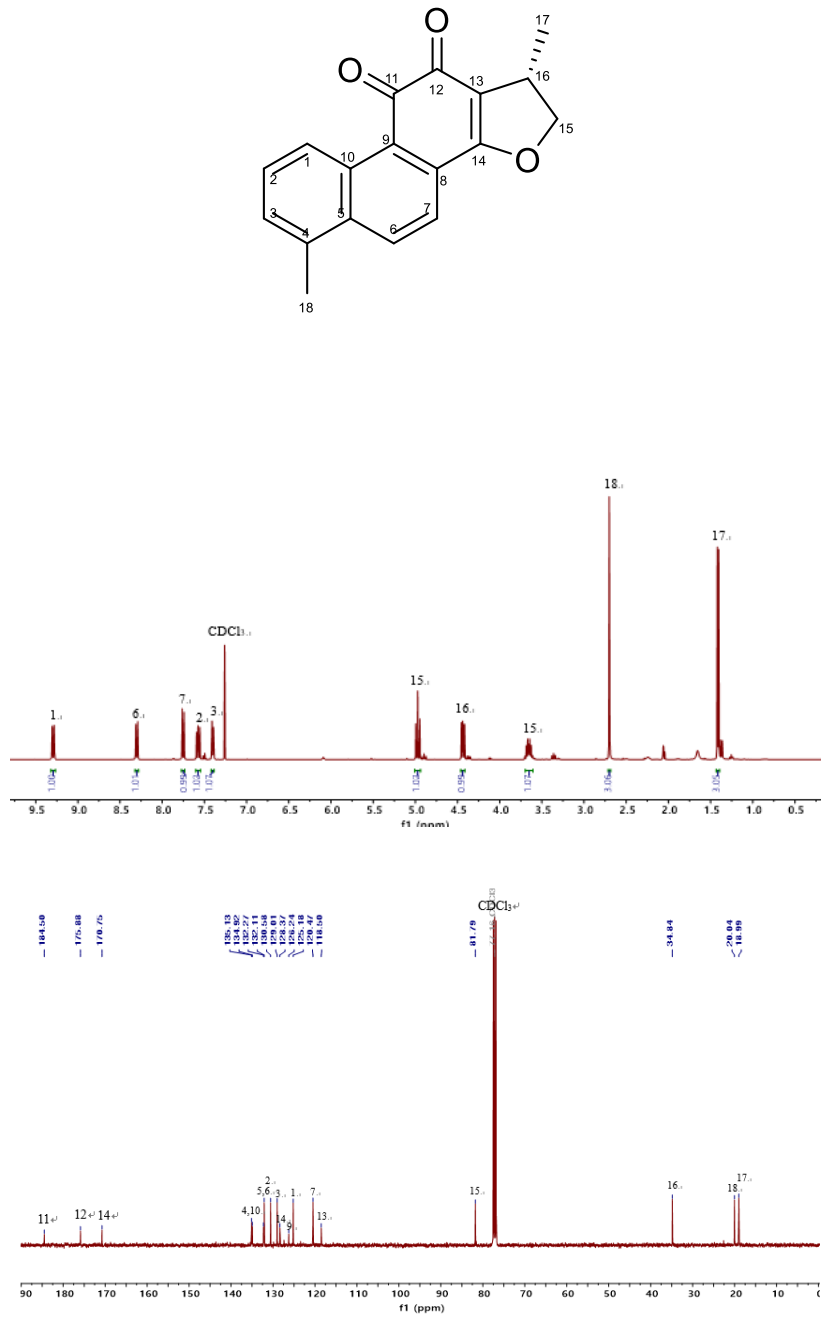



Figure 9.$^{1}$H, $^{13}$C NMR spectra of compound **5** in $CDCl_3$, 400 MHz

## 1-6 Compound **6**

Compound 6 was obtained as a colorless gum. Its molecular formula was established as $C_{18}H_{16}O_8$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 359.0761 $[M + H]^+$ (calcd for $C_{18}H_{15}O_8$, 359.0767) corresponding to eleven degrees of unsaturation. The $^1$H NMR spectrum of active compounds showed six aromatic protons which is part of ABX spin system. [($\delta_H$ 7.01, d, *J*=2.1 Hz. 1H), ($\delta_H$ 6.92, dd, *J*=8.2, 2.1 Hz, 1H), ($\delta_H$ 6.75, d, *J*=8.1 Hz. 1H), ($\delta_H$ 6.72, d, *J*=2.1 Hz. 1H), ($\delta_H$ 6.66, d, *J*=8.0 Hz, 1H), ($\delta_H$ 6.58, dd, *J*=8.1, 2.1 Hz, 1H)], olefinic protons indicating *trans* double bond by its coupling constant, [($\delta_H$ 7.52, d, *J*=15.9 Hz. 1H), ($\delta_H$ 6.24, d, *J*=15.9 Hz. 1H)], One proton attached to oxygenated carbon, ($\delta_H$ 5.15, dd, *J*=8.3, 4.3 Hz, 1H), and methylene protons. [($\delta_H$ 3.07, dd, *J*=14.3, 4.4 Hz, 1H), ($\delta_H$ 2.97, dd, *J*=14.3, 8.4 Hz, 1H)] Based on an analysis of the spectra and reported data[11], active compound was identified as Rosmarinic acid.

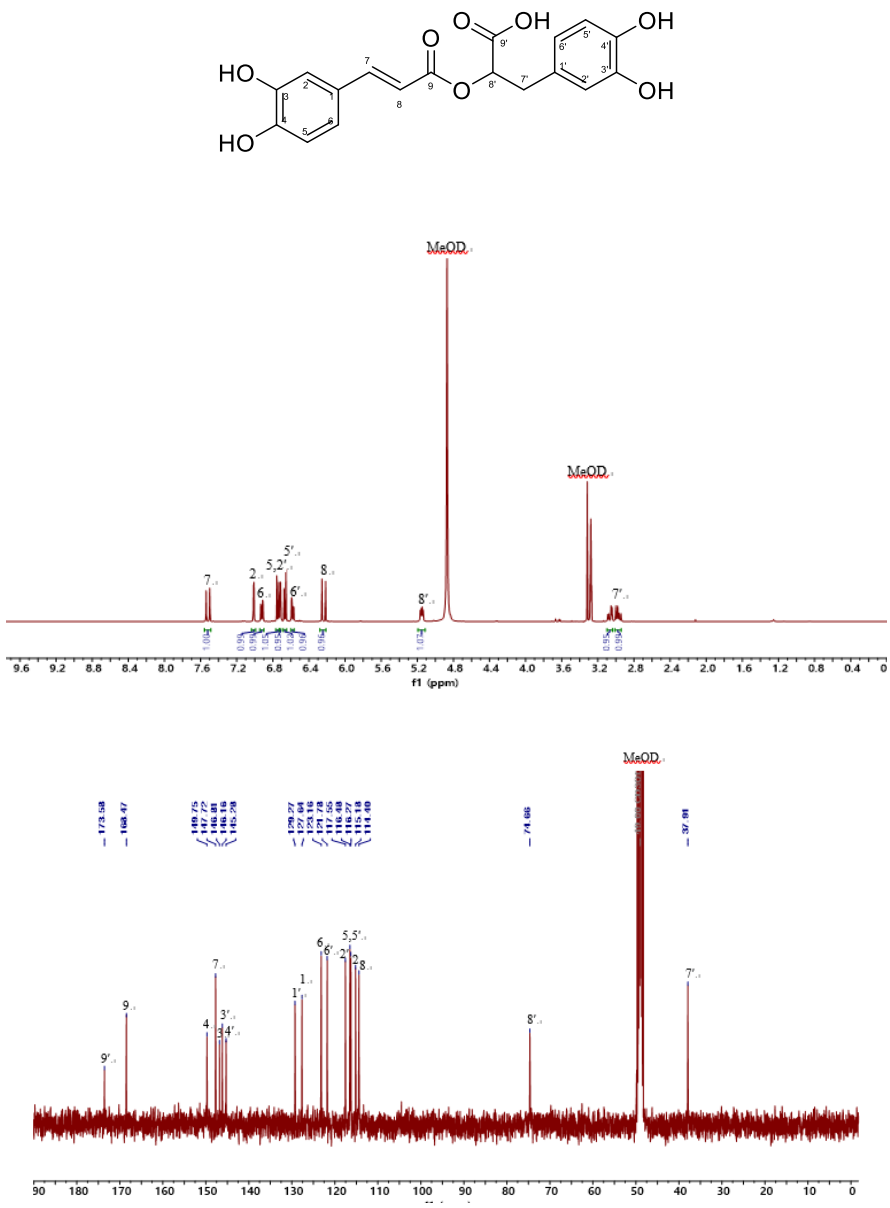
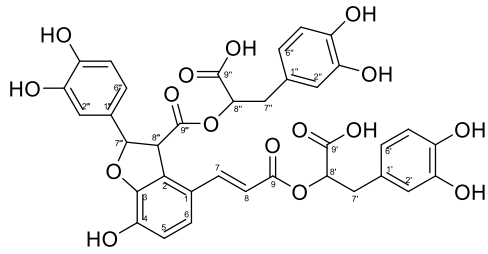
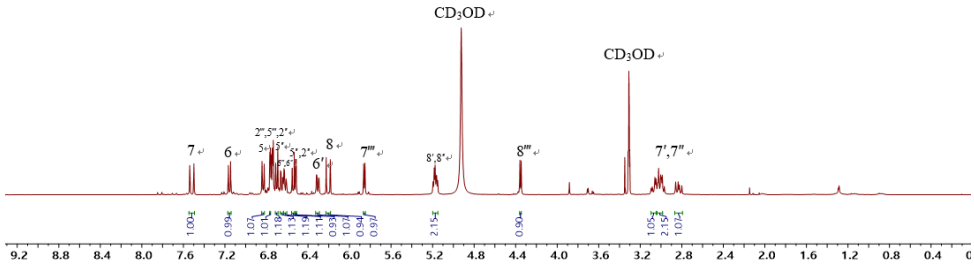



Figure 10.$^{1}$H, $^{13}$C NMR spectra of compound **6** in Methanol-*d4,* 400 MHz

## 1-7 Compound **7**

Compound 7 was obtained as a yellowish gum. Its molecular formula was established as $C_{36}H_{30}O_{16}$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 717.1447 $[M + H]^+$ (calcd for $C_{36}H_{29}O_{16}$, 717.1456) corresponding to twenty two degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed nine aromatic protons which is part of ABX spin system. [($\delta_H$ 6.76, d, *J*=2.1 Hz. 1H), ($\delta_H$ 6.75, d, *J*=8.1 Hz, 1H), ($\delta_H$ 6.74, d, *J*=2.2 Hz. 1H), ($\delta_H$ 6.70, d, *J*=8.0 Hz. 1H), ($\delta_H$ 6.65, dd, *J*=8.2, 2.1 Hz, 1H), ($\delta_H$ 6.62, dd, *J*=8.1, 2.0 Hz, 1H), ($\delta_H$ 6.54, d, *J*=8.1, Hz, 1H), ($\delta_H$ 6.52, d, *J*= 2.1 Hz, 1H), ($\delta_H$ 6.31, dd, *J*=8.1, 2.2 Hz, 1H)], and two more aromatic protons [($\delta_H$ 7.16, d, *J*=8.5 Hz. 1H), ($\delta_H$ 6.83, d, *J*=8.4 Hz, 1H),] olefinic protons indicating *trans* double bond by its coupling constant, [($\delta_H$ 7.52, d, *J*=15.9 Hz. 1H), ($\delta_H$ 6.20, d, *J*=15.9 Hz. 1H)], two protons attached to oxygenated carbon, ($\delta_H$ 5.17, m, 2H), and two sets of methylene protons. [($\delta_H$ 3.07, dd, *J*=14.3, 4.4 Hz, 1H), ($\delta_H$ 3.02, m, 2H), ($\delta_H$ 2.83, dd, *J*=14.3, 9.6 Hz 1H)] Based on an analysis of the spectra and reported data[11], active compound was identified as Salvianolic acid B.



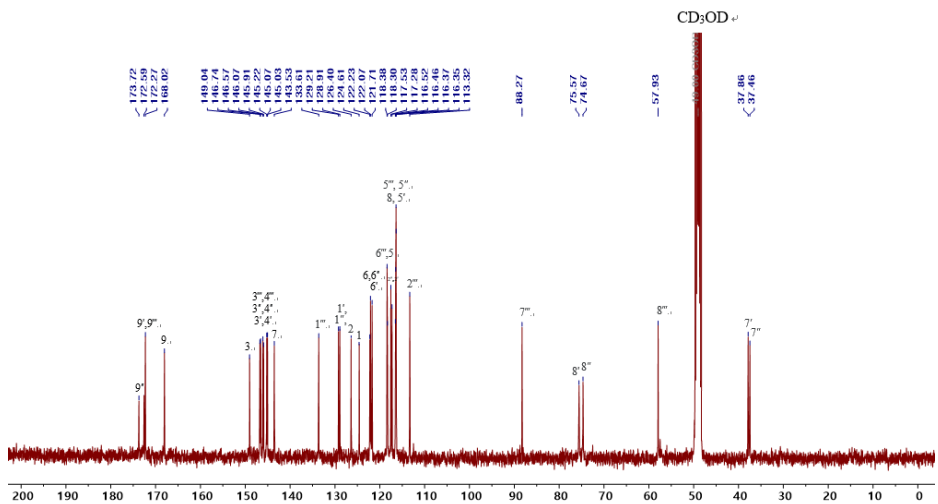




Figure 11.$^{1}$H, $^{13}$C NMR spectra of compound **7** in Methanol-*d4,* 400 MHz

## 1-8 Compound **8**

Compound **8** was obtained as a red powder. Its molecular formula was established as $C_{19}H_{22}O_2$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 283.1694 $[M + H]^+$ (calcd for $C_{19}H_{23}O_2$, 283.1698) corresponding to nine degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed three aromatic protons. [($\delta_H$ 7.59, d, *J*=8.0 Hz. 1H), ($\delta_H$ 7.10, d, *J*=8.0 Hz, 1H), ($\delta_H$ 7.07, s, 1H). six more protons attached to A ring of skeleton. [($\delta_H$ 3.17, t, *J*=6.4 Hz 2H), ($\delta_H$ 1.65, m, 2H), ($\delta_H$ 1.80, m, 2H)], Two sets of symmetric methyl protons, [($\delta_H$ 1.16, d, *J*=6.9 Hz, 6H), ($\delta_H$ 1.30, s, 6H)] and one proton showed septet between two symmetric methyls. ($\delta_H$ 3.02, sept, *J*= 6.9 Hz 1H). Based on an analysis of the spectra and reported data[12], active compound was identified as Sibiriquinone B.

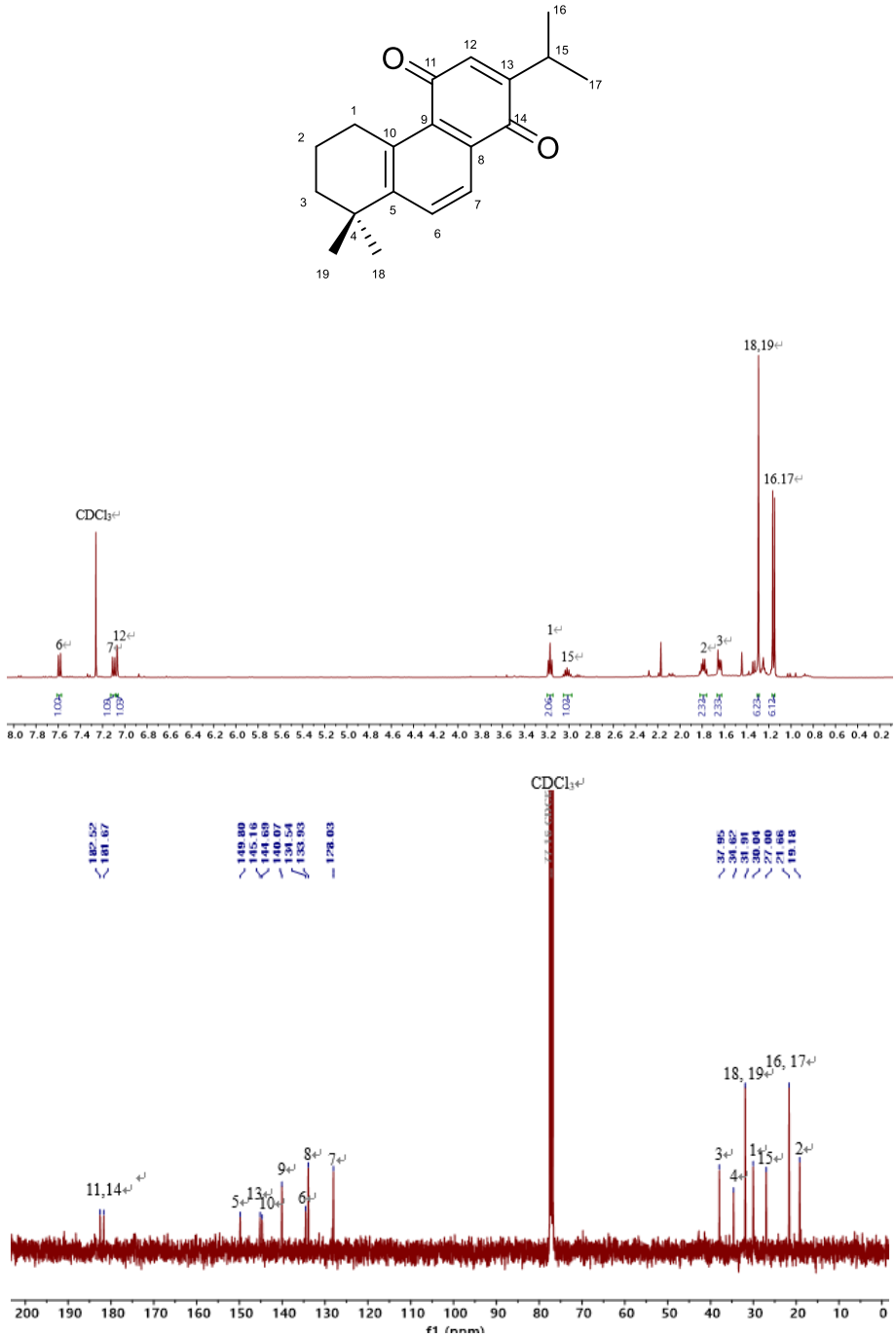



Figure 12.$^{1}$H, $^{13}$C NMR spectra of compound **8** in $CDCl_3$, 400 MHz

## 1-9 Compound **9**

Compound 9 was obtained as a yellowish gum. Its molecular formula was established as $C_{27}H_{22}O_{12}$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 537.1055 $[M - H]^-$ (calcd for $C_{27}H_{21}O_{12}$, 537.1033) corresponding to seventeen degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed six aromatic protons which is part of ABX spin system. [($\delta_H$ 6.71, d, *J*=8.1 Hz. 1H), ($\delta_H$ 6.67, d, *J*=2.1 Hz, 1H), ($\delta_H$ 6.60, d, *J*=2.1 Hz. 1H), ($\delta_H$ 6.56, d, *J*=8.6 Hz. 1H), ($\delta_H$ 6.54, dd, *J*=8.6, 1.9 Hz, 1H), ($\delta_H$ 6.32, dd, *J*=8.1, 2.1 Hz, 1H)] and two more aromatic protons [($\delta_H$ 7.22, d, *J*=8.5 Hz. 1H), ($\delta_H$ 6.82, d, *J*=8.4 Hz, 1H),] olefinic protons indicating *trans* double bond by its coupling constant, [($\delta_H$ 7.52, d, *J*=15.9 Hz. 1H), ($\delta_H$ 6.22, d, *J*=15.9 Hz. 1H)], one proton attached to oxygenated carbon, ($\delta_H$ 5.70, d, *J*=4.0 Hz, 1H), and one set of methylene protons. [($\delta_H$ 2.84, m, 1H), ($\delta_H$ 2.92, m, 1H)] Based on an analysis of the spectra and reported data[13], active compound was identified as Isolithospermic acid.

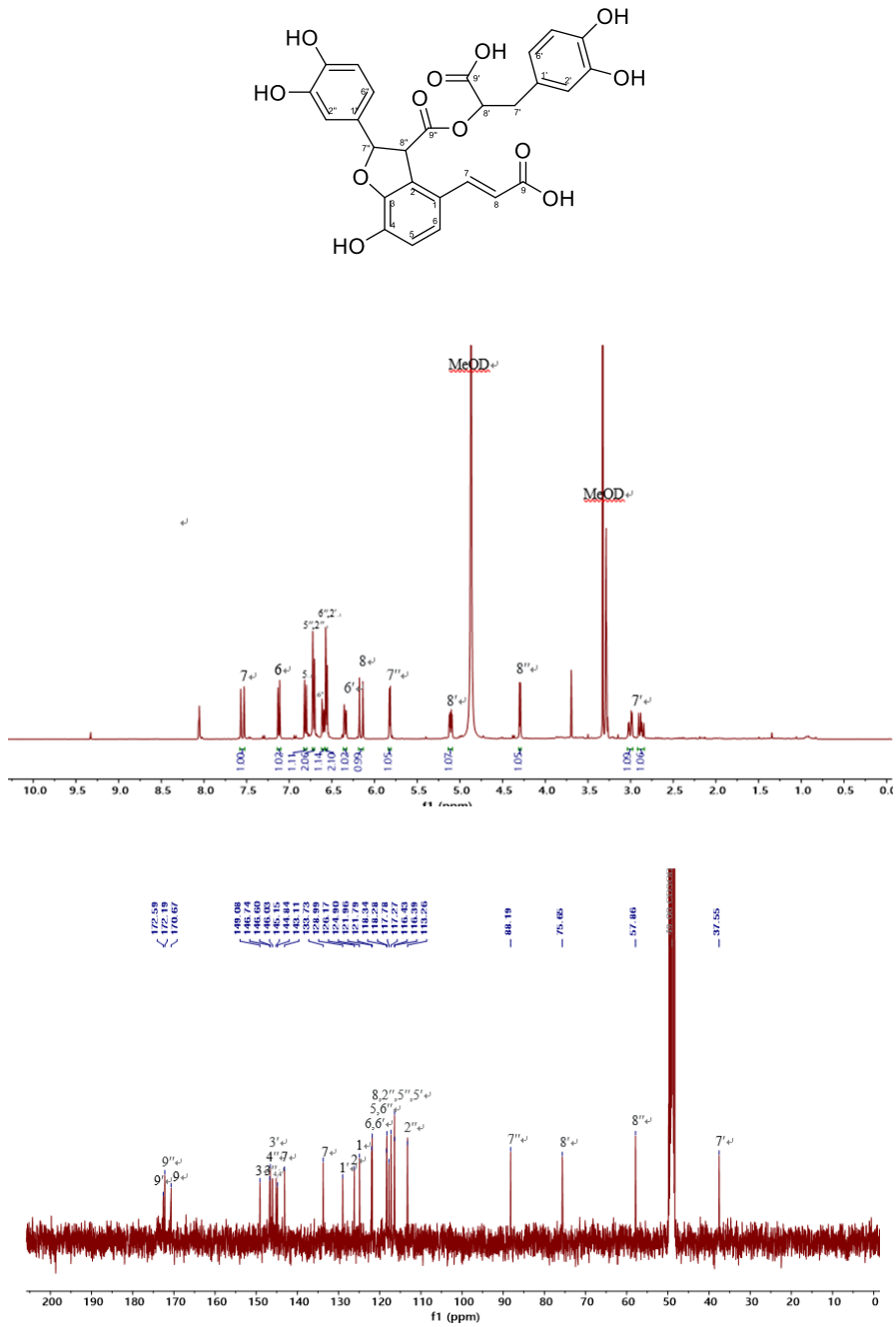



Figure 13.[1]H, [13]C NMR spectra of compound **9** in Methanol-*d4,* 400 MHz,

1-10 Compound **10**

Compound **10** was obtained as a colorless gum. Its molecular formula was established as $C_{18}H_{14}O_8$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 357.0598 $[M - H]^-$ (calcd for $C_{18}H_{13}O_8$, 357.0610) corresponding to twelve degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed three aromatic protons which is part of ABX spin system. [($\delta_H$ 6.78, d, *J*=8.5 Hz. 1H), ($\delta_H$ 6.68, dd, *J*=8.2, 2.0 Hz, 1H), ($\delta_H$ 6.73, d, *J*=8.1 Hz. 1H)] and two more aromatic protons. [($\delta_H$ 7.16, d, *J*=8.5 Hz. 1H), ($\delta_H$ 6.78, d, *J*=8.5 Hz, 1H),] Moreover, olefinic protons indicating *trans* double bond by its coupling constant, [($\delta_H$ 7.74, d, *J*=15.9 Hz. 1H), ($\delta_H$ 6.25, d, *J*=15.9 Hz. 1H)], one proton attached to oxygenated carbon, ($\delta_H$ 5.85, d, *J*=4.9 Hz, 1H), and one proton from furan ring. ($\delta_H$ 4.29, d, 1H), Based on an analysis of the spectra and reported data[14], active compound was identified as 8-epibechnic acid.

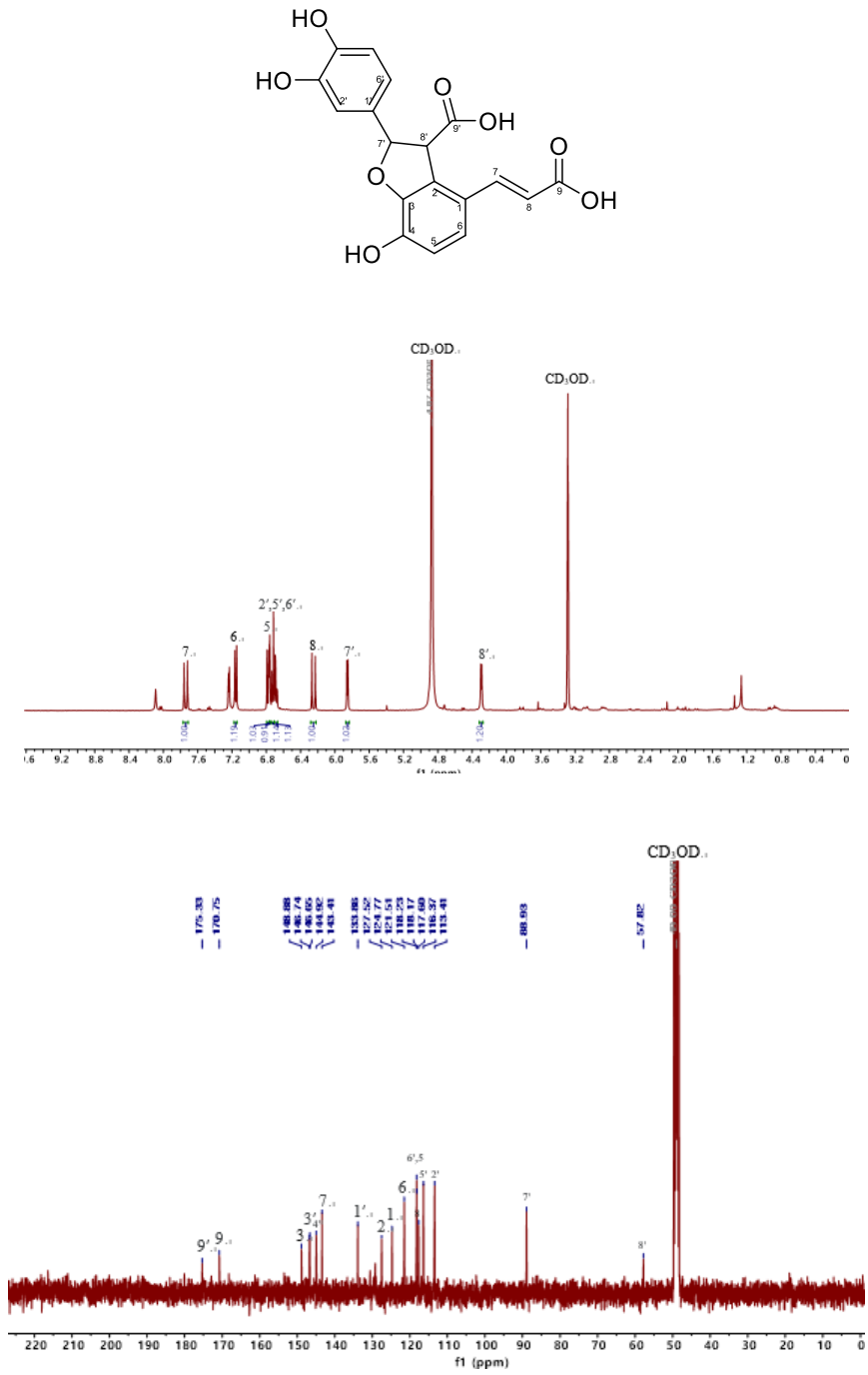



Figure 14.[1]H, [13]C NMR spectra of compound **10** in Methanol-*d4,* 400 MHz,

## 1-11 Compound **11**

Compound 11 was obtained as a red powder. Its molecular formula was established as $C_{19}H_{22}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 299.1653 $[M + H]^+$ (calcd for $C_{19}H_{23}O_3$, 299.1647) corresponding to nine degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed three aromatic protons. [($\delta_H$ 7.59, d, *J*=7.9 Hz. 1H), ($\delta_H$ 7.17, s, 1H), ($\delta_H$ 7.11, d, *J*=7.9, 1H)], Methylene protons attached to oxygenated carbon ($\delta_H$ 3.69, m, 2H), Three methyl protons, [($\delta_H$ 1.28, d, *J*=2.5 Hz, 6H), ($\delta_H$ 1.21, d, *J*=7.0 Hz, 3H), Based on an analysis of the spectra and reported data[11], active compound was identified as R-(+)-grandifolia D.

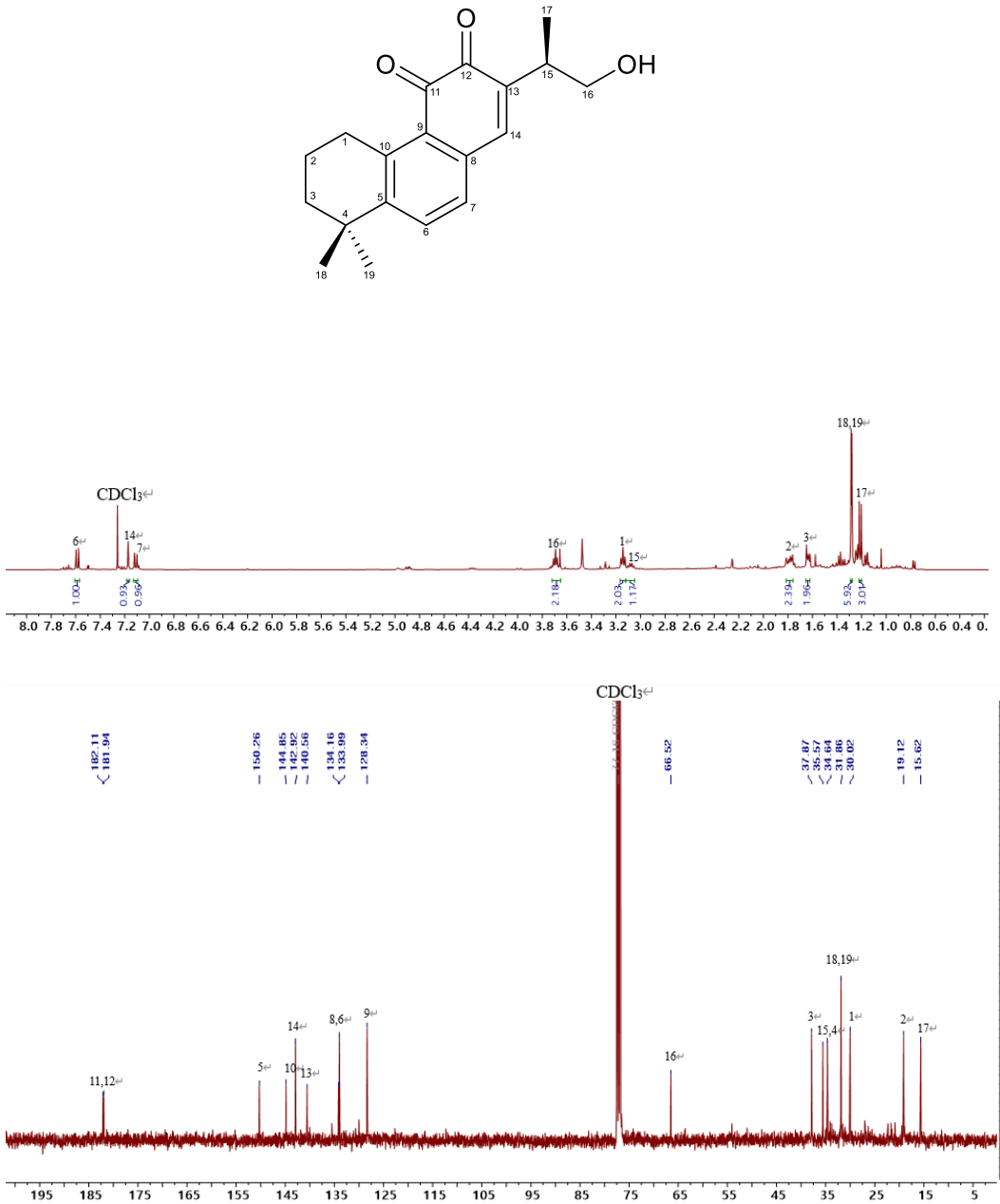



Figure 15.$^{1}$H, $^{13}$C NMR spectra of compound **11** in $CDCl_3$, 400 MHz,

## 1-12 Compound **12**

Compound 12 was obtained as a white amorphous solid. Its molecular formula was established as $C_{19}H_{24}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 301.1778 $[M + H]^+$ (calcd for $C_{19}H_{25}O_3$, 301.1725) corresponding to eight degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two aromatic protons. [($\delta_H$ 7.42, s, 1H), ($\delta_H$ 7.30, s, 1H)], protons attached to oxygenated carbon [($\delta_H$ 4.70, dd, *J*=3.8, 1.8 Hz, 1H), ($\delta_H$ 4.38, d, *J*=9.0 Hz, 1H), ($\delta_H$ 3.00, dd, *J*=9.1, 1.8 Hz, 1H)], six more protons attached to A ring of skeleton. [($\delta_H$ 1.73, m, 2H), ($\delta_H$ 1.63, m, 2H), ($\delta_H$ 1.23, m, 2H)], Three methyl protons, [($\delta_H$ 2.46, s, 3H), ($\delta_H$ 1.17, s, 3H), ($\delta_H$ 0.87, s, 3H)], Based on an analysis of the spectra and reported data[28], active compound was identified as Miltipolone.

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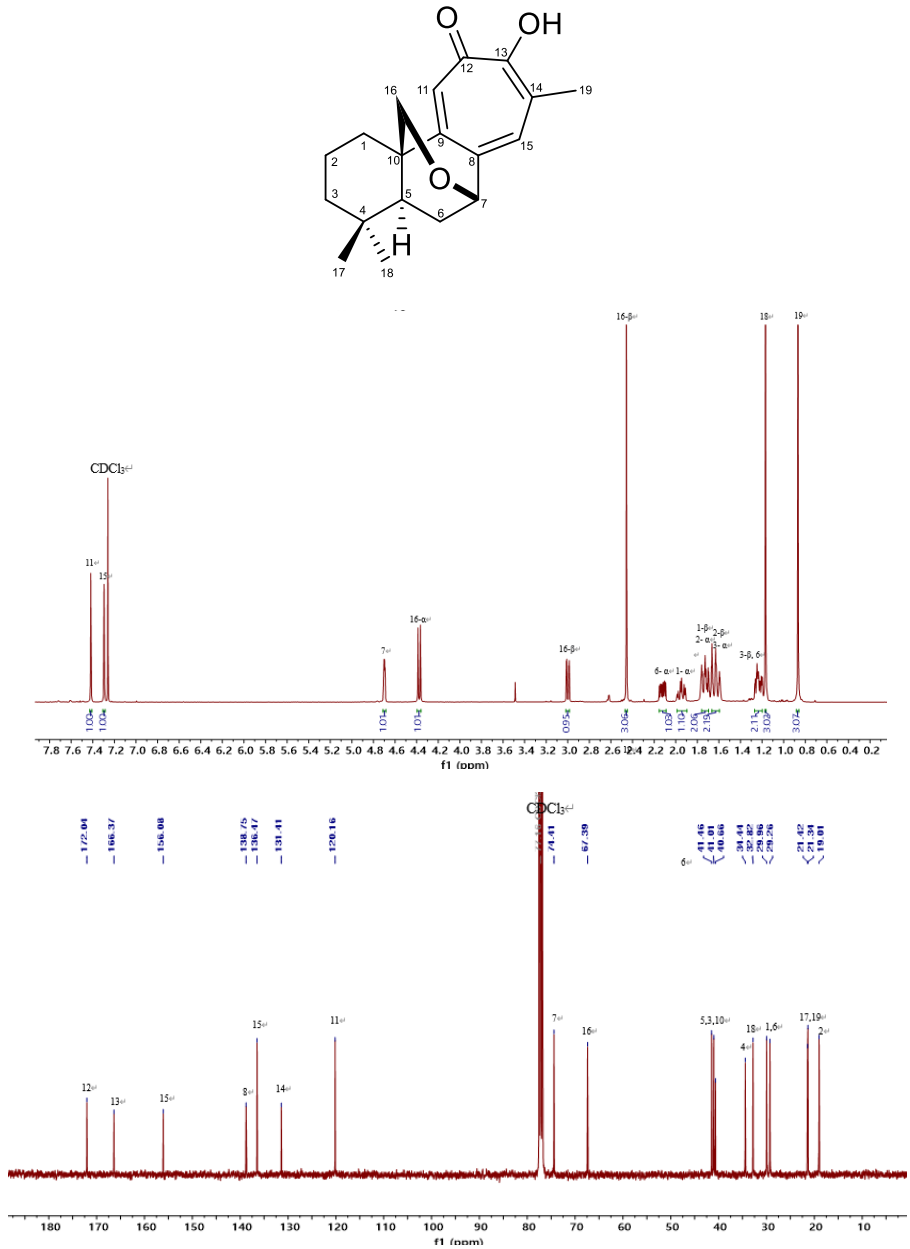


Figure 16.$^{1}$H, $^{13}$C NMR spectra of compound **12** in $CDCl_3$, 400 MHz

## 1-13 Compound **13**

Compound 13 was obtained as a red powder. Its molecular formula was established as $C_{18}H_{16}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 281.1191 $[M + H]^+$ (calcd for $C_{18}H_{17}O_3$, 281.1178) corresponding to nine degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two aromatic protons. [($\delta_H$ 7.53, d, *J*=7.9 Hz, 1H), ($\delta_H$ 7.43, d, *J*=7.9 Hz, 1H)], four protons attached to oxygenated carbon or near olefinic bond [($\delta_H$ 4.91, t, *J*=9.5 Hz, 1H), ($\delta_H$ 4.37, dd, *J*=9.3, 6.0 Hz, 1H), ($\delta_H$ 3.62, m, 1H), ($\delta_H$ 3.38, t, *J*=7.9 Hz, 2H), Two methyl protons, [($\delta_H$ 2.07, q, *J*=1.7 Hz, 3H), ($\delta_H$ 1.38, d, *J*=7.0 Hz, 3H)], Based on an analysis of the spectra and reported data[29], active compound was identified as Trijuganone B.

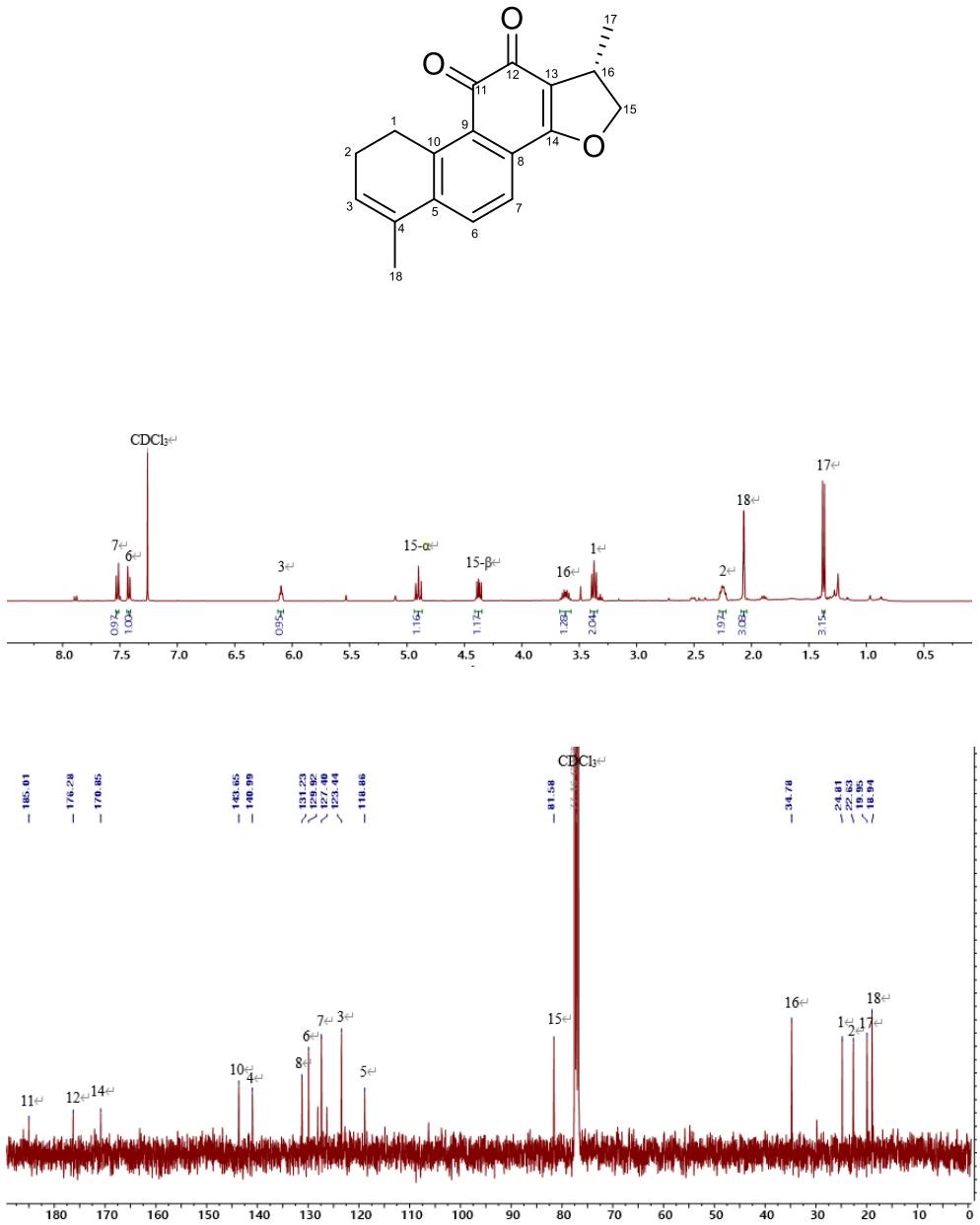


Figure 16.$^{1}$H, $^{13}$C NMR spectra of compound **13** in $CDCl_3$, 400 MHz

## 1-12 Compound **14**

Compound **14** was obtained as a orange powder. Its molecular formula was established as $C_{19}H_{26}O_3$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 303.1906 $[M + H]^+$ (calcd for $C_{19}H_{27}O_3$, 303.1960) corresponding to seven degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed two aromatic protons. [($\delta_H$ 7.61, s, 1H), ($\delta_H$ 7.42, s, 1H)], methylene protons attached to oxygenated carbon [($\delta_H$ 3.99, d, *J*=11.4, 1H), ($\delta_H$ 3.88, d, *J*=11.4 Hz, 1H)], six more protons attached to A ring and B ring of skeleton. [($\delta_H$ 3.03, ddd, *J*=17.0, 8.0, 2.6 Hz 1H), ($\delta_H$ 2.95, dd, *J*=17.0, 8.0 Hz, 1H), ($\delta_H$ 2.67, dd, *J*=12.7, 3.0 Hz, 1H), ($\delta_H$ 1.90, m, 2H), ($\delta_H$ 1.67, m, 2H)], Three methyl protons, [($\delta_H$ 2.38, s, 3H), ($\delta_H$ 0.97, s, 3H), ($\delta_H$ 0.96, s, 3H)], Based on an analysis of the spectra and reported data[30], active compound was identified as Isograndifoliol.

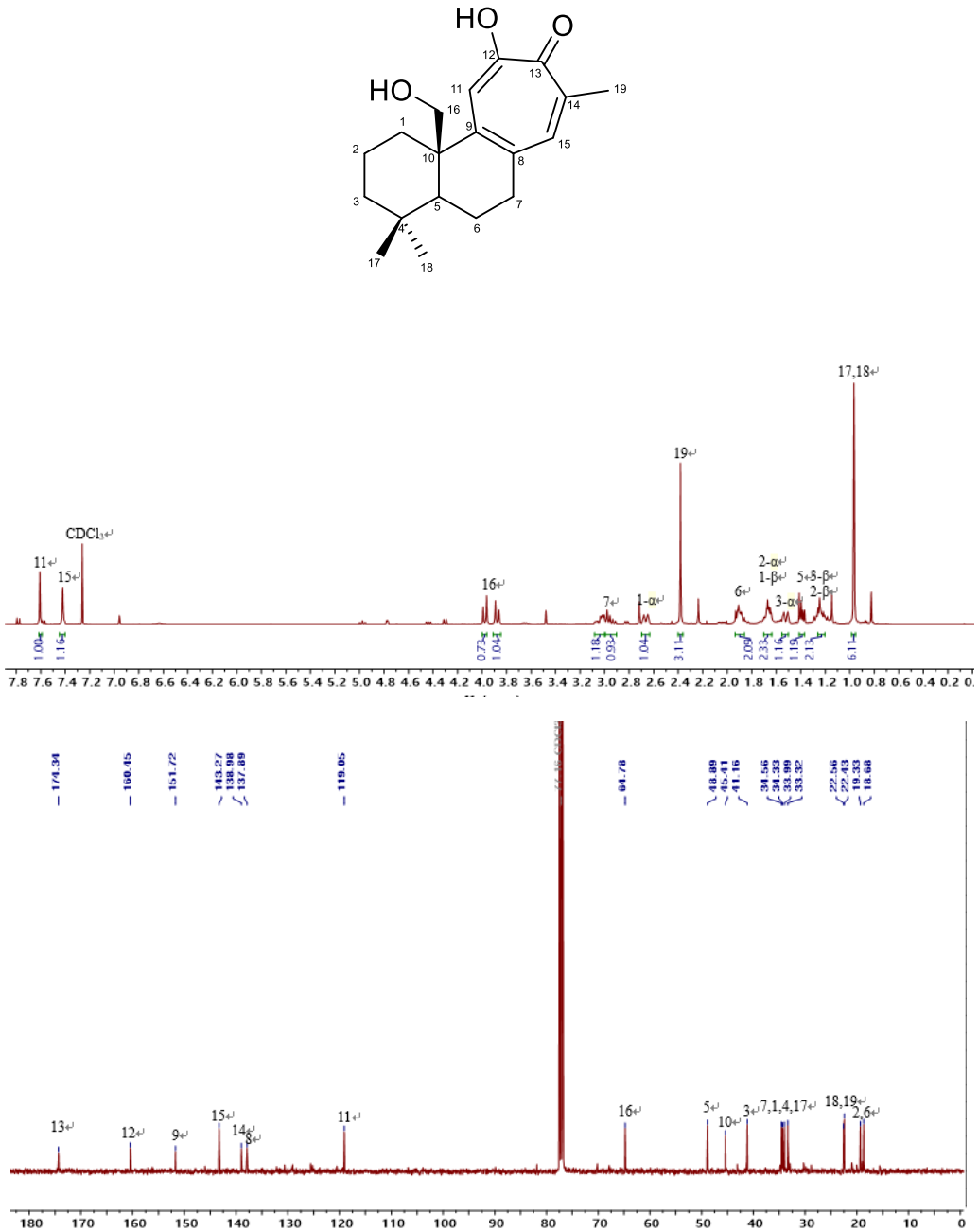


Figure 17.[1]H, [13]C NMR spectra of compound **14** in $CDCl_3$, 400 MHz

## 1-15 Compound **15**

Compound 15 was obtained as a red powder. Its molecular formula was established as $C_{18}H_{16}O_4$ based on its protonated high-resolution electrospray ionization mass spectrometry (HRESIMS) ion peak at *m/z* 297.1498 $[M + H]^+$ (calcd for $C_{18}H_{17}O_4$, 297.1169) corresponding to eleven degrees of unsaturation. The $^1H$ NMR spectrum of active compounds showed five aromatic protons. [($\delta_H$ 9.42, d, *J*=8.8 Hz. 1H), ($\delta_H$ 8.42, d, *J*=8.8 Hz. 1H), ($\delta_H$ 8.26, d, *J*=8.8 Hz. 1H), ($\delta_H$ 7.63, dd, *J*=8.9, 7.0 Hz. 1H), ($\delta_H$ 7.47, d, *J*=6.8, 1H)], Methylene protons attached to oxygenated carbon [($\delta_H$ 4.00, dd, *J*=10.7, 7.8 Hz. 1H), ($\delta_H$ 3.89, dd, *J*=10.7, 5.3 Hz. 1H), Two methyl protons, [($\delta_H$ 1.33, d, *J*=7.1 Hz, 3H), ($\delta_H$ 2.74, s, 3H), Based on an analysis of the spectra and reported data[31], active compound was identified as Danshenxinkun A.

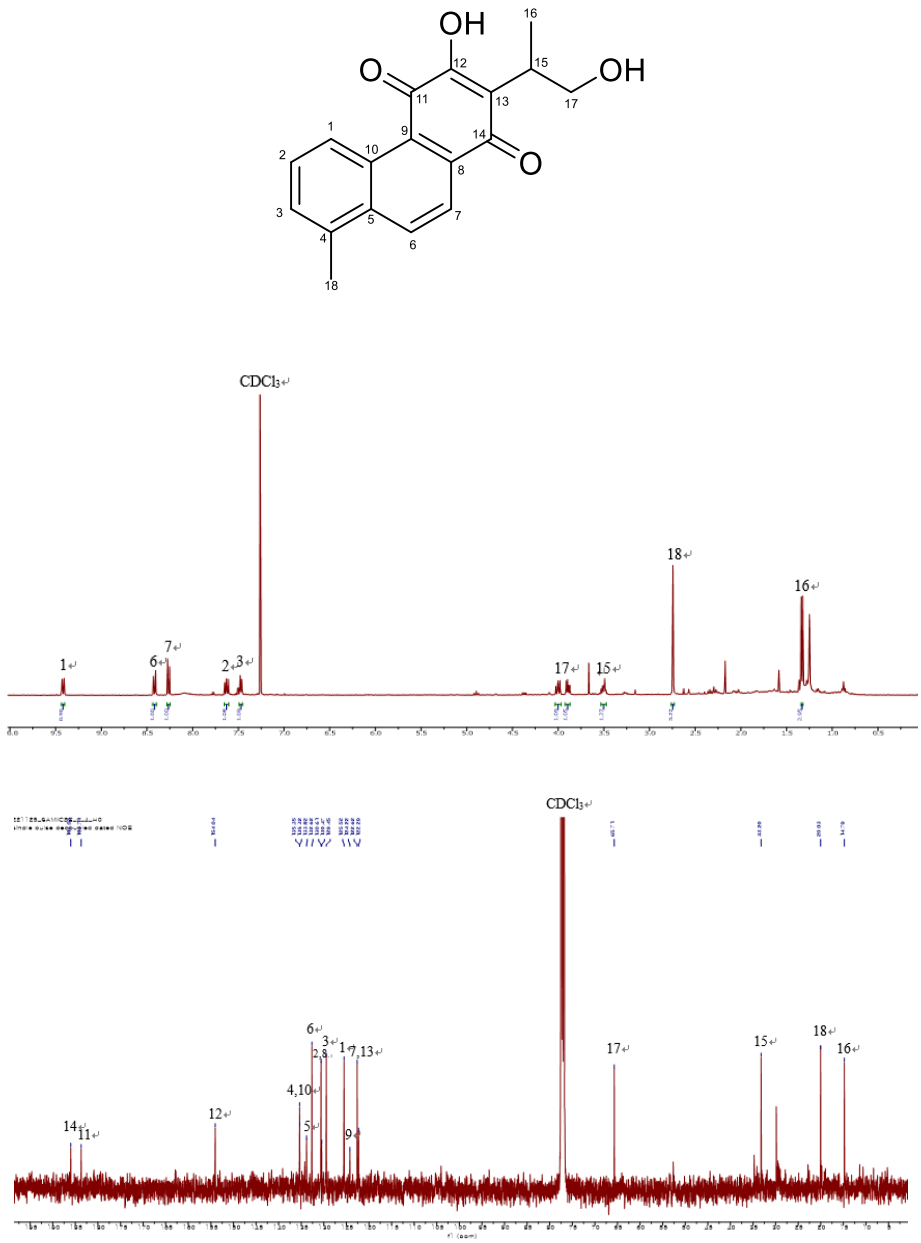


Figure 18.[1]H, [13]C NMR spectra of compound **15** in $CDCl_3$, 400 MHz

## 2. Bioactivity of isolated compounds from the roots of *Salvia miltiorhiza*

### 2-1 Anti-inflammation effects of isolated compounds on LPS-induced RAW264.7 cells

Thirteen single compounds (**1**-**13**) were isolated from the various fractions of Chinese and Korean roots of *salvia miltiorrhiza*. The effects of anti-inflammation effects on LPS-induced RAW264.7 cells of all isolated diterpenoids and phenolic acids were determined. Tanshinone I (**3**), Cryptotanshinone (**4**), Sibiriquinone B (**8**), showed similar or more activity then positive control Quercetin, 15,16-dihydrotanshinone (**5**) showed cytotoxicity on concentration of 20 μM. NO assay was done again with those four compounds to see their activity based on concentration. 15,16-dihydrotanshinone (**5**), which showed cytotoxicity on 20 μM showed activity on concentration of 5 μM.

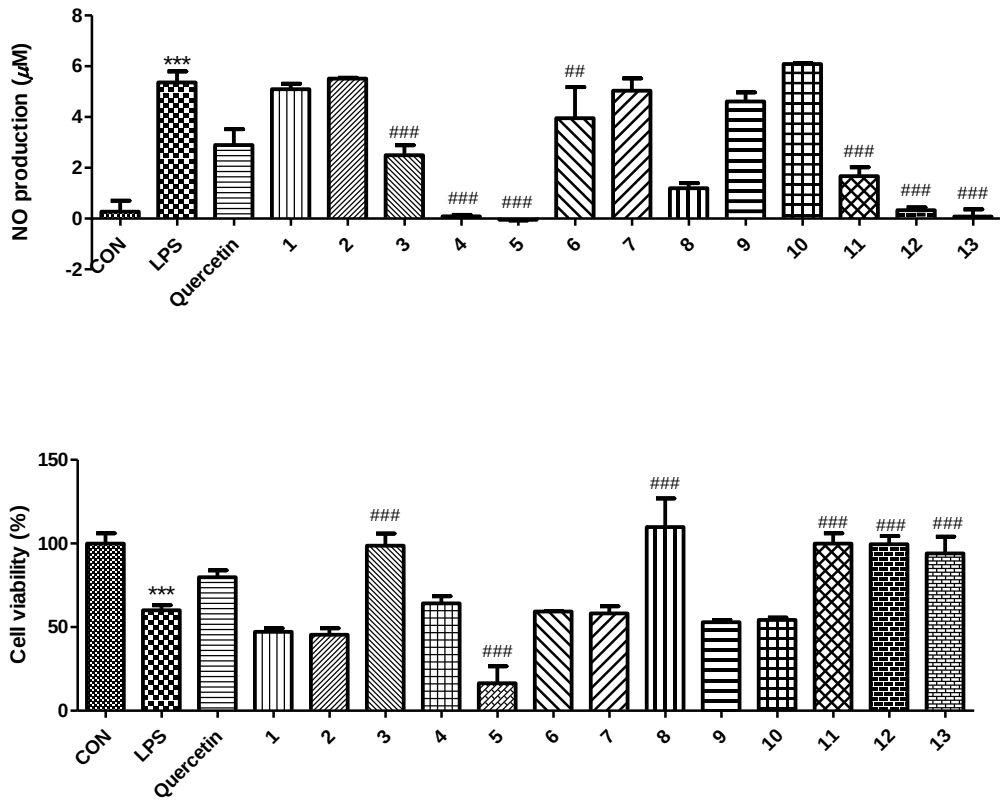


Figure 19. The effects of anti-inflammation effects on LPS-induced RAW264.7 cells of all isolated diterpenoids and phenolic acids were determined. Cells were treated with compounds 20 μM then treated with 1 ng/mL of LPS 1 hours later. After 4 hours of incubation, the MTT assay was performed as described in the experimental section. DMSO and Quercetin were used to control and positive control respectively. Data are represented as mean ± SD of three independent assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$ compared to control (CON), #$p < 0.05$; ##$p < 0.01$ compared to LPS-exposed samples (LPS).

2-2 Effects of isolated compounds on C2C12 cells.

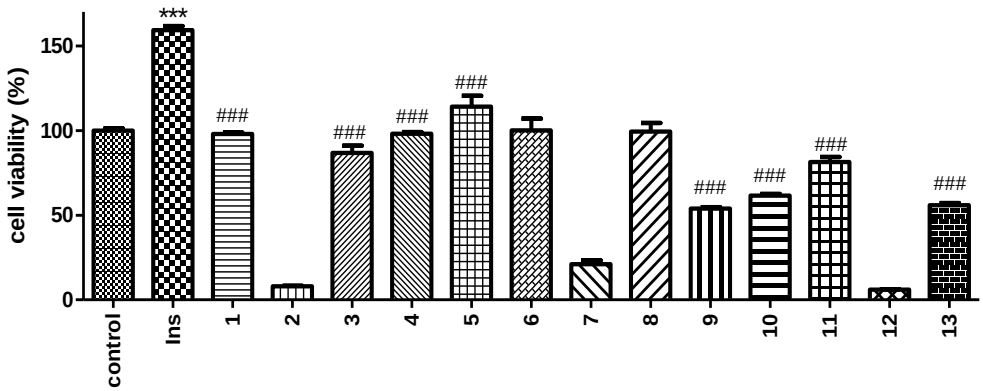


Figure 20. C2C12 cells were seeded in 96-well plates at density 5000 cell/well each sample treated triplicate. After overnight incubation, cells were treated 100 μL of sample containing serum free media for 24h. 20 μL of MTT solution (2mg/ mL) was directly added to each well. After four hours, purple MTT formazan precipitate was then dissolved in 100 μL of DMSO, and the absorbance at 540 nm was measured on a multiwall reader, Compounds 1-13 were tested to see possibility of usage as a treatment of sarcopenia, but none of candidates showed bioactivity as positive control Insulin. Data are represented as mean ± SD of three independent assays. *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$ compared to control (CON), #$p < 0.05$; ##$p < 0.01$ compared to LPS-exposed samples (LPS).

# IV. Conclusion

Fifteen compounds (**1**-**15**) were isolated from the roots of *Salvia miltiorrhiza.* Polar phenolic acids were isolated from Korean *Salvia miltiorrhiza*, and nonpolar diterpenoids were isolated from Chinese *Salvia miltiorrhiza*. Fifteen isolated compounds, *(E)*-Caffeic acid (**1**), Tanshinone IIA (**2**), Tanshinone I (**3**), Cryptotanshinone (**4**), 15,16-dihydrotanshinone (**5**), Rosmarinic acid (**6**), Salvianolic acid B (**7**), Sibiriquinone B (**8**), Isolithospermic acid (**9**), 8-epibechnic acid (**10**), (+)-grandifolia D (**11**), Miltipolone (**12**), Trijuganone B (**13**), Isograndifoliol (**14**), Danshenxinkun A (**15**) were known compounds. Compounds **11** to **15** were isolated under avoidance of light and heat. Isolated compounds were tested for their anti-inflammatory activity upon LPS-induced RAW264.7 cells and proliferatory activity for C2C12 cells. Compound **3**,**4**,**8**,**11**,**12**,**13** showed anti-inflammatory activities. None of compounds showed proliferatory activities.

## 국문초록

단삼 (*Salvia miltiorrhiza* Bunge) 는 꿀풀과 (Lamiaceae) 배암차즈기속 (Salvia)에 속하는 여러해살이풀로 원산지는 중국이며 한국에도 자생하고 있다. 단삼의 뿌리는 전통적으로 다양한 뇌혈관 질환과 심혈관 질환에 사용되어 왔다. 다양한 연구에 의해 항염증, 항산화, 심혈관 보호 효과와 같은 생리활성을 가지는 것으로 보고되었다. 현재까지 70종이 넘는 물질이 단삼으로부터 분리되어 보고되었으며, Tanshinone IIA로 대표되는 친유성의 Diterpenoid와 Salvianolic acid B로 대표되는 친수성의 Pheolic acid 들이 그것이다. Tanshinone IIA의 경우, 항염증 활성과 항산화 활성이 보고되어 있으며, Salvianolic acid B의 경우 심혈관 보호 활성이 보고되었다. 단삼의 국내 사용량은 2010년 이전까지 전량 중국에서의 수입에 의존하였다. 국내에서 재배되기 시작한 것은 2010년부터이며, 2016년에 '다산'이라는 품종과 2018년에 '고산'이라는 품종이 개발되었다. 본 연구는 단삼에서 분리한 화합물로 Chemical library를 구성하고, RAW264.7 대식세포에서의 LPS 유도 염증 반응 억제 활성을 확인하여, 향후 단삼의 더 많은 품종 개발이나 개발 후 용도의 결정에 있어 도움이 되고자 한다. 서울의 덕현당 한약국에서 중국산 단삼과 서울대학교 약학대학 약초원에서 국내산 단삼을 구하여 각각 75% 메탄올과 10% 메탄올 3회, 90% 메탄올 3회로 추출하였다. 이를 물, n-헥산, 에틸아세테이트와 부탄올로 분획하였다. 중국산 단삼의 에틸아세테이트 분획과 국내산 단삼의 부탄올 분획으로부터 다양한 크로마토그래피 기법을 활용하여 15종의 화합물을 분리하였다. 15종의 화합물은 모두 1D NMR 및 HR-ESI MS 분석을 통해 기지화합물로 동정되었다. 화합물을 RAW264.7 대식세포에서 LPS로

유도된 염증 반응 억제 활성을 확인하였을 때, Diterpenoid 계열 화합물 3,4,8,11,12,13 번이 양성 대조군 Quercetin과 유사하거나 그보다 강한 활성을 보였다. 따라서 본 연구에서는 단삼으로부터 15종의 기지화합물을 분리하였으며 그 중 항염증 활성을 보이는 화합물을 파악하고, 향후 진행될 수 있을 단삼의 품종 개발 연구에서 항염증 활성과 연관된 품종 개발 시 주목해야 할 화합물에 대해 제시하였다.

**주요어**: 단삼, 꿀풀과, 다이터펜노이드. 페놀산, 항염증 활성, 품종 개발
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