Thesis for the Degree of Master of Science

Isolation, identification and quantitative analysis of the BACE1 active compounds from the flowers of *Cirsium maackii*

by

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(엉겅퀴 꽃으로부터 분리된 BACE1 억제 활성 성분의 분리, 동정 그리고 정량적 분석)

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by

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List of Abbreviations and Symbols

δ	: Chemical shift
^{1}H NMR	: ^{1}Proton nuclear magnetic resonance
^{13}C NMR	: ^{13}Carbon nuclear magnetic resonance
Aβ	: Amyloid-β
AChE	: Acetylcholinesterase
AD	: Alzheimer's disease
APP	: Amyloid precursor protein
BACE1	: β-Site amyloid precursor protein cleaving enzyme 1
BChE	: Butyrylcholinesterase
CAA	: Cerebral amyloid angiopathy
CH_2Cl_2	: Dichloromethane
C. maackii	: *Cirsium maackii*
DMSO	: Dimethyl sulfoxide
DMSO-*d6*	: Deuterated dimethyl sulfoxide
EtOAc	: Ethyl acetate
H_2O	: Water
HPLC	: High performance liquid chromatography
Hz	: Hertz
IC_{50}	: Half inhibitory concentration
J	: Coupling constant (Hz)

MeOH : Methanol

MeOH-*d4* : Deuterated methanol

n-BuOH : *n*-butanol

nm : Nanometer

Rel. int. : Relative intensity

RP : Reverse phase

RT : Retention time

SD : Standard deviation

TLC : Thin layer chromatography

UV : Ultraviolet

엉겅퀴 꽃으로부터 분리된 BACE1 억제 활성 성분의 분리, 동정 그리고 정량적 분석

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요 약

알츠하이머 질환은 만성 진행성 퇴행성 질환이며 주요 건강 문제 중 하나로 대두되고 있다. 여러 연구들이 식물의 꽃 부위가 신경 퇴행을 예방하기 위해 사용될 수 있을 것이라고 제안하였다. 이전의 연구에서 *Cirsium*의 꽃 추출물이 전인지적 효과를 가지고 있다고 보고되었다. 이러한 근거들에 의거하여, 우리는 엉겅퀴 꽃의 항알츠하이머 질환 효과를 측정하고 꽃의 항알츠하이머 질환 효과가 어떤 성분에 의한 것인지 찾기 위해서 구성 성분들에 대한 정성 및 정량 분석을 실시하고자 하였다. BACE1 억제 활성 측정 결과, 꽃의 메탄올 추출물은 농도 의존적으로 효소를 억제하였으며 76.47 ± 1.66 μg/ml의 반수저해농도 (IC_{50})를 가지는 것으로 나타났다. 다음으로, 메탄올 추출물의 극성에 따른 용매별 분획물들에 대한 BACE1 억제 활성을 측정하였다. 그 결과, 에틸아세테이트, 디클로로메탄 그리고 부탄올 분획물 순으로 높은 억제 활성을 보였으며 각각 22.98 ± 1.45, 8.65 ± 0.63 그리고 72.47 ± 3.04 μg/ml 의 IC_{50}를 보였다. 양성 대조군으로는 4.51 ± 3.63 μg/ml의 IC_{50}를 가지는 퀘르세틴을 사용하였다. 오픈 컬럼 크로마토그래피를 통해서 디클로로메탄 분획물로부터 β-amyrenone, lupeol acetate, lupeol, β-sitosterol 그리고 β-sitosterol glucoside를 분리하였고, 에틸아세테이트 분획물로부터 apigenin, luteolin, apigenin 7-*O*-β-D-glucuronide methyl ester, tracheloside 그리고 luteolin 5-*O*-β-D-glucoside를 분리하였다. 또한, apigenin 5-*O*-β-D-glucoside 그리고 apigenin 7-*O*-β-D-glucuronide를 부탄올 분획물로부터 분리하였고 luteolin 7-*O*-β-D-glucuronide를 물 분획물로부터 분리하였다. 특히, 디클로로메탄 분획물의 lupeol과 lupeol acetate은 다른 성분들과 비교해서 상당히 많이 분리되었다. 게다가, 고속 액체 크로마토그래피 (HPLC)를 이용하여 350nm의 파장에서의 꽃 성분의 정량 및 정성분석을 실시하였다. 그 결과, apigenin, luteolin 그리고 apigenin 7-*O*-β-D-glucuronide가 메탄올

추출물, 에틸아세테이트 분획물 그리고 부탄올 분획물의 주요 성분인 것으로 나타났다. 특히, 에틸아세테이트 분획물에 apigenin과 luteolin이 각각 162.27 mg/g 그리고 97.17 mg/g으로 상당히 많이 함유되어 있음을 확인하였고, 부탄올 분획물에는 apigenin 7-*O*-β-D-glucuronide가 106.38 mg/g으로 많이 함유되어 있는 것을 HPLC를 통해 확인하였다. 이전의 연구에서 lupeol과 luteolin이 BACE1 저해제로써 보고된 바 있으므로, 엉겅퀴 꽃의 항알츠하이머 질환 효과는 이러한 주요 화합물들의 존재에 의한 것으로 사료된다. 따라서, 이번 연구를 통에서 엉겅퀴 꽃의 알츠하이머 질환에 대한 역할이 주요 triterpenoid와 flavonoid에 의한 것을 확인하였다

I. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease where brain regions, particularly neocortex and hippocampus are affected by deposition of amyloid-β (Aβ) plaques and neurofibrillary tangles, accompanied by synaptic dysfunction and neurodegeneration (Francis et al., 1999). The term AD was coined by Dr. Alois Alzheimer. It is estimated that 5.5 million Americans had AD dementia in 2017. By 2050, one new case of AD dementia is expected to develop every 33 seconds, resulting in nearly 1 million new cases per year (Alzheimer's Association 2017). Along with intracellular and extracellular accumulation of Aβ peptides, cholinesterases are key enzymes formed through the amyloidogenic pathway in which amyloid precursor protein (APP) is sequentially cleaved. Increased proteolytic processing of APP leads to production and accumulation of neurotoxic forms of Aβ in the brain (Fedele et al., 2015; Pereira et al., 2005). Alongside, it adds up a large economic burden costing worldwide US$818 billion, and will become a trillion dollar disease by 2018 (Prince et al., 2016). Due to the result of its disabling nature, chronicity, and high prevalence in older segments of the population together with inability of available treatments to justify their costs, current treatments of AD have been criticized for providing insufficient benefit. AD is characterized by amyloid plaques that are extracellular deposits of Aβ in the brain parenchyma and cerebral blood vessels where this form is known as congophilic angiopathy or cerebral amyloid angiopathy (CAA). Neurofibilary tangles are made of helical filaments that are paired with hyperphosphorylated tau proteins, responsible for both neuronal and synaptic loss in brain. According to the amyloid cascade hypothesis, APP is generally cleaved by α-secretase and simultaneously processed by β- and γ-secretases. This

disparity between production and clearance of Aβ peptide results in aggregation of Aβ peptide into soluble oligomers and forms fibrils insoluble beta-sheet conformation and are eventually deposited in diffused senile plaques (Kumar and Singh 2015). β-Site amyloid precursor protein cleaving enzyme 1 (BACE1) is the β-secretase enzyme that leads to the production of the neurotoxic Aβ in the pathogenesis of AD. Emerging researches have thus been directed towards BACE1 inhibition due to their potential to lower cerebral Aβ concentrations and to treat and prevent Alzheimer's disease. Moreover, BACE1 initiates the production of the toxic Aβ that plays a crucial role in early part in AD pathogenesis. Due to its apparent rate limiting function, BACE1 appears to be a prime target to prevent and lowering the Aβ generation in AD (Yan and Vassar 2014). Similarly, the cholinergic hypothesis where inhibition of enzymes, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are widely accepted and has been the focus of many investigations targeting AD (Francis et al., 1999).

Natural products have provided a plethora of opportunities in medicine throughout human evolution. Our earliest ancestors, Neanderthals (Solecki 1975), and the earliest human civilizations, such as traditional Chinese medicine (TCM) and Ayurveda (Patrick 2013), have used the various plants to relieve pain and treat wounds. However, it was only around the 18th century that specific chemicals with therapeutic effects were identified in natural products to result in advancements in analytical and structural chemistry (Ji et al., 2009). The prime focus for treating and preventing diseases has led to the development of new molecules for synthesis, development, and testing of potential pharmacologic compounds (Amatori et al., 2012). However, lack of understanding regarding underlying mechanisms necessitates further research to develop novel therapies inspired by these products. Several plant-based and synthetic

compounds have been discovered or developed in the last decade. Recently, natural products derived lead compounds to treat AD are in increase, as they are free of any potential life threatening side effects. Different generations of BACE1 inhibitors like, E2609 and verubecestat, show effect like liver toxicity, low oral bioavailability, and low efficacy, thus limiting their use (Hung and Fu 2017). The use of natural products in the health care can be evidenced by a report that describes that 80% of the global population still rely on plant derived medicines to address their health care needs. Also, 50% of all drugs in clinical use are natural products, and 74% of the most important drugs consist of plant-derived active ingredients (Rasul et al., 2013).

Cirsium maackii (*C. maackii*), one of the milk thistle is a herbaceous perennial plant belonging the compositae family and is native to Korea (Shin et al., 2017). The thistle genera includes *Carduus*, *Cirsium*, and *Silybum* species. All thistle have 0.5-2.0 m height with lanceshaped, spiny-toothed leaves and white to purple flowers. The thistles are present in an abundant amount in Korea, Japan, and China. Among 250 species of thistles have been identified worldwide, 10 of these species have been found in Korea (Jung et al., 2009). *C. maackii* plant can be as tall as 100 cm with the appearance of its upright stem being covered with white feathers. Its leaves have oblong and sharp needles shape, and are about 15–30 cm long and 6–15 cm wide. The flowers are reddish-purple in color with 3–5 cm diameter. The flower blooms between June and August (Jung et al., 2015).

C. maackii as well as other *Cirsium* species (Cirsii Radix et Herba) roots or whole plant are used as a folk medicine in the treatment of hemorrhage, diuresis, inflammation of the liver and kidney, and a variety of abdominal and intestinal disorder. The *Cirsium* species have

also been identified to exert various kinds of bioactivities, including anti-microbial (Nazaruk and Jakoniuk 2005), anti-proliferative (Sahli et al., 2017), anti-diabetic (Yin et al., 2008), anti-oxidant (Nazaruk et al., 2012), anti-inflammatory (Shin et al., 2017), vasorelaxant (Kim et al., 2008), anti-cancer (Liu et al., 2007), and hepatoprotective (Ma et al., 2016) activities. In addition, numerous phytochemicals, including flavonoids and phenolic acids (Nazaruk and Jakoniuk 2005; Jung et al., 2015); lignans, neolignans, and sesquineolignans (Könye et al., 2018; Jung et al., 2015); phenylacrylic acid esters and polyacetylenes (Lai et al., 2014); and sterols and triterpenes (Chung et al., 2016; Luan et al., 2016) have been isolated from these species.

Different extracts and isolated compounds from flowers like *Gossypium herbaceum* (Zhao et al., 2013A), *Lonicera japonica* (Wang et al., 2014; Liu et al., 2018), *Humulus lupulus* (Sasaoka et al., 2014), *Nelumbo nucifera* (Prabsattroo et al., 2016) and *Rosa damascene* (Esfandiary et al., 2015) are reported to be active against amyloid-beta (Aβ) aggregation, a hallmark of Alzheimer's disease. In a study conducted by Walesiuk et al. (2010), *Cirsium rivulare* flowers extract showed pro-cognitive and leaves extract showed an anxiolytic effect on a rat model. Kim and Kim (2003) showed that among methanol extract of root, stem, leaves, and flowers of *Cirsium japonicum*, flowers and leaves had optimum peaks when analyzed through high-performance liquid chromatography (HPLC). Our previous study (Jung et al., 2015) focused on *C. maackii* leaves for the inhibition of glycated end products (AGEs). Considering the mounting interest in assessing the anti-AD capacity from the natural source, this time, we attempted to present the phytochemicals from *C. maackii* flowers. Since the quantitative information about the distribution of chemical components on the extract

provides an important basis for building new chemical models to treat ailments, HPLC quantitative analysis was also performed.

C. maackii is a common perennial plant that can be easily cultivated into lowland, grassland, forest margins, meadows, and low elevations in mountains and on a sandy, loamy, and heavy soil (Ohwi 1965). This ease of access of *Cirsium* species have enticed researchers to further explore its anti-AD potentials. Therefore, our current findings tried to highlight the role of *C. maackii* flowers in the alleviation of AD through the evaluation of BACE1 inhibition and to clarify the activity through the quantitative analysis of the compounds present in different extract and fractions by HPLC.

II. Materials and Methods

1. Plant materials

The flower of *C. maackii* was collected from the southeast area of Gyeongsangnam-do Province, Korea in 2016 and authenticated by Professor Jae Sue Choi. A voucher specimen (No.20160901) was deposited in the laboratory of Professor J.S. Choi.

Figure 1. The flower of *C. maackii*

2. General experimental procedures

The NMR study, ^{1}H- and ^{13}C-NMR spectra were measured by a JEOL JNM ECP-400 spectrometer (Tokyo, Japan) at 400 MHz for ^{1}H-NMR and 100 MHz for ^{13}C-NMR in deuterated dimethylsulfoxide (DMSO-d_6) and methanol (MeOH-d_4) depending upon compound solubility.

Chemical shifts were referenced to the residual peaks of respective solvents (2.50 ppm for ^{1}H-NMR and 39.5 ppm for ^{13}C-NMR). HMQC and HMBC spectra were recorded using pulsed field gradients. Thin layer chromatography (TLC) was conducted for normal phase was conducted on precoated Merck Kieselgel 60 F_{254} plates (20 x 20 cm, 0.25 mm) and and reverse phase on RP-18 F_{254S} plates (5 x 10 cm, Merck) using 50% H_2S0_4 as the spray reagent. All solvents for column chromatography were of reagent grade and were purchased from commercial sources.

3. Chemicals and reagents

BACE1 kit was obtained from Biomol International LP (Plymouth Meeting, PA, USA). Ultra-pure grade water was used through and all graded chemicals/solvents used all over the experiment were purchased from the commercial sources. All solvents used in HPLC were of HPLC grade. Unless otherwise stated, all the solvents and chemicals were acquired from Sigma-Aldrich (St. Louis, MO, USA).

4. Experimental methods

4-1. Extraction, fractionation, and isolation

The dried flowers of *C. maackii* (2.8 kg) was extracted 4 times with MeOH at 70 and the final yield was 168.28 g extract. The obtained extract was then suspended in H_2O and successively partitioned with dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), and *n*-butanol (*n*-BuOH) to yield CH_2Cl_2 (34.78 g), EtOAc (13.33 g), and *n*-BuOH (16.63 g) fractions, as well as H_2O residue (102.00 g).

Isolation of compounds from CH_2Cl_2 fraction of *C. maackii*

The CH_2Cl_2 fraction was chromatographed over silica gel column and eluted with *n*-hexane:EtOAc 25:1 to EtOAc to get 5 subfractions (DCM-1 to DCM-5). Repeated chromatography of DCM-1 (7.55 g) using *n*-hexane:EtOAc 50:1 as isocratic eluent gave two compounds, namely β-amyrenone (49.70 mg) and lupeol acetate (1.43 g). Lupeol (1.22 g) was obtained from DCM-2 by the application of silica gel column chromatography by using *n*-hexane:EtOAc 30:1 as the mobile phase. DCM-3 (3.41 g) was subjected to silica gel column using *n*-hexane:EtOAc 25:1 as eluent which afforded 3 subfractions (DCM-3-1 to DCM-3-3). Among these subfractions, continual column chromatography of DCM-3-2 on silica gel column using combination of solvents like *n*-hexane:EtOAc (7:1) and *n*-hexane:acetone:methanol (9:1:1) yielded β-sitosterol (1.01 g). Further, DCM-5 (1.73 g) was eluted with *n*-hexane:EtOAc (3:1 to 1:1) that gave 4 subfractions (DCM-5-1 to DCM-5-4). DCM-5-2 (201 mg) contained a mixture of chlorophyll derivatives along with yellowish compound. Chlorophyll derivatives were dissolved in methanol and the mixture was filtered to separate the compound, which was later identified as apigenin (12.00 mg). Another compound, β-sitosterol-D-glucoside (13.00 mg) was isolated from DCM-5-4 (200.00 mg) through repetitive column chromatography using silica gel and Sephadex LH-20 columns with *n*-hexane:EtOAc 1:1 and 100% MeOH as respective eluents.

Isolation of compounds from EtOAc fraction of *C. maackii*

The EtOAc fraction was subjected to silica gel column chromatography eluted with CH_2Cl_2:MeOH:H_2O (7:1:0.1 to 3:1:0.1) to give 7 different subfractions (Et-1 to Et-7). Et-2 gave a yellow precipitate when MeOH was added. This peculiar compound was washed with *n*-hexane, CH_2Cl_2, and MeOH that gave a clean compound which was later found to be apigenin (100.00 mg). Repetitive chromatography of Et-3 over silica gel column with *n*-hexane:EtOAc (1:1) and CMW (10:2:0.2) as a mobile phase yielded luteolin (19.00 mg). Tracheloside (53.70) was obtained from Et-4 by continuously using silica gel column with solvent CMW (7:1:0.1) and RP-C18 column with 70% MeOH. Similarly, apigenin 7-*O*-β-D-glucuronide methyl ester (21.30 mg) was obtained from Et-5 by the use of CMW (7:1:0.1) and MeOH over silica gel and sephadex LH-20 column, respectively.

Isolation of compounds from *n*-BuOH fraction of *C. maackii*

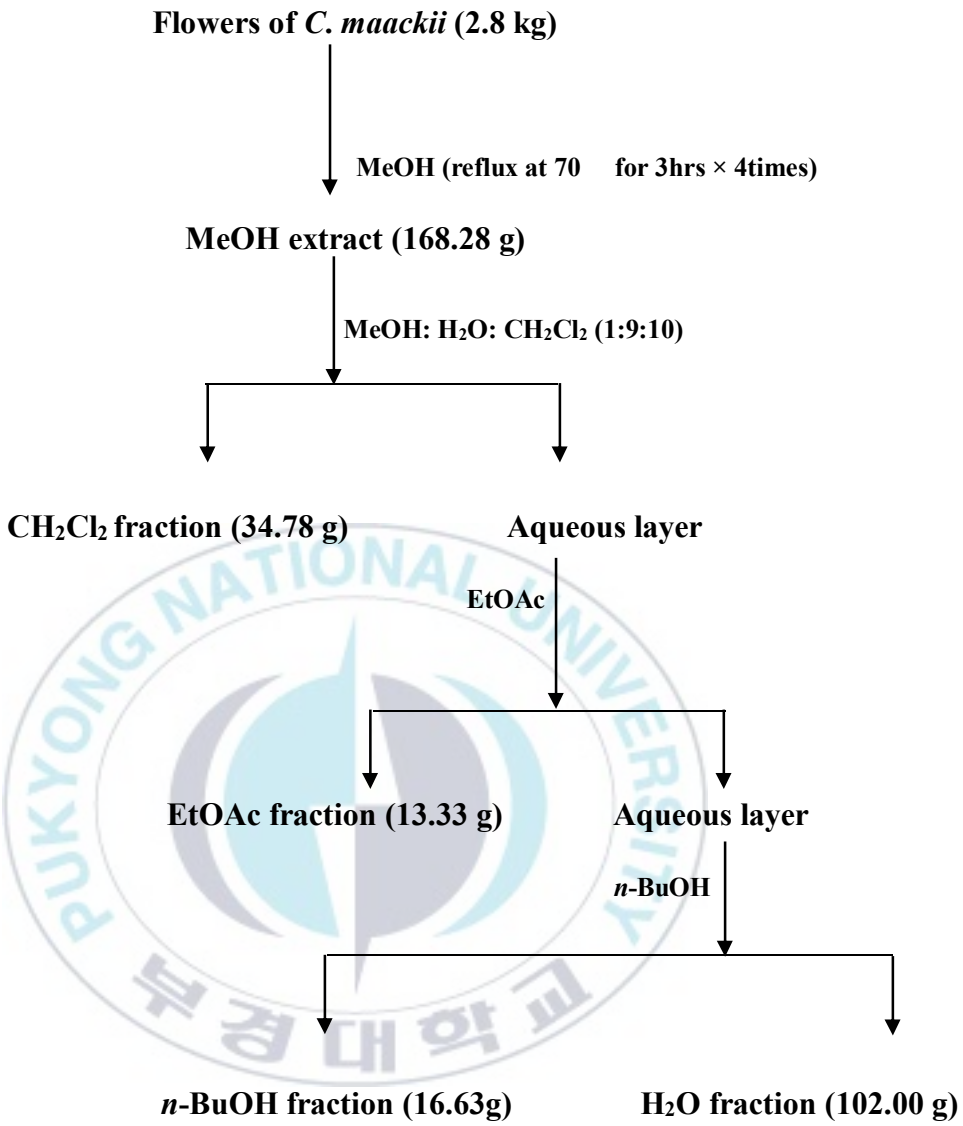
The *n*-BuOH fraction was proceeded to chromatography on Sephadex LH-20 column using 100% MeOH as a mobile phase solvent to yield 8 different subfractions (Bu-1 to Bu-8). The elution of Bu-4 (4.96 g) with EtOAc:MeOH:H_2O (24:4:3) over silica gel column gave 6 different subfractions (Bu-1-1 to Bu-1-6). Repetitive chromatography of the Bu-1-6 on silica gel and RP-C_{18} column gave one of the compounds, namely apigenin 7-*O*-β-D-glucuronide (91.00 mg). Bu-7 (2.33 g) was chromatographed over silica gel column to yield 4 different subfractions (Bu-7-1 to Bu-7-4). Bu-7-3 gave an insoluble precipitate on addition of MeOH. This precipitate was separated through filtration and later identified as luteolin 5-*O*-β-D-glucopyranoside (11.00 mg). Repeatative column chromatography of Bu-7-4 over silica gel,

RP-C_{18} and Sephadex LH-20 yielded apigenin 5-*O*-β-D-glucopyranoside (11.00 mg).

Isolation of compound from H_2O fraction of *C. maackii*

Firstly, about quarter of the H_2O fraction (40 g) was subjected to Diaion HP-20 column and eluted with 100% H_2O and 100% MeOH. The 100% MeOH subfraction (7 g) was eluted with EtOAc:MeOH:H_2O (24:4:3) to give 3 different subfraction (H2-1 to H2-3). H2-2 (1.19 g) was again chromatographed over EtOAc:MeOH:H_2O (24:4:3) that gave rise to 3 subfractions (H2-2-1 to H2-2-3). H2-2-2 (300 mg) was repetitively chromatographed over Sephadex LH-20 and MCI CHP-20P column using 30% MeOH as a mobile phase that gave luteolin 7- *O*-β-D-glucuronide (22.00 mg).

All the isolated compounds from CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O fractions were identified by spectroscopy, including 1D (^{1}H- and ^{13}C) and 2D (HMQC, HMBC, and DEPT) NMR along with TLC analysis and comparison with published spectral data with standard compounds.



Scheme1: Extraction and fractionation scheme of *C. maackii* flowers.

4-2. HPLC quantitative analysis

HPLC was performed on the Reverse phase JASCO HPLC system (Tokyo, Japan), comprising of PU-1580 Intelligent HPLC pump, LG-1580–04 quaternary gradient unit, UV-1575 intelligent UV/Vis detector, PG-1580–54 4-line degasser, and CO-1560 intelligent column thermostat. Using the BORWIN chromatographic system (Le Fontanil, France), HPLC data was analyzed. The chromatography was accomplished on a Phenomenex C18 reverse phase column (Phenomenex, 4.6 × 250 mm, 5 μm) at 30 °C and monitored at 350 nm. An isocratic solvent system consisting 0.2% phosphoric acid and MeOH in ratio 55:45 was used for 80 min at the flow rate of 0.5 ml/min. For preparation of stock solutions, extracts and seven flavonoids were dissolved in 100% MeOH at concentrations of 2 mg/ml and diluted to 1, 0.5, and 0.25 μg/ml, respectively. After filtration through a centrifugal filter device (0.45 μm, Millipore Co., Bedford, MA, USA), 10 μl of each sample was injected. The calibration curves of all 7 compounds were drawn with 3 standards at concentrations ranging from 62.5 to 500 μg/ml. The regression equations were calculated in the form of $y = ax + b$, where y and x correspond to the peak area and concentration, respectively. The relative quantity of 7 flavonoids in the MeOH extract, EtOAc and *n*-BuOH fraction (mg/g of the extract) was calculated from each equation.

4-3. Assay for BACE1 inhibition

BACE1 inhibitory assay was carried out according to the supplied manual with selected modifications. Briefly, mixtures of 10 μL of assay buffer (50 mM sodium acetate, pH 4.5), 10 μL of BACE1 (1.0 U/ml), and 10 μL of the substrate (750 nM Rh-EVNLDAEFK-Quencher in

50 mM, ammonium bicarbonate), and 10 μL of samples (final concentration, 250 μg/ml for the extracts/fractions) were incubated at 25 for 60 min in the dark. The proteolysis of two fluorophores (Rh-EVNLDAEFK-Quencher) by BACE1 was observed by exciting the mixtures at 545 nm and collecting the emitted light at 585 nm. Fluorescence was recorded with a microplate spectrofluorometer (Molecular Devices). The percent inhibition (%) was obtained by the following equation: % Inhibition = [1-(S - S_0)/(C - C_0)] X 100, where C was the fluorescence of the control (enzyme, buffer, and substrate) after 60 min of incubation and C_0 was the initial fluorescence of the control, S was the fluorescence of the tested samples (enzyme, sample solution, and substrate) after 60 min of incubation, and S_0 was the initial fluorescence of the tested samples. The BACE1 inhibitory activity of compounds was expressed in terms of the IC_{50} value (μg/ml required to inhibit proteolysis of the substrate, BACE1, by 50 %). Quercetin was used as a positive control.

4-4. Statistics

Data are presented as the mean ± SD of at least four independent experiments. Statistical comparisons between groups were performed using one-way ANOVA followed by Student's *t* test.

III. Results

1. Isolation

1-1. Isolation of compounds

By continual column chromatography of the EtOAc and *n*-BuOH fractions from the flowers, luteolin 5-*O*-β-D-glucopyranoside, luteolin 7-*O*-β-D-glucuronide, apigenin 5-*O*-β-D-glucopyranoside, apigenin 7-*O*-β-D-glucuronide, apigenin 7-*O*-β-D-glucuronide methyl ester, luteolin, and apigenin were obtained while CH2Cl2 fraction yielded lupeol, lupeol acetate, β-sitosterol, and β-sitosterol glucoside. Following NMR and TLC analysis with the standard and comparison with the reported data, all isolated compounds were confirmed. The structure of compounds is shown in figure 2 and the respective spectral data are as follows:

β-Amyrenone: EIMS for $C_{30}H_{48}O$ m/z (rel. int.): m/z at 409 [M-CH_3] (4.59%), 218.25 (100%), 203.15 (34%),189.20 (24.21%),147.15 (11.88%). (Quintao et al., 2014).

Lupeol acetate: EIMS for $C_{32}H_{52}O_2$ m/z (rel. int.): m/z at 468 [M^+] (20.99%), 408.35 [M-CH_3COO]$^+$ (8.36%), 189.15 (100%). (Tahany et al., 2010; Fernández-Martínez et al., 2018)

Apigenin 5-*O*-β-D-glucoside: ^{1}H NMR (DMSO-d_6, 600MHz) δ_H 7.88 (1H, d, J=9, 2.2Hz, H-2′/6′), 6.91 (1H, d, *J*=8.9Hz, H-5′), 6.79 (1H, d, *J*=2.0Hz, H-8), 6.65 (1H, s, H-3), 6.73 (1H, d, *J*=2.5Hz, H-6), 4.70 (1H, d, *J*=7.7Hz, H-1″), ^{13}C NMR (DMSO-d_6, 150MHz) δ_C 176.99 (C-4), 162.69 (C-2), 161.18 (C-7), 158.64 (C-9), 158.32 (C-5), 160.76 (C-4′), 115.87 (C-3′/5′), 128.08 (C-2′/6′), 121.16 (C-1′), 108.17 (C-10), 104.56 (C-3), 104.40 (C-6), 98.30 (C-8), 75.57 (C-3″),

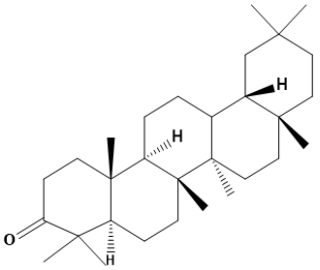
77.57 (C-5″), 73.62 (C-2″), 69.66 (C-4″), 60.82 (C-6″). (Veit et al., 1990).

Apigenin 7-*O*-β-D-glucuronide: ^{1}H NMR (DMSO-d_6, 400MHz) δ_H 7.85 (2H, d, *J*=8.9 Hz, H-2′/6′), 6.88 (2H, d, *J*=8.9Hz, H-3′, 5′), 6.77 (1H, d, *J*=4.2Hz, H-8), 6.40 (1H, s, H-3), 6.40 (1H, d, *J*=2.0Hz, H-6), 5.05 (1H, d, *J*=6.6Hz, H-1″).^{13}C NMR (DMSO-d_6, 100MHz) δ_C 181.92 (C-4), 172.45 (C-6″), 164.41 (C-2), 163.03 (C-7), 156.94 (C-9), 162.51 (C-5), 161.0 (C-4′), 116.16 (C-3′/5′), 128.44 (C-2′/6′), 120.16 (C-1′), 105.26 (C-10), 102.64 (C-3), 99.61 (C-6), 94.65 (C-8), 73.75 (C-3″), 76.48 (C-5″), 72.95 (C-2″), 71.98 (C-4″). (Ma et al., 2018; Ozgen et al., 2010)

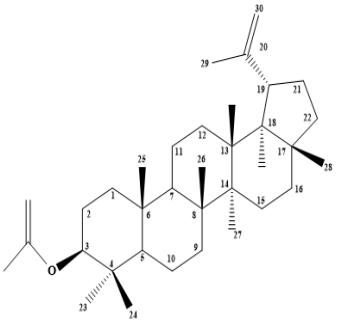
Luteolin 7-*O*-β-D-glucuronide: ^{1}H NMR (DMSO-d_6, 600MHz) δ_H 7.42 (1H, s, H-2'), 7.38 (1H, d, *J*=8.7 Hz, H-6'), 6.86 (1H, d, *J*=7.86 Hz, H-5'), 6.76 (1H, d, *J*=1.4 Hz, H-8), 6.69 (1H, s, H-3), 6.40 (1H, d, *J*=2.1 Hz, H-6), 5.09 (1H, d, *J*=7.6 Hz, H-1"). ^{13}C NMR (DMSO-d_6, 150MHz) δ_C 181.80 (C-4), 172.70 (C-6"), 162.95 (C-2), 164.46 (C-7), 160.98 (C-5), 156.89 (C-9), 150.34 (C-4'), 145.96 (C-3'), 118.96 (C-1'), 120.90 (C-6'), 116.04 (C-5'), 113.11 (C-2'), 105.23 (C-10), 102.83 (C-3), 99.54 (C-1"), 99.54 (C-6), 94.51 (C-8), 76.39 (C-3"), 77.48 (C-5"), 72.90 (C-2"), 73.80 (C-4"). (Ma et al., 2018; Ozgen et al., 2010)

Apigenin 7-*O*-β-D-glucuronide methyl ester: ^{1}H-NMR (DMSO-d_6, 600 MHz)) δ_H 7.95 (2H,d, J=8.9 Hz, H-2', 6'), 6.94 (2H, d, J=8.8 Hz, H-3', 5'), 6.86 (1H, s, H-3), 6.85 (1H, d, J=2 Hz, H-8), 6.47 (1H, d, J=2.7 Hz, H-6), 5.32 (2H, d, J=7.6 Hz, H-1", 4.21 (1H, d, J=9.6 Hz, H-5", 3.67 (3H, s, OCH_3). ^{13}C-NMR (DMSO-d_6, 150 MHz) δ_c 181.95 (C-4), 169.16 (C-6"), 164.30 (C-2), 162.39 (C-7), 161.46 (C-4', 161.16 (C-5), 156.93 (C-9), 128.59 (C-2', 6'), 120.90 (C-1'), 116.00 (C-3', 5'), 105.96 (C-10), 103.08 (C-3), 99.28 (C-6), 94.60 (C-8), 75.38 (C-5"), 75.13 (C- 2"), 72.69 (C-3"), 71.28 (C-4"), 51.95 (OCH_3). (Ma et al., 2018; Mostafa et al., 2017)

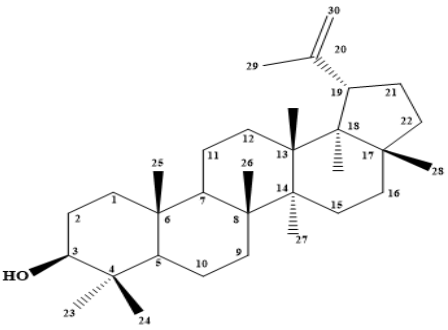
Tracheloside: ^{1}H-NMR (MeOH-*d*$_4$, 600 MHz)) δ_H 7.06 (1H,d, J=8.28 Hz, H-5'), 6.85 (1H, d, J=7.56 Hz, H-5), 6.70 (1H, d, J=2.1 Hz, H-2), 6.68 (3H, d, H-6), 6.67 (2H, d, J=2.1 Hz, H-2', 6'), 3.98 (2H, d, J=8.94 Hz, H-9), 3.14 (1H, d, J= 13.74 Hz, H-7'), 2.88 (1H, d, J= 13.74 Hz, H-7'), 2.79 (1H, dd, J=13.74, 5.52 Hz, H-7), 2.51 (1H, dd, J=13.8, 9.63 Hz, H-7), 2.43 (1H, m). ^{13}C-NMR (MeOH-*d*$_4$, 150 MHz) δ_c 180.37 (C-9'), 150.61 (C-4'), 150.58 (C-4), 149.18 (C-3), 147.17 (C-3'), 133.34 (C-1), 131.71 (C-1'), 124.13 (C-6'), 122.23 (C-6), 117.82 (C-5'), 115.94 (C-2'), 113.96 (C-2), 113.25 (C-5), 102.85 (C-1''), 77.27 (C-8'), 77.84 (C- 3''), 74.89 (C-2''), 71.78 (C-9), 71.30 (C-4''), 44.60 (C-8), 41.83 (C-7'), 32.18 (C-7). (Johansen et al., 2011)



β-Amyrenone



Lupeol acetate



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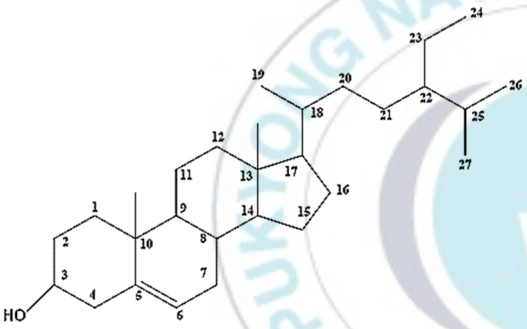
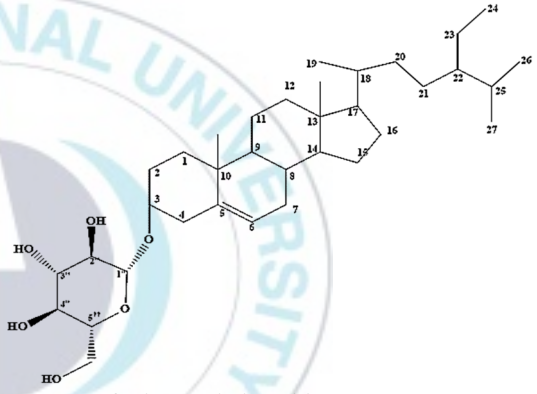
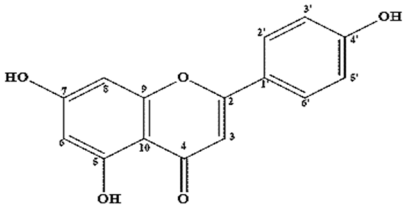


β-Sitosterol



β-Sitosterol glucoside



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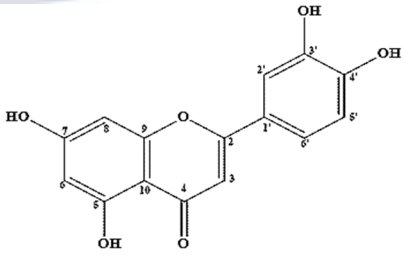
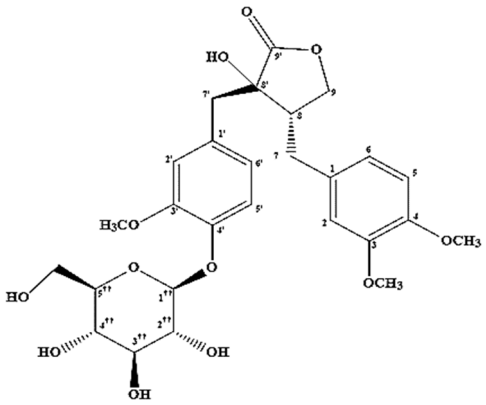
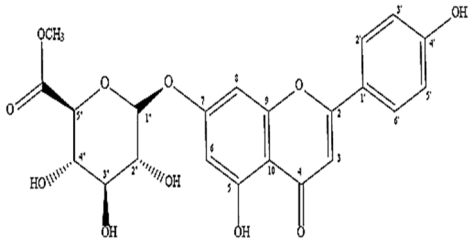


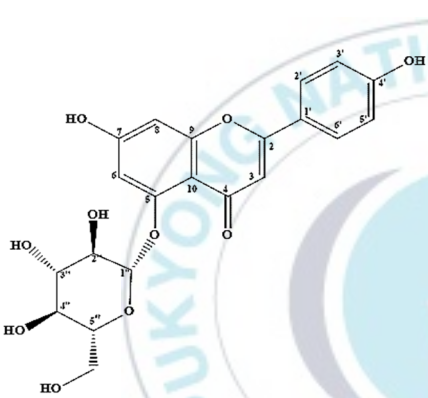
Luteolin



Tracheloside



Apigenin 7-*O*-β-D-glucuronide methyl



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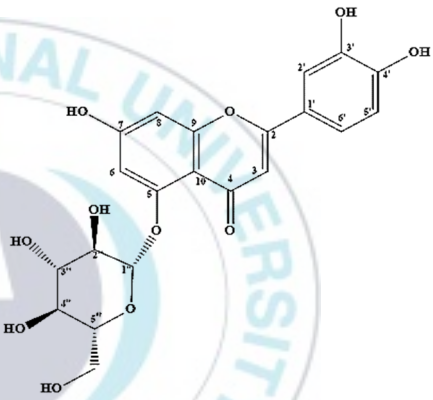
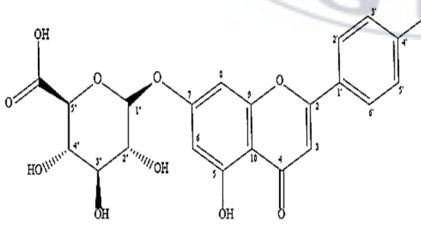
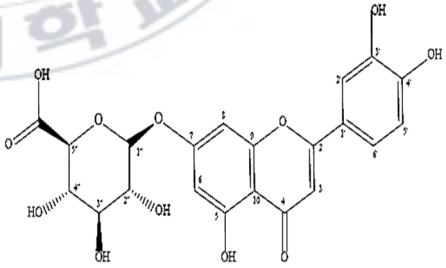


Luteolin 5-*O*-β-D-glucoside



Apigenin 7-*O*-β-D-glucuronide



Luteolin 7-*O*-β-D-glucuronide

Figure 2. Structures of the compounds isolated from *C. maackii* flower

2. HPLC analysis

2.1 Quantitative HPLC analysis in different fraction of *C. maackii* flowers

In order to determine the part-specific preponderance of all the isolated flavonoids, HPLC quantitative analysis of MeOH extract, EtOAc, and *n*-BuOH fraction of *C. maackii* flowers were performed. The retention times (RT) obtained for luteolin 5-*O*-β-D-glucoside, luteolin 7-*O*-β-D-glucuronide, apigenin 5-*O*-β-D-glucoside, apigenin 7-*O*-β-D-glucuronide, apigenin 7-*O*-β-D-glucuronide methyl ester, luteolin, and apigenin were approximately 9.73, 12.40, 13.32, 17.44, 33.29, 41.52, and 66.23 min, respectively. The regression equations and correlation coefficients (r^2) of 1, 2, 3, 4, 5, 6, and 7 are as follows: y = 2227168.42x + 504059.63, r^2 = 0.996; for luteolin-5-*O*-β-D-glucoside, y = 632193.91x + 549692.85, r^2 = 0.995; for luteolin7-*O*-β-D-glucuronide, y = 1404260.47x + 687709.38, r^2 = 0.998; for apigenin 5-*O*-β-D-glucoside, y = 4546174.33x + 405951.05, r^2 = 0.998; for apigenin 7-*O*-β-D-glucuronide, y = 2304160.44x - 303419.83, r^2 = 0.998; for apigenin 7-*O*-β-D-glucuronide methyl ester, y = 9784471.21x - 5398920.96, r^2 = 0.999; for luteolin, and y = 9953690.54x - 5317178.62, r^2 = 0.999; for apigenin. The MeOH extract had highest content of apigenin and luteolin followed by apigenin 7-*O*-β-D-glucuronide. By the help of calibration curve, the amount of individual compounds in the extract and fractions were calculated. The relative amount of apigenin 7-*O*-β-D-glucuronide, apigenin 7-*O*-β-D-glucuronide methyl ester, luteolin, and apigenin in the MeOH extracts from *C. maackii* flowers were 16.65 mg/g, 6.66 mg/g, 31.79 mg/g, and 33.11 mg/g, respectively. The yield of the EtOAc fraction for luteolin 5-

O-β-D-glucoside, apigenin 5-*O*-β-D-glucoside, apigenin 7-*O*-β-D-glucuronide, apigenin 7-*O*-β-D-glucuronide methyl ester, luteolin, and apigenin were 16.09 mg/g, 8.32 mg/g, 73.43 mg/g, 26.50 mg/g, 97.17 mg/g, and 162.27 mg/g indicating that the indicating that EtOAc fraction has the highest yield of apigenin. The contents of luteolin 7-*O*-β-D-glucuronide, apigenin 5-*O*-β-D-glucoside, apigenin 7-*O*-β-D-glucuronide, apigenin 7-*O*-β-D-glucuronide methyl ester, luteolin, and apigenin in the *n*-BuOH fraction of flower were 61.55, 22.80, 106.38, 13.35, 44.13, and 31.92 mg/g respectively, showing that the presence of apigenin 7-*O*-β-D-glucuronide is abundant in *n*-BuOH fraction.

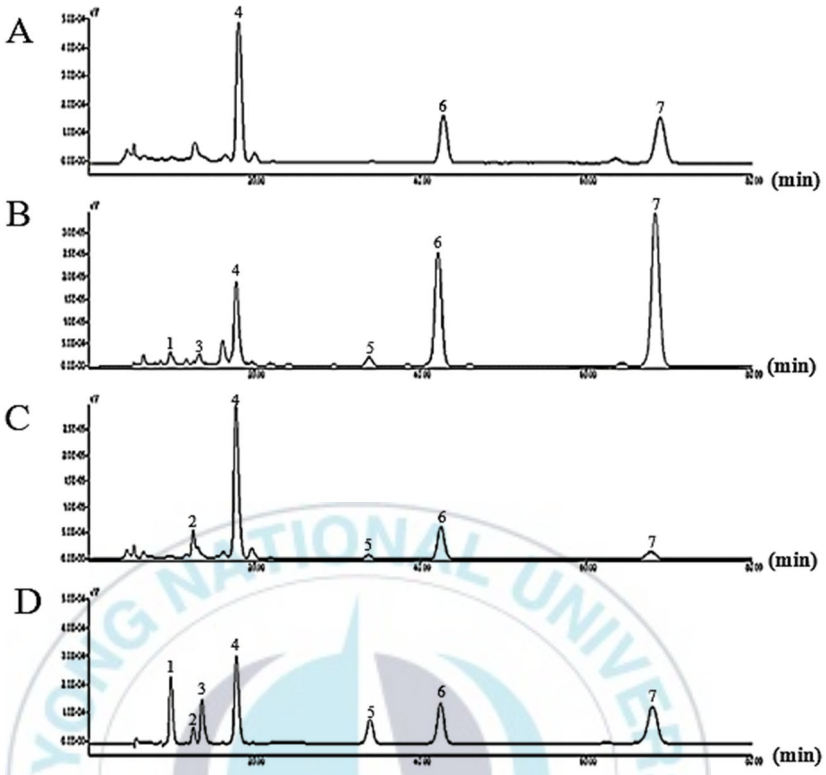


Figure 3. HPLC chromatograms of MeOH extract and different fractions of *C. maackii* flower. (A) MeOH extract, (B) ethyl acetate fraction, (C) *n*-BuOH fraction along with (D) standard compounds: peak **1** (RT: 9.84 min, luteolin 5-*O*-β-D-glucoside), peak **2** (RT: 12.57 min, luteolin 7-*O*-β-D-glucuronide); peak **3** (RT: 13.60 min, apigenin 5-*O*-β-D-glucoside) ; peak **4** (RT: 17.74 min, apigenin 7-*O*-β-D-glucuronide) ; peak **5** (RT: 33.87 min, apigenin 7-*O*-β-D-glucuronide methyl ester) ; peak **6** (RT: 42.38 min, luteolin) ; peak **7** (RT: 67.93 min, apigenin).

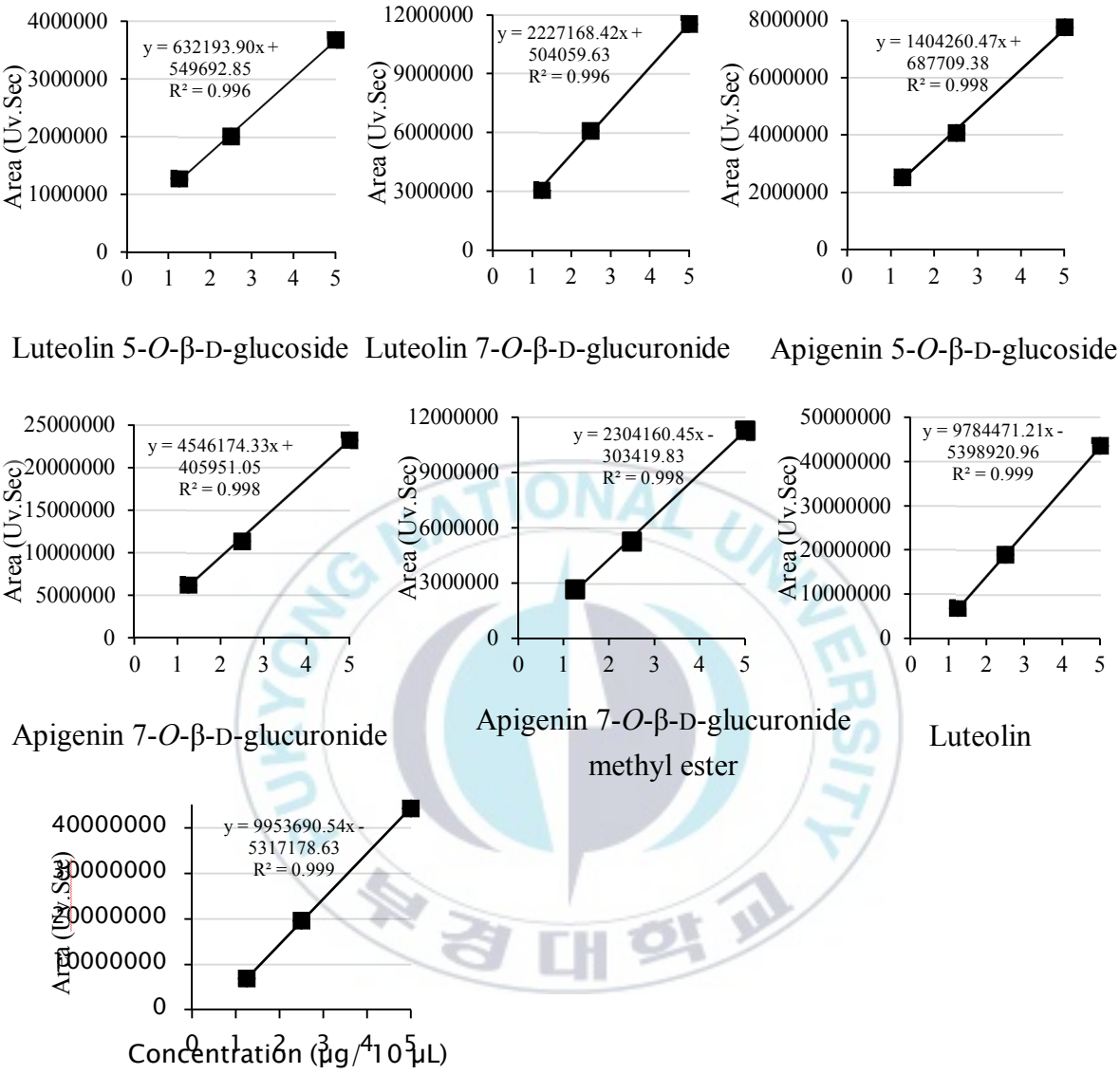


Figure 4. Calibration curve of luteolin 5-*O*-β-D-glucoside, luteolin 7-*O*-β-D-glucuronide, apigenin 5-*O*-β-D-glucoside, apigenin 7-*O*-β-D-glucuronide, apigenin 7-*O*-β-D-glucuronide methyl ester, luteolin, and apigenin.

Table 1. Relative amount of isolated compounds in extract and fractions of *C. maackii* flowers

Extract/ Fraction	Amount of compounds in the extract/fraction (mg/g)						
	Luteolin 5-*O*-β-D-glucoside	**Luteolin 7-*O*-β-D-glucuronide**	**Apigenin 5-*O*-β-D-glucoside**	**Apigenin 7-*O*-β-D-glucuronide**	**Apigenin 7-*O*-β-D-glucuronide methyl ester**	**Luteolin**	**Apigenin**
MeOH	-	-	-	16.65	6.66	31.79	33.11
EtOAc	16.09	-	8.32	73.43	26.50	97.17	162.27
n-BuOH	-	61.55	22.80	106.38	13.35	44.53	31.95

3. Anti-Alzheimer's disease activity

3-1. BACE1 inhibitory activities of different fractions extracts of *C. maackii* flowers

Initially, the anti-AD potential of MeOH extract of *C. maackii* showed dose dependent inhibition of BACE1 by 16.50, 43.45, and 59.48% at 20, 50, and 100 μg/ml, respectively. Since MeOH extract showed modest inhibition, it was further portioned into different fraction and the BACE1 inhibitory potential of different fraction were determined. The results obtained from different assays are summarized in Table 3 and Figure 5. Dose dependent inhibition by CH_2Cl_2 (86.58% at 50 μg/ml), EtOAc (65.45% at 25 μg/ml), and *n*-BuOH (59.63% at 100 μg/ml) fraction against BACE1 by with respective IC_{50} values of 22.98 ± 1.45, 8.65 ± 0.63, and 72.47 ± 3.04 μg/ml was observed compared to quercetin (IC_{50} = 4.51 ± 3.63 μg/ml). The H_2O fraction did not show BACE1 inhibition in the tested concentration (4-100 μg/ml) denoting the absence of BACE1 inhibitory potential. The inhibition order is as EtOAc > CH_2Cl_2 > *n*-BuOH. From this result it is evident EtOAc and CH_2Cl_2 fraction have a remarkable inhibitory potential against BACE1.

Table 2. BACE1 inhibitory activities of MeOH extract and different fractions from *C. maackii* flowers.

Extract/ Fraction	**IC_{50}(μg/ml, Mean ± SEM)** [a]
MeOH	76.47 ± 1.66
CH_2Cl_2	22.98 ± 1.45
EtOAc	8.65 ± 0.63
n-BuOH	72.47 ± 3.04
H_2O	>100
Quercetin[b]	4.51 ± 3.63

[a]The 50% inhibition concentrations (IC_{50}, μg/ml) are expressed as the mean ± SEM of triplicates

[b]Positive control

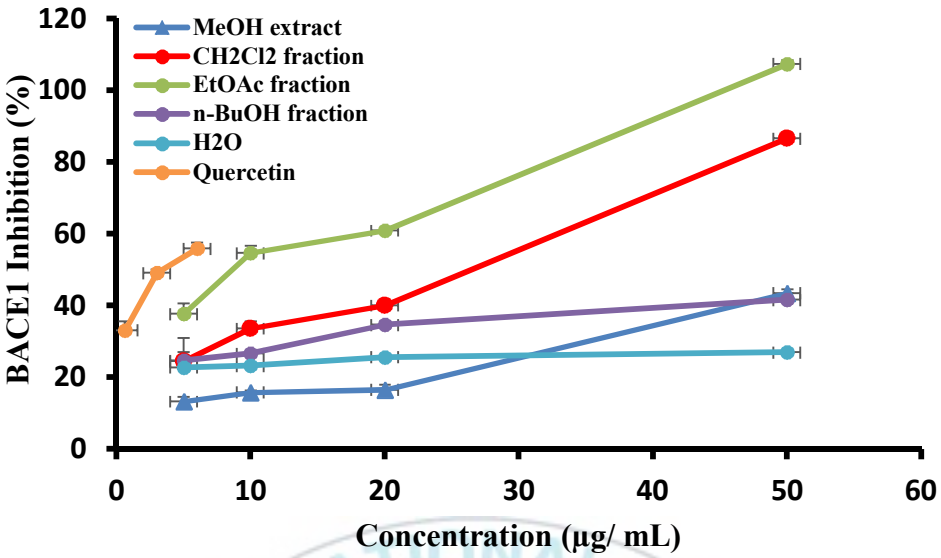


Figure 5. BACE1 inhibitory activities of MeOH extract and different fractions from *C. maackii* flowers. Error bars indicate standard error of the mean (SEM).

IV. Discussion

BACE1 is an aspartyl protease regulates the cleavage of APP as the first rate limiting step of Aβ formation. The design and development of BACE1 inhibitor provides a promising therapeutic target for the elucidation of AD pathology. Several BACE1 inhibitors have therefore been identified and are now being investigated in the clinical trials (De Strooper et al., 2010). The flower extract of one of the milk thistle *Cirsium rivulare* has already been reported to reduce mental confusion and delirium mentioned as pro-cognitive effect on a rat model (Walesiuk et al., 2010). Evidences suggest a close relation between delirium and dementia (Fick et al., 2009). Therefore, prevention of dementia might be useful in reduction or prevention of the occurrence of delirium. With this in mind, in this study we aimed to target the anti-AD potential of *C. maackii* flower.

In our previous study (Jung et al., 2009), the MeOH extract of *C. maackii* leaves along with the isolated major compounds, luteolin and luteolin 5-*O*-β-D-glucoside were shown to posess rat lens aldose reductase (RLAR) activity. In the same study, the MeOH extract of flower showed good activity against RLAR with an IC_{50} of 7.98 ± 0.48 μg/ml and the inhibitory activity against DPPH, $ONOO^-$ and total ROS for the same flower extract was 51.90, 11.27 ± 0.24, 33.42 ± 0.95 μg/ml. Also, in the DPPH radical scavenging activity, *n*-BuOH (12.80 μg/ml) fraction followed by EtOAc (17.10μg/ml) and H_2O fraction (78.40 μg/ml) showed the highest inhibition. In case of inhibition against $ONOO^-$ and total ROS, EtOAc fraction showed high activity of 1.37 ± 0.10 and 1.79 ± 0.14 μg/ml, respectively. Also, it was important to note that CH_2Cl_2 fraction was only active against $ONOO^-$ (13.94 ± 0.19 μg/ml). In one of our reports,

luteolin 5-*O*-β-D-glucoside was also reported to have anti-inflammatory activity (Jung et al., 2012). Moreover, compounds isolated from the EtOAc fraction of *C. maackkii* leaves showed inhibition against advanced glycated endproducts (AGE) formation, with the prominent activity of luteolin, quercetin, cernuoside, luteolin 5-*O*-β-D-glucoside, luteolin 7-*O*-β-D-glucoside, luteolin 4'-*O*-β-D-glucoside, quercetin 3-*O*-β-D-glucoside, and chlorogenic acid (Jung et al., 2015). Thus from our previous reports it is evident that the flower of *C. maackii* has an anti-oxidant potential.

In this study, the EtOAc fraction showed the highest BACE1 inhibitory activity. From the HPLC analysis, it was evident that the presence of two flavonoids, apigenin and luteolin is in the highest amount in the EtOAc fraction. These two compounds might have the highest contribution in the EtOAc fraction to show anti-AD property. As evidenced by one of our works (Choi et al., 2014), luteolin showed an incredible BACE1 inhibitory potential with an IC_{50} of 1.95 ± 0.02 μM, which is higher even than the positive control, quercetin (3.17 ± 0.12 μM). Similarly, in cerebral cortex in a mouse model, luteolin has been reported to exert neurovascular protection in $A\beta_{25-35}$-induced amnesia, improve the spatial learning and memory capabilities, modulate the microvascular function, increase regional cerebral blood flow values, combat reactive oxygen species, improve cholinergic neuronal system, and the increase of the brain-derived neurotrophic factor level and its receptor tyrosine kinase B expression (Liu et al., 2009). Wang et al. (2016) also highlighted the potential role of luteolin on learning defects and hippocampal structured in AD. Moreover, apigenin was shown to posess scavenging effects against superoxide anion, improve anti-oxidative enzyme activity of superoxide dismutase and glutathione peroxidase, and restore the neurotrophic ERK/CREB/BDNF pathway in the

cerebral cortex, suggesting its ability to ameliorate AD-associated learning and memory impairment by relieving Aβ burden (Zhao et al., 2013B). Since the presence of luteolin and apigenin was in the highest amount in EtOAc fraction, it is possible that apigenin synergized the BACE1 inhibitory activity in the luteolin in EtOAc fraction of *C. maackii* flower.

Another fraction that showed a notable degree of inhibition against BACE1 was CH_2Cl_2 fraction. Column chromatography of *C. maackii* flower revealed that this fraction was rich in lupeol and lupeol acetate. Upon analysis of lupeol against BACE1 in our previous report (Koirala et al., 2017), it was found that this compound could effectively inhibit BACE1 enzyme (IC_{50} of 5.12 ± 0.30 μM). This result was further proved by enzyme kinetics and molecular docking where lupeol showed competitive inhibition with the binding energy of -8.2 kcal/ mol on amino acid residues. Further, in the rat brain model, lupeol was reported to restore $A\beta_{(1\text{-}42)}$ induced behavioral and biochemical abnormalities (Kaundal et al., 2017). Thus, it can be suggested that the activity of CH_2Cl_2 fraction of *C. maackii* flower against BACE1 might be correlated with the inhibitory activity of lupeol.

The association of cellular oxidative stress resulting in the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) to AD has been reported (Butterfield et al., 2007; Smith et al., 1994). Since, the potent RLAR and DPPH inhibiting activity of luteolin has already been reported (Hwang et al., 2018), it can be evident that the anti-BACE1 activity of EtOAc fraction of *C. maackii* flower might be associated with the anti-oxidative potential of this compound whose presence is in the highest amount. Moreover, the *n*-BuOH fraction of the flower had modest activity compared to two other nonpolar fractions. From HPLC analysis it can be seen that the presence of two glucuronides, apigenin 7-*O*-β-D-glucuronide

and luteolin 7-*O*-β-D-glucuronide is in the highest amount. In an article published by Iakovleva et al. (2015), only luteolin but not luteolin 7-*O*-β-D-glucoside showed attenuation of the cytotoxic response to transthyretin, a plasma protein with amyloidogenic property, in cultured neuronal cells and rescued the phenotype of a *Drosophila melanogaster* model. Thus, it is can be seen that the aglycone has higher inhibiting potential than the glucoside during amyloidogenesis. The presence of glucoronidated forms of apigenin and luteolin in a large amount in the *n*-BuOH fraction could possibly be responsible for the lower activity of this fraction.

Over the period of time, flowering part of a plant is the most underexploited part with respect to other counterparts like leaves, root, and stem. Only few out of many has been investigated against different diseases. In this study, we can see that the CH_2Cl_2 and EtOAc fraction of *C. maackii* flower is highly inhibited BACE1 enzyme; this anti-AD effect may also be associated with an array of phytochemicals present in those fractions. Characterization of the extract with HPLC revealed that the major constituents of the EtOAc fraction were apigenin 7-*O*-β-D-glucuronide, luteolin and apigenin (Figure 3, Table 2) and the substantial amount of lupeol and lupeol acetate were obtained from the CH_2Cl_2 fraction. One of the major flavonoids that has been quantified in this study for the EtOAc fraction, luteolin, is acknowledged as the anti-AD and anti-oxidative agent. Similarly, the presence of lupeol in the CH_2Cl_2 fraction parallels with the moderate activity of CH_2Cl_2 fraction. The presence of therapeutic levels of lupeol and luteolin in *C. maackii* flower provides a chemical rationale for its use in the treatment and prevention of AD. Further, the cultivation of C. *maackii* plant flower may allow the flowering part to be used as a source of the important bioactive molecule, lupeol and luteolin,

or to be develop it as a nutraceutical. The flowers of the *C. maackii* are available in plenty during flowering season at fairly low or free of cost, suggesting that the thistle flower can be an important source of lupeol and luteolin and it does not only have aesthetic but also therapeutic potential to ameliorate AD.

V. Conclusions

Different fractions *C. maackii* flower showed significant inhibitory activity against BACE1. Column chromatography and HPLC revealed the presence of high amount of triterpenoids (CH_2Cl_2 fraction) and flavonoids (EtOAc fraction). Moreover, the presence major reference compounds, lupeol and luteolin from this plant may have the potential in the treatment of AD as the corresponding fraction exhibited anti-BACE1 activity. Our current findings suggests that flowers of *C. maackii* may be therapeutically beneficial in treatment of patients with AD. Since these results are based on in-vitro tests, further experiments needs to verify the mechanism of *C. maackii* flower.

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