A THESIS FOR THE DEGREE OF MASTER OF SCIENCE

Effects of lotus-derived constituents on learning and memory activity of scopolamine-induced mice

Scopolamine 처리 생쥐에 대한 연 유래 화합물의 기억력 증진효과

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UNDER THE DIRECTION ADVISER YOUNG-JOON AHN
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Various reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radical, hydrogen peroxide, and hydroxyl radical, are generated as by-products during the aerobic metabolisms in cells. If the production of free radicals exceed the antioxidant capacity of a living system, free radicals not only attack to cell's enzymes and DNA, protein, and lipid, but also cause numerous disorders such as brain dysfunction immune system decline, heart disease, or Alzheimer's disease. Antioxidants can prevent oxidative damages, increased intakes from the diet will also reduce the risks of numerous diseases.

An assessment was made of the antioxidant activity of the constituents derived from Indian lotus, *Nelumbo nucifera* Gaertner (Nelumbonaceae), using 2,2-diphenyl-1-picrylhydrazyl assay. Results were compared with those of a commonly used antioxidant ascorbic acid. The biologically active constituents were determined to be the simple

benzoic acid 3,4-dihydroxy benzoic acid and the flavone luteolin from the roots and the flavonol isoquercetin from the leaves. Based on fifty percent inhibition concentration (IC_{50}), 3,4-dihydroxy benzoic acid (10.90 μM) was the most antioxidant compound and was ≈5 times more active than ascorbic acid (58.19 μM). The antioxidant activity of luteolin and ascorbic acid did not differ significantly. IC_{50} of isoquercetin was 123.23 μM. In addition, the Morris water maze is used for examining the effects of luteolin and tacrine on the learning and memory activity of scopolamine-induced cognitive impairment mice. The scopolamine-treated group (0.5 mg/kg, i.p.) exhibited longer latency time than the saline-treated control group during the 5 days. However, luteolin (20 mg/kg) or tacrine (10 mg/kg) plus scopolamine-treated groups showed significantly shorter latency time than the scopolamine-treated group during the experimental period. Results of distance to platform was similar with those of latency time. Further studies will warrant possible applications of luteolin as potential antioxidant products for the eradication from oxidative stress and increasing learning and memory.

Key words: Natural antioxidant, Alzheimer's disease, *Nelumbo nucifera*, 3,4-dihydroxy benzoic acid, luteolin, isoquercetin

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INTRODUCTION

Life under aerobic conditions is characterized by continuous production of free radicals. Various reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radical, hydrogen peroxide, and hydroxyl radical, are generated as by-products during the aerobic metabolisms in cells (Apel, 2004). Reactive oxygen is essential to energy supply, detoxification, chemical signaling, and immune function (Pai, 2014). However, if the production of free radicals exceeds the antioxidant capacity of a living system, free radicals attack targets in biological cells and cause numerous disorders and diseases such as brain dysfunction, immune system decline, heart disease, or Alzheimer‘s disease (AD) (Tuppo, 2001). Antioxidants can prevent oxidative damages and also reduce the risks of numerous diseases (Radimer et al., 2004). They are molecules which can safely interact with free radicals and terminate the chain reaction before vital molecules are damaged. Antioxidants inhibit oxidation of other molecules by limiting the initiation. Because oxidative stress has been highly correlated with AD (Zhao et al., 2013), an age-related neurological disorder, it also plays a major role in the learning and memory. Although acetylcholinesterase (AChE) inhibitors, such as tacrine, donepezil, rivastigmine, and galantamine, are being successfully used for AD, they have side effects, such as nausea, diarrhea, and vomiting (Terry and Buccafusco, 2003; Cummings et al., 2008). Natural antioxidants has been proposed as an effective therapeutic approach for learning and memory (Williams et al., 2008). Their protective effects have been attributed to their scavenging free radicals (Kameoka et al., 1999). There is a pressing need for the

development of new improved antioxidants with novel target sites.

Natural compounds extracted from natural products such as animals, plants, and minerals have been suggested as alternative sources for antioxidant products. This approach is appealing, in part, because the natural products constitute a potential source of bioactive secondary substances that have been perceived by the general public as relatively safe and often act at multiple and novel target sites (Raskin et al., 2002; Jassim and Naji, 2003). Certain plant preparations and their constituents are regarded as potential sources for developing commercial antioxidants. In the screening of plant materials for antioxidant activity, a methanol extract of the root from the Indian lotus, *Nelumbo nucifera* Gaertner (Nelumbonaceae), was shown to have good antioxidant activity. Large number of references has mentioned the antioxidant activity of different parts of *N. nucifera* in *in vitro* studies (Phonkot et al., 2007). Little work has been done to assess its potential use to treat or prevent brain disorders, although historically *N. nucifera* has been used as a traditional folk medicine for the treatment of diarrhea, gastritis, insomnia, and nervous prostration as well as a hemostatic in Korea, China, and India (Cour et al., 1995).

In this study, an assessment was made of the antioxidant activity of luteolin, isoquercetin, and 3,4-dihydroxy benzoic acid from *N. nucifera* using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The antioxidant activity of the constituents was compared with that of a commonly used broad-spectrum antioxidant ascorbic acid. Acethylcholinesterase (AChE) inhibitory activity of the constituents were evaluated and compared with that of AChE inhibitor tacrine. In addition, the Morris water maze is used for examining the effects of *N. nucifera* root ethanol extracts, luteolin, ascorbic acid, and

tacrine on learning and memory activity of scopolamine-induced cognitive impairment mouse model.

Literature review

1. Free radical and oxidative stress

A free radical is an atom, molecule, or ion that contains one or more unpaired electrons and is more reactive than their parent species (Halliwell, 2001). In living systems, free radicals are generated as part of the body's normal metabolic process, and the free radical chain reactions are usually produced in the mitochondrial respiratory chain, liver mixed function oxidases, through xanthine oxidase activity, atmospheric pollutants and from transitional metal catalysts, drug (Tiwari, 2001). ROS, such as superoxide radicals, hydroxyl radicals, and peroxyl radicals, are natural byproducts of the normal metabolism of oxygen in living organisms with important roles in cell signaling (Aruoma et al., 1997). Reactive oxygen is essential to energy supply, detoxification, chemical signaling, and immune function. Although antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase play an important role in scavenging oxidants and preventing cell injury, these defense mechanisms are not adequate (Kuriakose, 2014). Excessive amounts of ROS may be a primary cause of biomolecular oxidation and may result in significant damage to cell structure, contributing to various diseases, such as cancer, stroke, diabetes and degenerative processes associated with aging (Ames, 1983). Oxidative stress has been considered a mechanism involved in the pathogenesis of Alzheimer’s disease. It has also played a major role in the aging process (Grassi et al., 2009; Stocker et al., 2004). Epidemiological research reveals that the consumption of antioxidant phytochemicals shows benefits for oxidative stress-induced damage (Dia et

al., 2006).

2. Antioxidants

Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause pathological conditions such as ischemia, anaemia, asthma, arthritis, inflammation, neurodegeneration, Parkinson's diseases, mongolism, aging process, and perhaps dementias (Shwetha et al., 2011). Antioxidants are important inhibitors of lipid peroxidation not only for food protection but also as a defense mechanism of living cells against oxidative damage (Halliwell, 1991). Antioxidants have been shown to prevent the destruction of β-cells (Slonim et al., 1983), and to prevent or inhibit oxidation processes in human body and food products (Diaz et al., 1997). Several studies have revealed that a major part of the antioxidant activity may be from compounds such as flavonoids, isoflavones, flavones, anthocyanins, and catechins and other phenolics with mechanisms involving both free radical-scavenging and metal chelation (Lien et al., 1999). Flavonoids are classified to wide group of chemical compounds – polyphenols, and they are produced mainly by plants. Amongst the most studied categories of antioxidants, dietary polyphenols and other natural antioxidants have rapidly gained attention as viable candidates for clinical testing in neurodegeneration and acute neuronal injury such as stroke (Mandel, 2004; Simonyi, 2005). It is produced in response to the immoderate UV radiation, ions of transition metals, thermal shock, or as the reaction to fungal infection (Wojcieszyńska, 2006). The most important sources of flavonoids are drinks such as coffee or tea, fruits, and vegetables (Habauzit and Morand, 2012). The

antioxidant properties of flavonoids are the result of presence in their molecular structure a hydroxyl groups, associated with the benzene ring (Gawlik-Dziki, 2004). The most important mechanisms of their action consist: deactivation of active forms of oxygen or nitrogen, interruptions free radical chain reactions, reduction of reactive oxygen, or nitrogen species through giving an electron of the hydrogen atom (Habauzit and Morand, 2012).

2.1. Natural antioxidants

In nature, there are a wide variety of naturally occurring antioxidants which are different in their composition, physical and chemical properties, mechanisms, and site of action (Naik, 2003). Natural phenolic antioxidants, such as synthetics, can effectively scavenge free radicals, absorb light in the ultraviolet (UV) region (100 to 400 nm), and chelate trasition metals, thus stopping progressive oxidative damage (Brewer, 2007). Food tissues, because they are living, are under constant oxidative stress from free radicals, ROS, and prooxidants generated both exogenously and endogenously (Brewer, 2007). For this reason, many of these tissues have developed antioxidant systems to control free radicals, lipid oxidation catalysts, oxidation intermediates, and secondary breakdown products (Agati et al., 2007; Brown and Kelly 2007; Chen 2008; Iacopini et al., 2008). Species and herbs, used in foods for their flavor and in medicinal mixtures for their physiological effects, often contain high concentrations of phenolic compounds that have strong H-donating activity (Lugasi et al., 1995; Muchuweti et al., 2007).

2.2. Synthetic antioxidants

Most important and widely available synthetic antioxidants as well as their uses, showing that the main focus of synthetic antioxidants is the prevention of food oxidation, especially fatty acids (Carocho, 2013). Almost all processed foods have synthetic antioxidants incorporated, which are reported to be safe, although some studies indicate otherwise. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are the most widely used chemical antioxidants (Carocho, 2013). They effectively can inhibit oxidationl chelating agents, such as ethylene diamine tetra acetic acid, and bind metals reducing their contribution to the process (Brewer, 2011). Octyl gallate is considered safe to use as a food additive because after consumption it is hydrolyzed into gallic acid and octanol, which are found in many plants and do not pose a threat to human health (Joung et al., 2004). Nordihydroguaiaretic acid, despite being a food antioxidant, is known to cause renal cystic disease in rodents (Evan and Gardner, 1979).

2.3. Endogenous antioxidants

Endogenous antioxidants are essentially enzymes that catalytically remove oxidants (Pouillot et al., 2011). Major endogenous antioxidants are SOD, superoxide reductase, catalase, and glutathione peroxidase. These enzymes play a key role in decreasing the content of oxidants and preventing oxidative damage (Pouillot et al., 2011). The human antioxidant system is divided into two major groups, enzymatic antioxidants and nonenzymatic oxidants (Pouillot et al., 2011).

2.3.1. Enzymatic antioxidant

Regarding enzymatic antioxidants, they are divided into primary and secondary enxymatic defences (Carocho, 2013). With regard to the primary defense, it is composed of three important enzymes that prevent the formation or neutralize free radicals: glutathione peroxidase, which donates two electrons to reduce peroxides by forming selenoles and also eliminates peroxides as potential substrate for the Fenton reaction (Carocho, 2013). Catalase converts hydrogen peroxide into water and molecular oxygen and has one of the biggest turnover rates known to man, allowing just one molecule of catalase to convert 6 billion molecules of hydrogen peroxide. SOD converts superoxide anions into hydrogen peroxide as a substrate for catalase (Rahman, 2007). The secondary enzymatic defense includes glutathione reductase that reduces glutathione from its oxidized form to its reduced form, thus recycling it to continue neutralizing more free radicals (Carocho, 2013).

2.3.2. Nonenzymatic antioxidant

Considering the nonenzymatic endogenous antioxidants, there are quite a number of them, such as vitamins, enzyme cofactors (Q10), nitrogen compounds (uric acid), and peptides (glutathione) (Carocho, 2013). β-Carotene is an excellent scavenger of singlet oxygen (Gupta et al, 2006). Vitamin A is carotenoid produced in the liver and results from the breakdown of β-carotene (Carocho, 2013). There are about a dozen forms of vitamin A. It is known to have beneficial impact on the skin, eyes, and internal organs (Carocho, 2013). Glutathione is a good scavenger of many free radicals like O_2^-, HO, and various

lipid hydroperoxides and may help to detoxify many inhaled oxidizing air pollutants like ozone, NO_2, and free radicals in cigarette smoke in the respiratory tract (Gupta et al, 2006).

2.4. Exogenous antioxidants

Exogenous antioxidants include antioxidants that cannot be synthesized by our body, such as vitamins, trace elements, and phytoantioxidants (Thingle and Nitave, 2014). Vitamin E (tocopherol) is the most powerful liposoluble antioxidant (Thingle and Nitave, 2014). It inhibits the peroxidation of membrane lipids. It reacts with free radicals to form the radical tocopheryl, a stable substance that stops the chain reaction of the membrane lipids. The chain reaction is propagation of free radicals (Thingle and Nitave, 2014).

3. Risk factors of synthetic antioxidant.

Synthetic antioxidants, such as BHA and BHT, have been used as antioxidants since beginning of this century (Hall and Cuppett, 1997). Synthetic antioxidants Trolox and *tert*-butylhydroquinone (TBHQ) are also widely used. TBHQ is a derivative of hydroquinone, substituted with a *tert*-butyl group (Nunes et al., 2012). It is a highly effective antioxidant used in foods as a preservative for unsaturated vegetable oils and many edible animal fats. In the food industry, synthetic antioxidants such as ascorbic acid and BHT have been widely used as additives to preserve and stabilize foods and animal feed products for freshness, nutritive value, flavor, and color (Nunes et al., 2012). Yet, one study has shown BHT to be potentially toxic, especially in high doses, making it important to consider health risks associated with long-term dietary intake of BHT (De Oliveira et

al., 2009). For this reason, it has become very necessary to derive antioxidants from natural sources for use as supplements to human health.

4. Antioxidant activity of natural products

The growing relevance of medicinal plants as possible sources for the discovery of novel antioxidant molecules is often based on their long historical utilization in folk medicine, especially in developing countries. The 21 commonly used South African medicinal plants were investigated for their phytochemical antioxidants after 12 or 16 years storage in comparison to freshly harvested material (Hutchings et al., 1996; Jager et al., 1996; Duncan 1999). Extensively studied sources of natural antioxidants are fruits and vegetables, seeds, cereals, berries, wine, tea, onion bulbs, olive oil, and aromatic plants (EI-Gharras, 2011). Herein is rich in nutraceuticals including phenolic compounds and flavonoids, could protect the liver from age-associated antioxidant decline and protect the brain from cognitive deficits in SAMP8 mice (Shih et al., 2010). Food antioxidants such as α-tocopherol, ascorbic acid, carotenoids, amino acids, peptides, proteins, flavonoids and other phenolic compounds might also play a significant role as physiological and dietary antioxidants (Shahidi, 2000). Plant polyphenols with antioxidant capacity could scavenge reactive chemical species as well as minimize oxidative damage resulting from excessive light exposure (Oliveira et al., 2012). Some plant polyphenols are important components of both human and animal diets and are safe to be consumed (Gulçin et al., 2005). Several plants are reported to be produced various biological active compounds (Paudel et al., 2014). Lichens from the extreme environments such as high altitude, high

UV, drought, and cold are believed to be synthesized unique types of secondary metabolites than the other one (Table 1). Natural antioxidants are known to exhibit a wide range of biological effects including antibacterial, antiviral, antiinflammatory, antiallergic, antithrombotic, and vasodilatory activities (Cook and Samman, 1996). Figure 1 revealed natural antioxidants separated in classes.

Table 1. IC_{50} values of natural plant's extract*

Natural product	IC_{50} (μg/mL)	Natural product	IC_{50} (μg/mL)
Rhododendron dauricum	0.45±0.02	*Ranunculus reptans*	7.3±0.23
Dryas grandis	0.52±0.03	*Thymus pavlovii*	7.5±0.45
Rhododendron redowskianum	0.61±0.02	*Sparganium hyperboreum*	8.2±0.43
Dryopteris fragrans	1.2±0.08	*Saxifraga punctata*	8.2±0.25
Saxifraga bronchialis	1.8±0.07	*Pinus pumila*	9.2±0.64
Aconogonon tripterocarpum	1.9±0.17	*Ribes fragrans*	9.5±0.38
Chamerion angustifolium	2.1±0.17	*Sedum sukaczevii*	9.8±0.49
Salix pulchra	2.1±0.15	*Thalictrum foetidum*	10.3±0.31
Chamerion angustifolium	2.2±0.06	*Sorbaria sorbifolia*	11±0.88
Betula divaricate	2.5±0.15	*Ptarmica salicifolia*	11.1±0.67
Artemisia vulgaris	2.8±0.14	Rheum compactum	11.2±0.34
Rhododendron lapponicum	3.1±0.31	*Artemisia lagocephala*	11.6±1.1
Andromeda polifolia	3.2±0.26	*Campanula rotundifolia* ssp. *langsdorffiana*	11.8±0.71
Vaccinium uliginosum	3.4±0.14	*Veratrum lobelianum*	12±0.72
Ribes triste	3.5±0.07	*Oxycoccus microcarpus*	12.1±0.48
Comarum palustre	3.6±0.18	*Beckmannia syzigachne*	13±0.39
Salix reptans	3.7±0.15	*Empetrum nigrum*	15.1±0.9
Ledum palustre	3.8±0.27	*Euprasia hyperborea*	15.1±0.45
Rosa acicularis	3.9±0.19	*Cassiope tetragona*	15.2±0.76
Pyrola rotundifolia	3.9±0.16	*Alopecurus roshevitzianus*	18±1.44
Sanguisorba officinalis	4.1±0.33	*Chosenia arbutifolia*	18.1±1.25
Carex aquatilis	4.1±0.08	*Dryas punctata*	18.2±0.91
Rubus matsumuranus	4.3±0.22	*Achillea millefolium*	19±1.33
Vaccinium vitis-idaea	4.7±0.19	*Thamnolia vermicularis*	5.2±0.16
Rubus chamaemorus	4.7±0.38	*Peltigera didactyla*	5.7±0.46

Veronica incana	4.8±0.14	*Peltigera malacea* 6.1±0.31
Pentaphylloides fruticosa	4.8±0.24	*Peltigera aphthosa* 14.7±1.03
Galium verum	4.9±0.34	*Sphagnum fuscum* 19.7±0.98
Cassiope ericoides	5.1±0.31	*Loeskypnum badium* 19.8±0.59
Parnassia palustris	5.4±0.11	*Hylocomium splendens* 19.8±1.19
Dracocephalum palmatum	6±0.48	*Polytrichastrum alpinum* 19.9±0.99
Orostachys spinosa	6.2±0.19	*Scorpidium scorpioides* >20
Salix tschuktschorum	6.3±0.25	*Paludella squarrosa* >20
Juniperus communis	6.8±0.14	

* From Paudel et al. (2014).

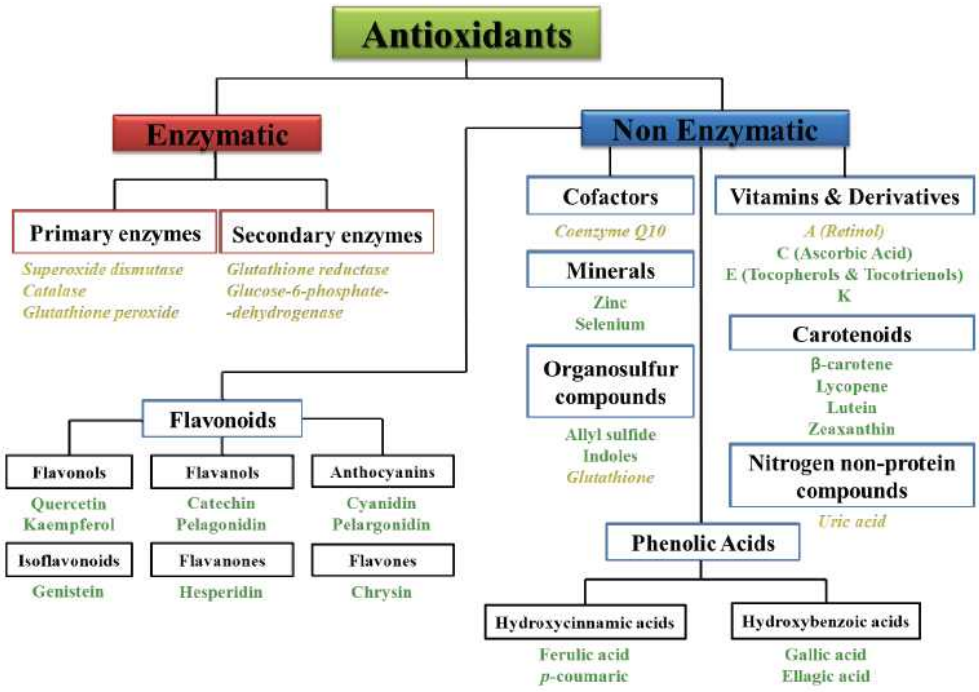


Fig. 1. Natural antioxidants separated in classes. Green words represent exogenous antioxidants, while yellow ones represent endogenous antioxidants.

4.1. Flavonoid polyphenols as neuroprotective agents

Phenolics are ubiquitous bioactive compounds and a diverse group of secondary metabolites universally present in higher plants (Robards et al., 1999). The action of phenolics as antioxidants is viewed as beneficial in both foods and the body where phenolics are oxidized in preference to other food constituents or cellular components and tissues (Robards et al., 1999). Quercetin is a flavonoid polyphenol found in many common foods such as apples and capers. *In vitro* studies show that quercetin increases cell survival in the presence of hydrogen peroxide (Dajas et al., 2003; Heo et al., 2004), linoleic acid hydroperoxide(Sasaki et al., 2003), and *tert*-butyl hydroperoxide (Silva et al., 2008). *In vivo* studies of quercetin effects on neurodegeneration have mostly focused on cognitive impairments, ischemia, and traumatic injury. Quercetin improves memory and hippocampal synaptic plasticity in models of impairment induced by chronic lead exposure (Hu et al., 2008). In addition, quercetin is neuroprotective against colchicine administration, which similarly causes cognitive impairments (Kumar et al., 2008). Epigallocatechin 3-gallate is a flavonoid polyphenol and the main antioxidant compound found in green tea and selectively protects cultured rat cerebellar granule neurons from oxidative stress (Schroeder, 2009).

4.2 Nonflavonoid polyphenols as neuroprotective agents

Resveratrol is a polyphenolic antioxidant found in many kinds of grapes and is known mostly for its cardiovascular benefits (Bertelli et al., 2009; Sadruddinet al., 2009). *In vivo*, resveratrol significantly attenuates hippocampal neurodegeneration and learning

impairment in the inducible p25 transgenic mouse model of Alzheimer's disease (AD) and tauopathy (Kim et al., 2007). Moreover, resveratrol also reduces oxidative damage and preserves striatal dopamine in the 6-OHDA rat model of PD (Khan et al., 2010).

Research into the neuroprotective effects of the nonflavonoid polyphenol curcumin is less extensive than that for resveratrol. However, in Neuro2a mouse neuroblastoma cells infected with Japanese encephalitis virus, curcumin enhances cell viability by decreasing ROS and inhibiting proapoptotic signals (Dutta, 2009). Furthermore, curcumin displays an additive protective effect to that of catalase and SOD activities in the striatum and midbrain of MPTP-treated mice (Rajeswari, 2006).

5. Learning and memory

Normal aging is associated with a slow decline in brain functions such as sensory and motor performance, and at times, this decline is accompanied by progressive memory loss, dementia, and cognitive dysfunctions, ultimately resulting in limited functionality (Papadreou et al, 2011). The learning and memory are closely related to the cholinergic and glutamatergic neurotransmitter systems in brain (Bartus et al., 1982; Durand et al., 1996). There are three stages for learning and memory processing including acquisition, consolidation, and retrieval processes (Abel et al., 2001). After detection by the sensory system, information is rapidly encoded and may pass into labile memory. During consolidation, this memory may then be consolidated into long-term storage. The retrieval process is the final stage which uses the consolidated and more fixed memory. It is important to know the effects of a compound on each stage of memory processing as the

compound which improves multiple cognitive processes will be most useful as a treatment for memory deficits (Prickaerts et al., 2005). However, it is difficult to differentiate each stage of memory processing experimentally because two or more stages of memory are affected by experimental techniques, depending on the time course of the manipulations and the neurotransmitter system(s) involved (Collinson et al., 2006).

5.1. Relationship of memory and antioxidant protection

In both aged humans and rodents, cognitive impairment has been correlated to the accumulation of oxidative damage to lipids, proteins, and nucleic acids (Forster et al., 1996; Butterfield et al., 2006; Murali et al., 2007) and the vulnerability of various neurotransmitters/neurotrophin systems activity to oxidative stress (Pradham et al., 1980; Mokrasch et al., 1984; Grotto et al., 2007). One of the major markers of cholinergic function is the activity of the enzyme AChE which is known to be decreased with aging in various cerebral areas (Papandreou et al., 2006) and synaptic plasma membranes (Gorini et al., 1996). AChE activity is also known to be decreased by free radicals and increased oxidative stress (Molochkina et al., 2005). This data has led to the suggestion that various antioxidant supplements and phytochemical components might be beneficial for preserving brain functions and forestalling the age-related deficits (Shukitt-Hale et al., 2008).

6. Cognitive impairment

Cognitive impairment is an inclusive term to describe any characteristic that acts as a

barrier to the cognition process (Coren et al., 1999). Cognitive impairment can be caused by normal aging and stress as well as by specific neurodegenerative and psychiatric disorder such as AD, vascular dementia, and schizophrenia (Hsiao et al., 1996; McEwen, 1999; Gooding et al., 2004; Haenschel et al., 2009). Therefore, the development of new side effect-free drugs to treat cognitive impairments would be highly desirable.

6.1. Alzheimer's disease

AD is a progressive neurodegenerative disorder associated with memory impairment and cognitive deficit, which is characterized with low levels of the neurotransmitter acetylcholine in the brain of the patients (Xiao, 2013). AD is single-handedly the main factor behind dysfunction among persons over age 85, and the major cause of dementia in old age (Larson et al., 1992). A report on Alzheimer's disease in 2009 estimates that people over the age of sixty-five become twice as vulnerable to AD every five years. According to epidemiological surveys, an estimated 7–10% of individuals over 65 and 50–60% over 85 suffer from AD (Evans et al., 1989; McKhann et al., 1984), reaching approximately 35 million people worldwide. In Europe, 7.3millions of citizens suffer from dementia (Ferri et al., 2005). Approximately 5.4 million people currently suffer from the disease, consisting of approximately one in every 8 older adults who are over 65 in the United States (US) (Alzheimer's Association, 2011). The oldest old age cohort of Koreans, which is greatly inclined to be afflicted with AD, is expected to grow, in accordance with the high rate of national population growth (Moon, 2006). The pathophysiology of AD is related to the injury and death of neurons, initiating in the

hippocampus brain region that is involved with memory and learning, then atrophy affects the entire brain (Shaffer et al. 2013).

7. Cause of cognitive impairment

Amyloid beta (Aβ) is a short peptide that is an abnormal proteolytic byproduct of the transmembrane protein amyloid precursor protein, whose function is unclear but thought to be involved in neuronal development. Aβ monomers are soluble and contain short regions of beta sheet at sufficiently high concentration, they undergo a dramatic conformational change to form a beta sheet-rich tertiary structure that aggregates to form amyloid fibrils. These fibrils deposit outside neurons in dense formations known as senile plaques or neuritic plaques, in less dense aggregates as diffuse plaques, and sometimes in the walls of small blood vessels in the brain in a process called amyloid angiopathy or congophilic angiopathie.

8. Therapy of cognitive impairment

The research of novel drug candidates has shown that natural products such as plant extracts and plant-originated compounds have enormous potential to become drug leads with neuroprotective activity (Xiao, 2013). The current pharmacologic therapy for AD only provides short-term improvement for a short period of time, six to eighteen months (Reynish et al., 2006). The only medicines approved in the US and several parts of Europe for short term alleviation of symptoms are cholinesterase inhibitors and *N*-methyl-D-aspartate (NMDA) receptor antagonist memantine. For example, AChE inhibitor

galantamine is a natural alkaloid first obtained from *Galanthus* spp. Huperzine A, an alkaloid found in *Huperzia* spp., is an AChE inhibitor commercialized as a dietary supplement for memory support, used to treat AD symptoms in China (Xiao, 2013). AChE inhibitors are used in the treatment of several neuromuscular disorders and have provided the first generation of pharmaceuticals for the treatment of AD. Tacrine, donepezil, rivastigmine, and galantamine, are being successfully used for the therapeutic approaches for AChE inhibitors. These drugs do not affect the pathology of AD, but allows the brain to compensate for the loss of neurons that communicate via acetylcholine, a neurotransmitter. These drugs are prescribed to treat symptoms related to memory, thinking, language, judgment, and other cognitive processes. Due to unfavorable pharmacological profile, tacrine is currently no longer marketed (Francis et al., 1999). This has been followed by the more recent products, donepezil, rivastigmine, and metrifonate. However, AChE inhibitors have side effects such as nausea, diarrhea, and vomiting (Terry et al., 2003; Cummings et al., 2008). Current therapies with AChE inhibitors and NMDA receptor antagonists are based on the cholinergic and glutamatergic hypothesis, respectively (Schmidt et al., 2008). Although active at ameliorating AD symptoms, none of the current drugs are able to modify disease progression, a fact that has provided the driving force behind the ongoing research for new and potent anti-Alzheimer compounds (Schmitt et al., 2004; Schmidt et al., 2008). These drugs offered a significant advance in the pharmacological management of dementia, yet the search for novel and potent AChE inhibitors to treat AD is still an ongoing endeavor and AChE remains a highly viable target for the symptomatic improvement in AD (Wilkinson et al.,

2004).

9. Morris water maze

The Morris water maze is widely used to study spatial memory and learning. Animals are placed in a pool of water that is colored with powdered nonfat milk or nontoxic tempera paint, where they must swim to a hidden escape platform. Because they are in opaque water, the animals cannot see the platform, and cannot rely on scent to find the escape route. Instead, they must rely on external/extra-maze cues. Spatial learning is assessed across repeated trials and reference memory is determined by preference for the platform area when the platform is absent (Vorhees et al., 2006). The principle of the test is that mouse can learn to swim, from any starting position, towards a hidden escape platform. They do this using distal extra-maze cues that are remote from the actual place in the pool to which the animal is heading. Therefore, the room containing the tank should have permanently positioned distinctive objects such as posters placed outside of the pool or on the walls. Developed by Richard G. Morris in 1981, this paradigm has become one of the "gold standards" of behavioral neuroscience.

Since its first application in 1981, the Morris water maze has become one of the most frequently used tool for analyzing spatial learning and memory (Morris, 1981). It has been used widely in investigations of different aspects of learning and memory in rodents and in investigations of the variables that may affect the animal's behavior in the task. It has also been used as a tool to investigate chemically induced effects on learning and memory.

10. *Nelumbo nucifera*

N. nucifera is a perennial aquatic plant with stout creeping yellowish white colored rhizomes. The roots of lotus are planted in the soil of the pond or river bottom, while the leaves float on top of the water surface or are held well above it. The flowers are usually found on thick stems rising several centimeters above the leaves. The plant normally grows up to a height of about 150 cm and a horizontal spread of up to 3 meters. The leaves may be as large as 60 cm in diameter, while the showy flowers can be up to 20 cm in diameter. Lotus is a rhizomatus aquatic, ornamental, edible, and medicinal plant which is grown as a nonconventional vegetable commonly in China, India, Japan, and Australia (Qichao, 2005).

10.1. Pharmacological effects of *Nelumbo nucifera*

All parts of lotus are used for various medicinal purposes in Oriental medicine. Pharmacological studies of the plant revealed that the whole plant possess antidiabetic, antipyretic, antiinflammatory, anticancerous, antimicrobial, antiviral, and antiobesity properties (Kashiwada et al., 2005). The seeds of lotus have been used in traditional medicine for the alleviation of fever and treatment of bleeding, dizziness, and hematuria (Bensky and Gamble, 1993). Experimental studies demonstrated that lotus seed has hepatoprotective and antifertility activities as well as free radical scavenging activity (Mazumder et al., 1992; Sohn et al., 2003). It has been also reported that the seed could suppress cell cycle progression, cytokine genes expression, and cell proliferation in human peripheral blood mononuclear cells (Liu et al., 2004). The leaves of lotus are

considered best for over-coming body heat and stopping bleeding (Bensky, 2004). They are also used as a drug for hematemesis, epistaxis, hemoptysis, hematuria, and metrorrhagia in traditional Chinese medicine (Ono, 2006). Especially, they have been applied in Chinese herbal prescriptions to alleviate tissue inflammation, cancer, and liver cirrhosis for a long time (Mukherjee, 1997). The rhizome extracts also possess antiobesity (Ono, et al., 2006) and antidiabetic properties (Mukherjee et al., 1997). The rhizomes are used as popular vegetable and exhibit multiple nutritional and medicinal properties (Ono et al., 2006).

10.2. Constituents of *Nelumbo nucifera*

Much works have been done on the phytochemical constituents of lotus. Mainly, various alkaloids, such as nuciferine, *N*-nornuciferine, roemerine, liensinine, neferine, and (−)-1-(*S*)-norcoclaurine, have been reported from this herb (Furukawa, 1996; Kashiwada et al., 2005; Luo et al., 2005; Wu et al., 2004; Agnihotri et al., 2008). The major constituents present in the seeds are alkaloids like dauricine, lotusine, nuciferine, pronuciferine, liensinine, isoliensinine, roemerine, nelumbine, and neferine (Tomita et al., 1961; Furukawa et al., 1965; Wang et al., 1991; Anonymous, 1992; Qian, 2002). Lotus leaves contain several flavonoids and alkaloids and is effective in the treatment of hyperlipidemia in rodents (Onishi, 1984). Biochemically, the rhizomes are composed of proteins, fats, carbohydrates and minerals and are good source of energy (Sridhar et al., 2007). The alkaloid liensinine extracted from rhizomes is effective to treat arrhythmia (Ling et al., 2005), sunstroke, fever, dysentery, diarrhea, dizziness, and stomach problems

(Lee et al., 2005).

11. Perspectives

Natural products and their derivatives have been and continue to be rich sources for drug discovery. More than 60% of the drugs that are in the market are derived from natural sources (Molinery, 2009). Natural product resource industry has many potential possibilities to grow up developing valuable sources of new medicinal compounds for human. For many decades, humans were in search of effective drugs that will combat deadly diseases without any side effects. The world's biodiversity has been tested for biological activity and have demonstrated a distinct difference in the structural properties of natural products relative to synthetic compounds. Compared with synthetic compounds, they generally have higher molecular weights and exhibit a different distribution of heteroatoms. Well-known examples of natural products that are part of today's armament against diseases include antibiotics, immune-suppressive agents, antitumor agents, and anticholesterolemic agents (Kai, 2001). During the past decades, a lot of research has been carried out around antioxidants and their effects on health. It is true that antioxidants are beneficial and display a useful role in human homeostasis. The academic community should search deeper into the kinetics and *in vitro* mechanisms of antioxidants to uncover the optimal concentrations for desired functions in order to push forward against cancer, neurodegenerative diseases, and cardiovascular diseases. Isolation and identification of antioxidants from natural products are the initial steps to understanding their significance and mode of action and assessing new chemical compounds families of antioxidant agents.

It can identify the limitations of antioxidants and provide a perspective on the likely future trends in this field.

MATERIALS AND METHODS

1. Instrumental analyses

^{1}H and ^{13}C NMR spectra were recorded in MeOD on a AVANCE 600 spectrometer (Bruker, Rheinstetten, Baden-Württemberg, Germany) at 600 and 150 MHz, respectively, using tetramethylsilane as an internal standard, and chemical shifts are given in δ parts per million (ppm). Distortionless enhancement by polarization transfer (DEPT) spectra was acquired using the Bruker software. Mass spectra were obtained on a GSX 400 spectrometer (Jeol, Tokyo, Japan). UV spectra were obtained in methanol with a V-550 spectrophotometer (Jasco, Tokyo, Japan). Silica gel 60 (0.063–0.2 mm) (Merck, Darmstadt, Germany) was used for column chromatography. Merck precoated silica gel plates (Kieselgel 60 $F_{254,}$ 0.20 mm) were used for analytical thin layer chromatography (TLC). An Isolera one medium-pressure liquid chromatograph (MPLC) (Biotage, Uppsala, Sweden) and an Agilent 1200 high-performance liquid chromatograph (HPLC) with binary solvent pump (Santa Clara, CA, USA) were used for isolation of active principles.

2. Materials

Organic pure 3,4-dihydroxy benzoic acid, luteolin, and isoquercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). A commercially-available antioxidant agent ascorbic acid was supplied by Sigma-Aldrich. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), scopolamine hydrobromide (S0929), 9-amino-1,2,3,4-tetrahydroacridine hydrochloride

hydrate (A3773) were purchased from Sigma-Aldrich. All of the other chemicals and reagents used in this study were of analytical grade quality and available commercially.

3. Plant materials

Root and leaves of *N. nucifera* were purchased from a Sinwon market (Sinwon, Gwanak Borough, Seoul, Republic of Korea (ROK)) in March 2013. They were washed by tap water, and then powdered using a blender. The voucher specimen of root (NN-R-01) and leaves (NN-L-01) were deposited in the Research Institute of Agriculture and Life Sciences, College of Agriculture and Life Sciences, Seoul National University.

4. Sample preparation

The air-dried root (18.1 kg) and leaves (1.72 kg) of *N. nucifera* was pulverized, extracted with ethanol (3 × 90 L) at room temperature for 2 days, and filtered through Whatman no.2 filter paper (Whatman, Maidstone, UK). The combined filtrate was concentrated to dryness by rotary evaporation at 35°C to yield ~324 g of a dark yellowish tar. The extract was sequentially partitioned into with hexane- (3.88 g), chloroform- (0.65 g), ethyl acetate- (2.79 g), butanol- (38.88 g), and water-soluble (276.69 g) portions for subsequent bioassay (Fig. 2). The air-dried leaves (1.72 kg) of the plant were pulverized, extracted with methanol (3 × 8.6 L) at room temperature for 2 days, and filtered, as stated previously. The combined filtrate was concentrated to dryness by rotary evaporation at 35°C to yield ~51.9 g of a dark greenish tar. The extract was sequentially partitioned into with hexane- (14.56 g), chloroform- (7.55 g), ethyl acetate- (2.59 g), butanol- (24.17 g),

and water-soluble (3.03g) portions for subsequent bioassay (Fig. 3). The organic solvent-soluble portions were concentrated under vacuum at 35°C, and the water-soluble portion was concentrated at 50°C. For isolation of active principles, 1000–500 mg/L of each *N. nucifera* root- and leaf-derived material were tested in a DPPH radical scavenging assay, as described previously by Oliveira et al. (2012).

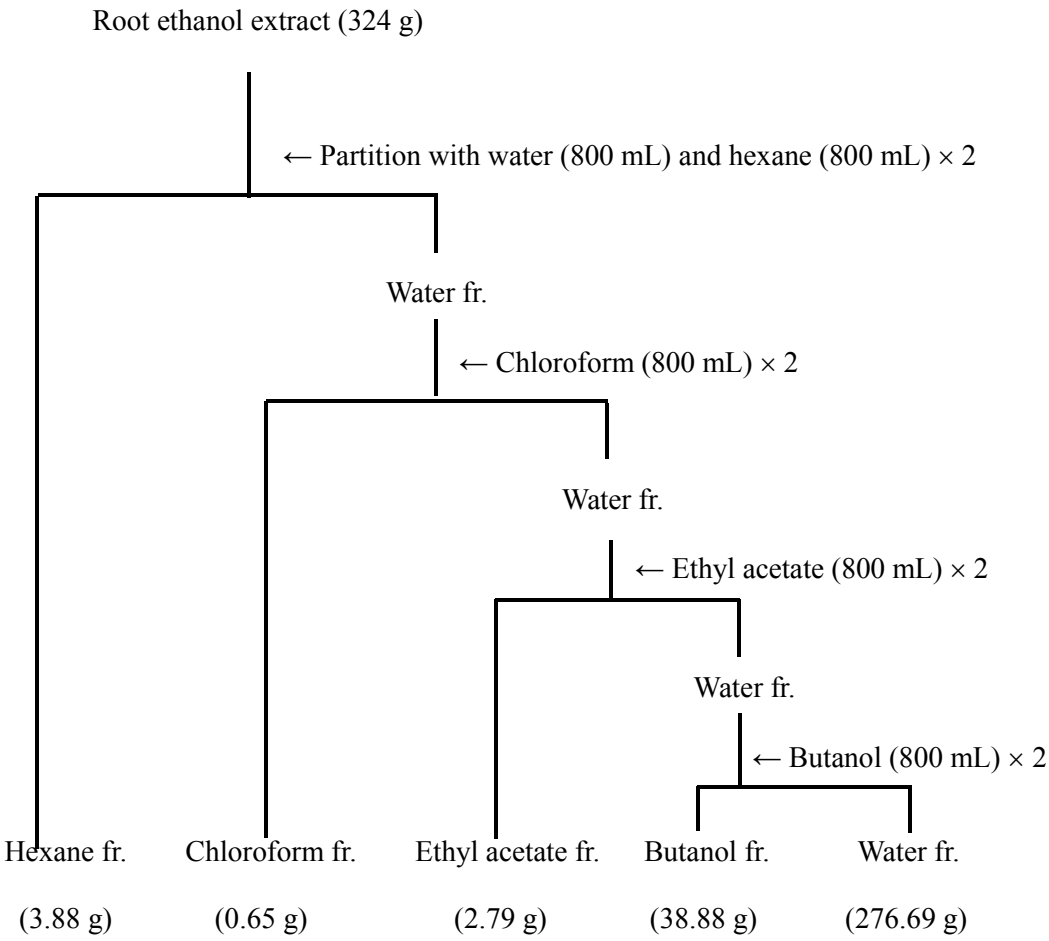


Fig. 2. Solvent fractionation procedures of ethanol extract from root of *Nelumbo nucifera*.

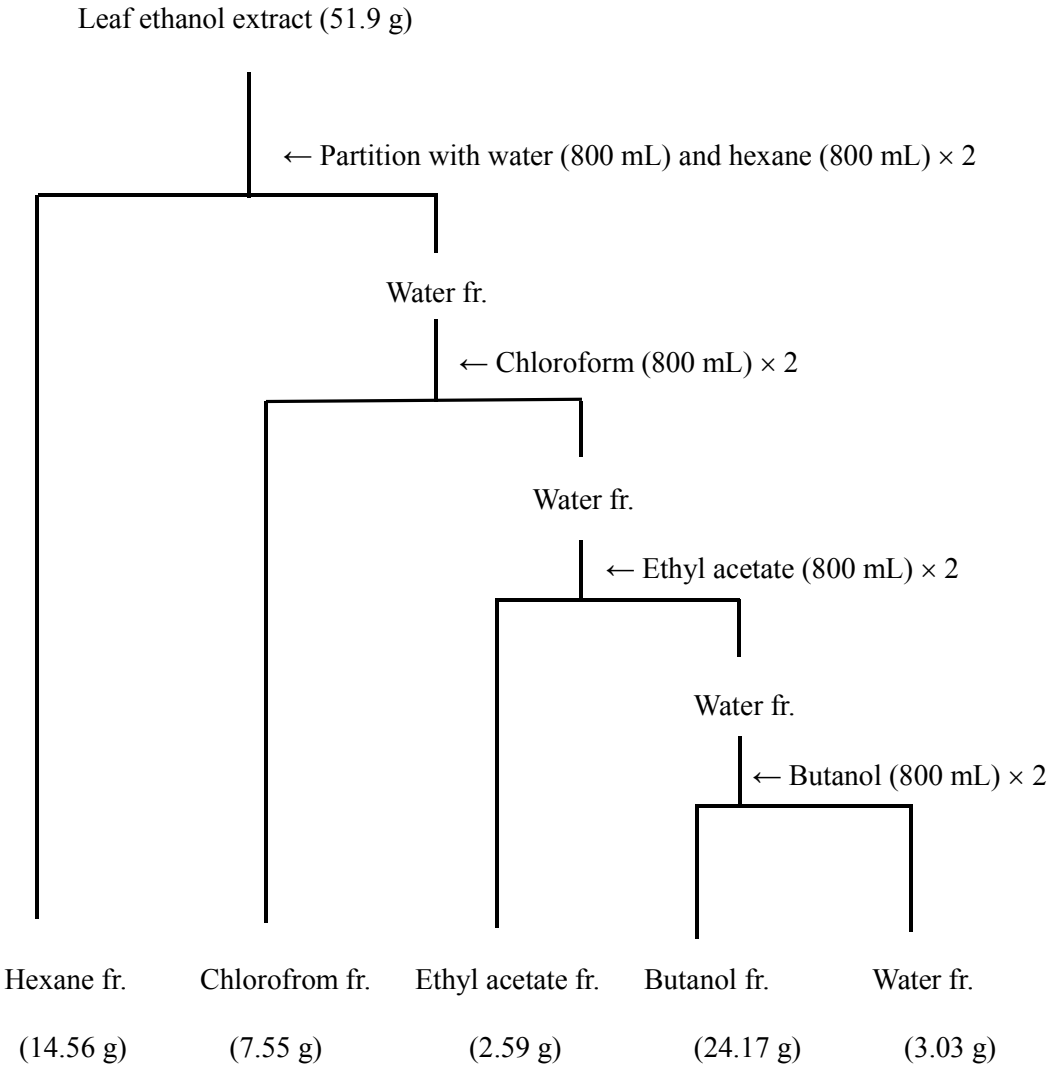


Fig. 3. Solvent fractionation procedures of ethanol extract from leaves of *Nelumbo nucifera*.

5. Isolation of active constituents from *Nelumbo nucifera*

The ethyl acetate-soluble fraction (2.79 g) from the root methanol extract of *N. nucifera* was most biologically active and MPLC was performed using an Isolera apparatus equipped with UV detector at 254 nm and a column cartridge SNAP (100 g silica gel) with column volume of 180 mL. Separation was achieved with a gradient of chloroform and methanol [100:0 (330 mL), 90:10 (440 mL), 80:20 (836 mL), 70:30 (924 mL), 40:60 (586 mL), 20:80 (982 mL), and 0:100 (1 L) by volume] at a flow rate 50 mL/min to provide 52 fractions (each ~22 mL) (Fig. 4). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (8:2 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 2% sulfuric acid and then heating on a hot plate. The active fractions 13 to 38 (E3) (940 mg) was obtained. Fraction E3 was separated by MPLC with a UV detector at 254 and 280 nm and column cartridge (100 g silica gel) by elution with a gradient of chloroform and methanol [100:0 (286 mL), 90:10 (968 mL), 85:25 (176 mL), 80:20 (176 mL), 70:30 (242 mL), 60:40 (1012 mL), and 0:100 (1 L) by volume] at a flow rate of 50 mL/min to provide 65 fractions (each ~22 mL). Column fractions were monitored by TLC on silica gel plates, as stated previously. Active fractions 34–49 (E34) was obtained. Fraction E34 (319 mg) was separated by MPLC with a UV detector at 254 and 280 nm and a column cartridge (25g silica gel) by elution with a gradient of chloroform and methanol [100:0 (44 mL), 90:10 (418 mL), 80:20 (902 mL), 75:25 (506 mL), 70:30 (902 mL), 60:40 (440 mL), 40:60 (462 mL), 20:80 (1056 mL), and 0:100 (500 mL) by volume] at a flow rate of 50 mL/min to provide 92 fractions (each ~22mL). The

active fractions 15 to 25 (E342) was obtained. Fraction E342 (260 mg) was separated by preparative TLC [chloroform:methanol (70:30) by volume] to provide four fractions. Of the four fractions, the active fractions E3422 (44 mg, $R_f = 0.51$) and E3423 (40 mg, $R_f = 0.76$) were obtained. A preparative HPLC was used for separation of the constituents from the active fractions. The column was a 7.8 mm i.d. × 300 mm Waters μBondapak C18 (Milford, MA, USA) with a mobile phase of methanol and water (80:20 by volume) at a flow rate 1 mL/min. Chromatographic separations were monitored using a UV detector at 259 and 345 nm, respectively. Finally, two active principles **1** (14.20 mg) from E3422 fraction and **2** (13.25 mg) from E3423 fraction were isolated at the retention time of 12.03 min (Fig. 5) and 11.25 min (Fig. 6), respectively.

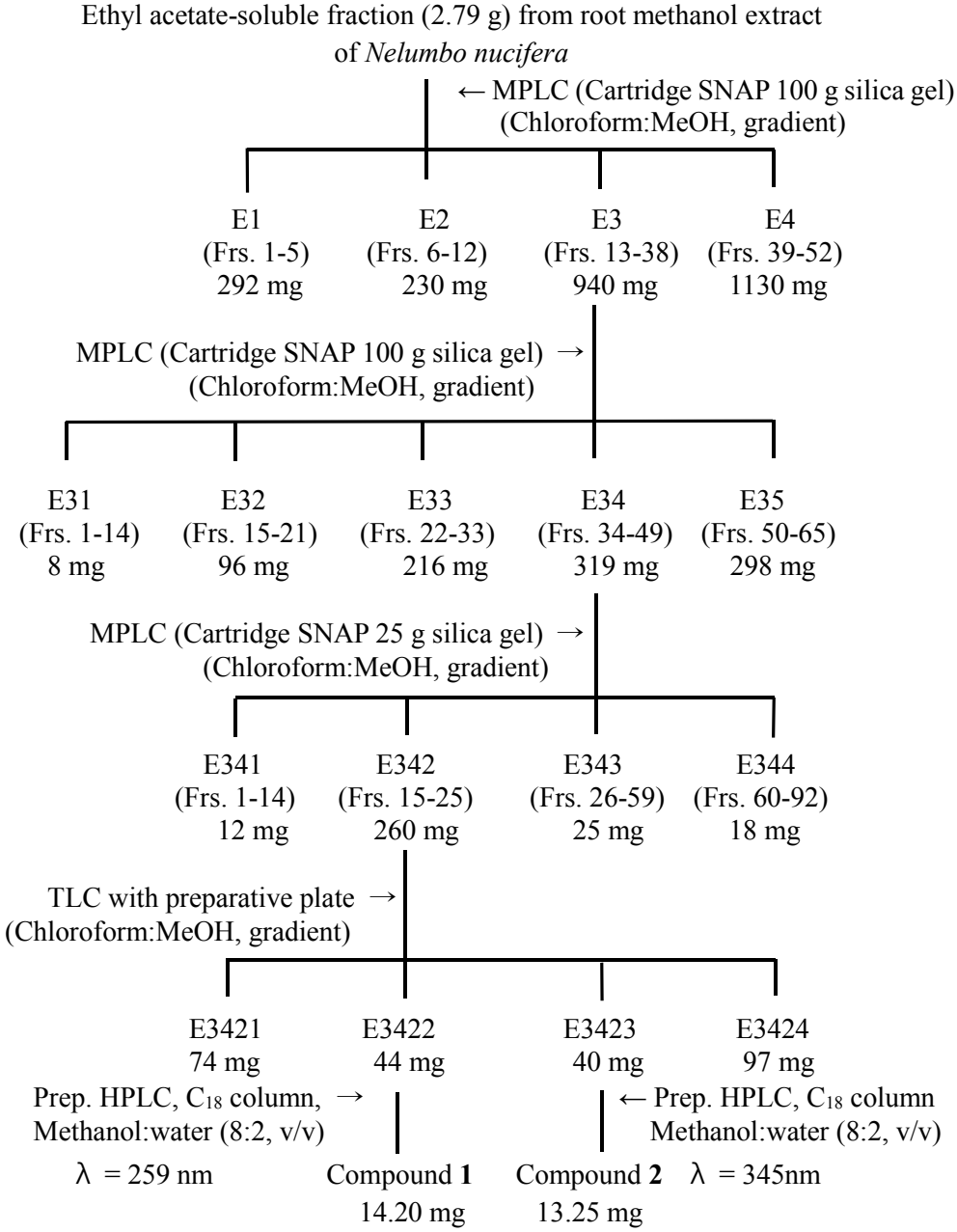


Fig. 4. Isolation procedures of *Nelumbo nucifera* root-derived constituents. For isolation of active principles from the hexane-soluble fraction, 1000–500 mg/L of each *N. nucifera* rhizome-derived material were tested in a DPPH radical scavenging assay.

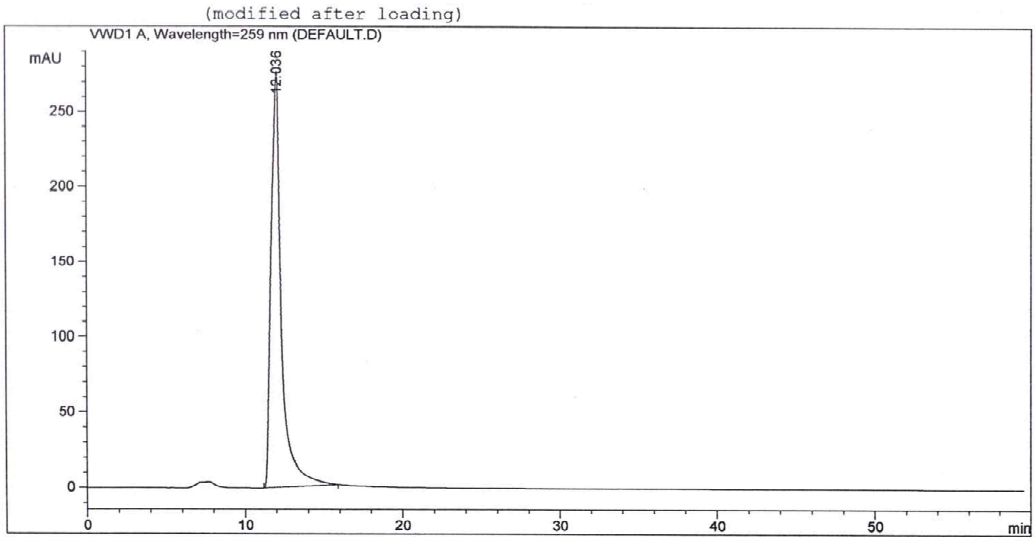


Fig. 5. HPLC chromatogram of compound 1.

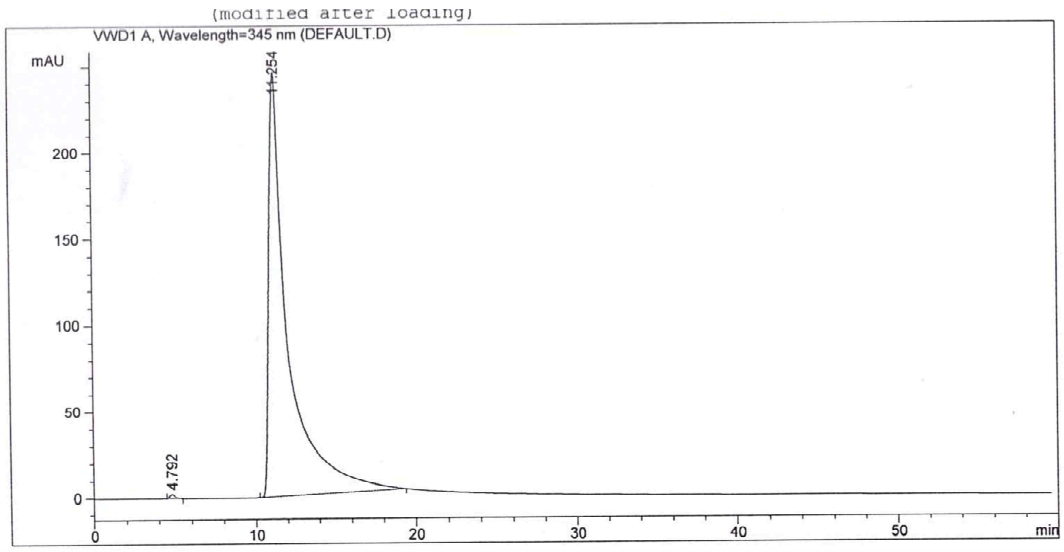


Fig. 6. HPLC chromatogram of compound 2.

The ethyl acetate-soluble fraction (2.59 g) from the leaf methanol extract of *N. nucifera* was most biologically active and MPLC was performed using an Isolera apparatus equipped with a UV detector at 245 nm and column cartridge SNAP (100 g silica gel) with column volume of 180 mL. Separation was achieved with a gradient of chloroform and methanol [100:0 (1464 mL), 90:10 (864 mL), 80:20 (568 mL), 70:30 (918 mL), 60:40 (1122 mL), 40:60 (516 mL), 80:20 (422 mL), and 0:100 (1 L) by volume] at a flow rate of 50 mL/min to provide 266 fractions (each ~22 mL) (Fig. 7). Column fractions were monitored by TLC on silica gel plates developed with chloroform and methanol (5:5 by volume) mobile phase. Fractions with similar R_f values on the TLC plates were pooled. Spots were detected by spraying with 2% sulfuric acid and then heating on a hot plate. Active fractions 119 to 168 (E4) (405 mg) was obtained. Fraction E4 was separated by MPLC with a UV detector at 245 and 280 nm and a column cartridge (100g silica gel) by elution with a gradient of chloroform and methanol [100:0 (156 mL), 90:10 (113 mL), 80:20 (86 mL), 70:30 (124 mL), 60:40 (479 mL), and 0:100 (1 L) by volume] at a flow rate of 50 mL/min to provide 89 fractions. Column fractions were monitored by TLC on silica gel plates with chloroform and methanol (7:3 by volume) mobile phase. The active fractions 26 to 78 (E42) (342 mg) was obtained. Fraction E42 was separated by MPLC with SNAP 25 g silica column and eluted with a gradient of chloroform and methanol [100:0 (54 mL), 90:10 (246 mL), 80:20 (190 mL), 75:25 (238 mL), 70:30 (246 mL), 60:40 (508 mL), 40:60 (260 mL) 20:80 (248 mL), and 0:100 (500 mL) by volume] at a flow rate of 50 mL/min to provide 113 fractions. Column fractions were monitored by TLC on silica gel plates with chloroform and methanol (80:20 by volume) mobile phase. The

active fractions 35 to 57 (E423) (241 mg) was obtained. Fraction E423 was separated by preparative TLC [chloroform:methanol (80:20) with 4% formic acid by volume] to provide 5 fractions. Of the five fractions, the active fraction E4232 (26 mg, $R_f = 0.42$) was obtained. A preparative HPLC was used for separation of the constituents from the active fractions E4232. The column was a 7.8 mm i.d. × 300 mm Waters μBondapak C_{18} with a mobile phase of acetonitrile and water (7:3 by volume) at a flow rate 1 mL/min. Chromatographic separations were monitored using a UV detector at 257 nm. Finally, a potent active principle **3** (13.10 mg) was isolated at the retention time of 10.22 min. (Fig. 8).

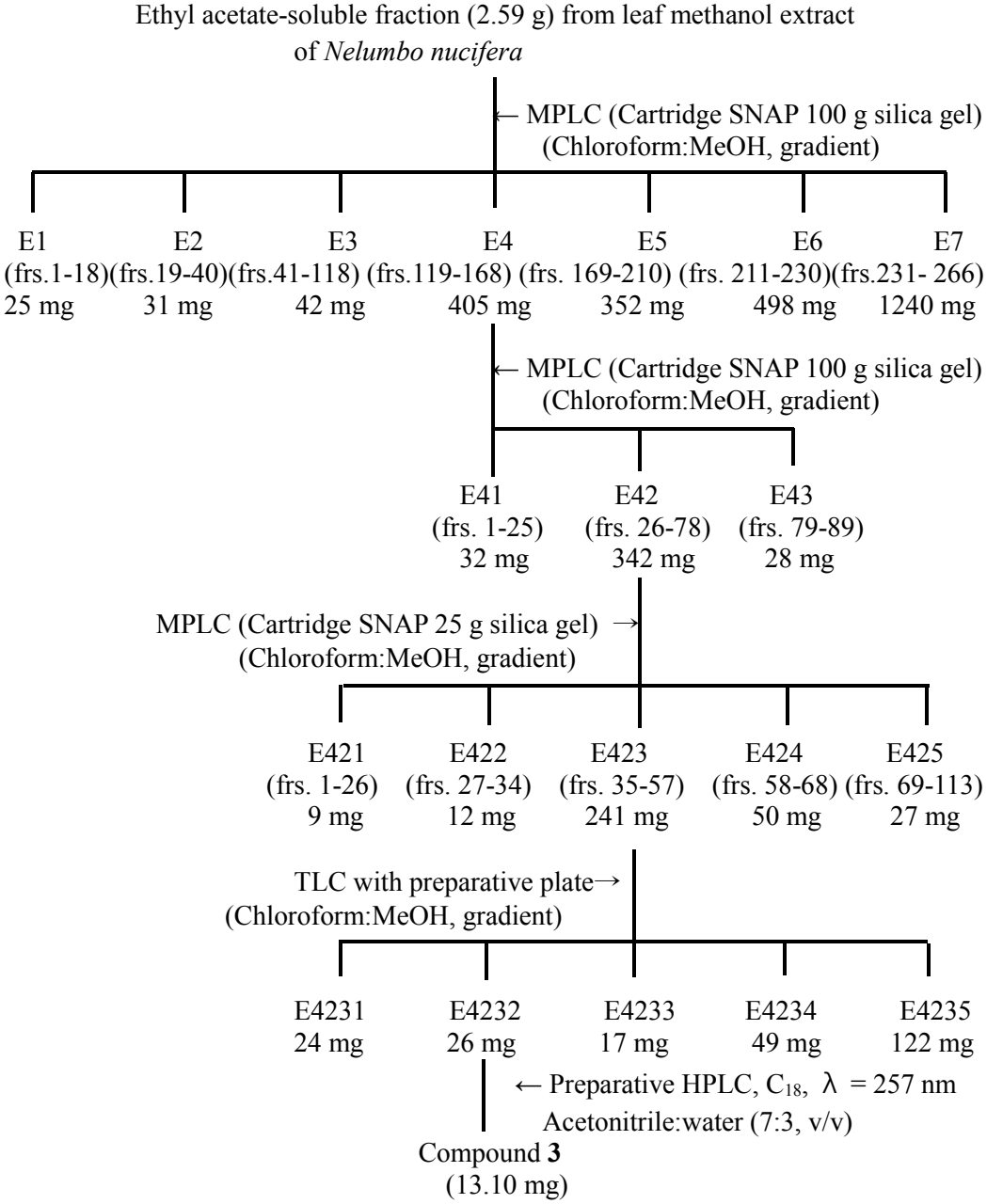


Fig. 7. Isolation procedures of *Nelumbo nucifera* leaf-derived constituents. For isolation of active principles from the hexane-soluble fraction, 1000–500 mg/L of each *N. nucifera* leaf-derived material were tested in a DPPH radical scavenging assay.

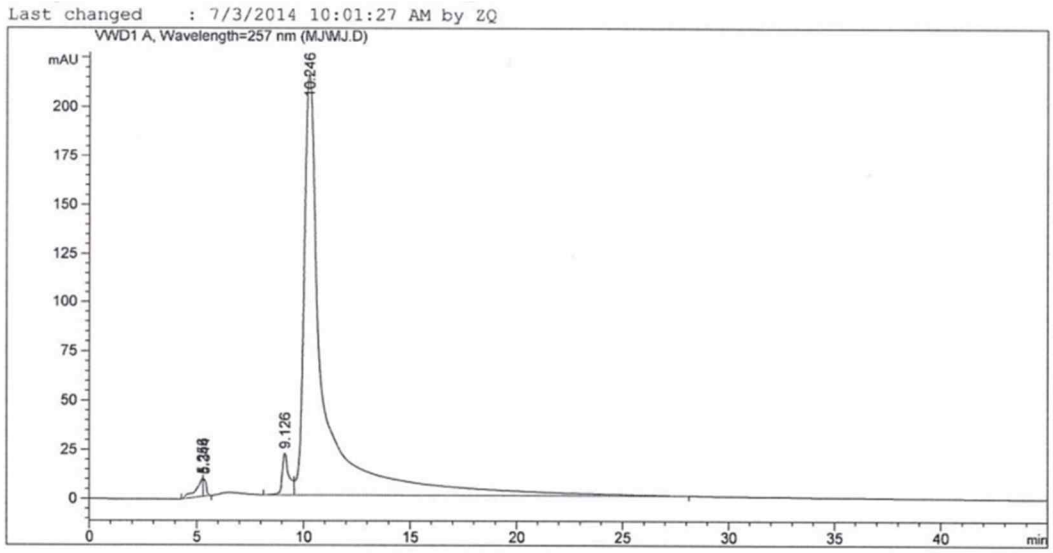


Fig. 8. HPLC chromatogram of compound 3.

6. DPPH radical scavenging activity assay *in vitro*

DPPH radical scavenging activity was evaluated according to the method described previously by Oliveira et al. (2012). The electron donation ability of natural products can be measured by DPPH radical purple-colored solution bleaching. Each concentration of the test sample in 50 μL of methanol was added to 100 μL of 0.4 mM DPPH solution in 96-well microplates. After incubation at room temperature for 30 min, the mixture was determined by measuring the absorbance at 518 nm using a VersaMAx microplate reader (Molecular Devices, Sunnyvale, CA, USA). Ascorbic acid served as a positive control and was likewise formulated. Negative controls consisted of methanol solution only.

7. Acetylcholinesterase inhibitory activity assay *in vitro*

The 96-well microplate AChE assay was carried out following the method of Kennedy et al. (2006) adapted from Ellman et al.(1961) with a slight modification. 3,4-Dihydroxy benzoic acid, luteolin, and isoquercetin were used to detect AChE inhibition *in vitro*. The reaction mixture consisted of 20 μL of electric eel AChE (0.1 U/mL), 140 μL of 0.1 M phosphate buffer (pH 8.0), 10 μL of various concentration of each test sample in 1% ethanol or 1% DMSO, 10 μL of 1 mM DTNB in 0.1 M phosphate buffer (pH 7.0), and 10 μL of 5mM acetylcholine iodide. After incubation for 30 min at 37°C, the absorbance was recorded at 412 nm using a VersaMax microplate reader.

8. Animals

The animals used in this study were female ICR mice (25–30g). Mice were kept in polyacrylic cage (38 × 23 × 10 cm) with ten animals per cage and were available food and water ad libitum and kept under a 12 h light:dark cycle at room temperature. Animal procedures were conducted in accordance with the Principle of Laboratory Animal Care (NIH publication No. 85-23, revised 1985) and with the Animal Care and Used Guidelines issued by Kyung Hee University. Mouse were subdivided into six groups consisting of 10 animals each: control group, scopolamine group, *N. nucifera* root ethanol extract, ascorbic acid group, tacrine group, and luteolin group. *N. nucifera* root ethanol extract (60 mg/kg), tacrine (10 mg/kg), luteolin (20 mg/kg), or ascorbic acid (20 mg/kg) were administered daily one hour before the starting. Memory impairment was induced by scopolamine (1 mg/kg, i.p) 30 min after luteolin, tacrine, or ascorbic acid treatment. The control group

received 0.9% saline solution only. Each animal was treated with 0.02 mL/g of body weight.

9. Morris water maze

Morris water maze (Harvard Bioscience, Holliston, Massachusetts, USA) is consisted of a circular pool (90 cm in diameter and 45 cm in height) (Fig. 9). The pool is filled to a depth of 25 cm with water (24–25°C) that is made opaque by the addition of nontoxic squid ink. A black platform (10 cm in diameter and 23 cm high) was placed in one of the pool quadrants and submerged 1 cm below the water surface so that it was not visible. The first experimental day was allowed to swimming freely to the escape platform for 60 s. If mouse did not find the platform in 60 s, it was placed on the platform for 10 s. It was removed from the pool for 20 s before being placed at the next starting point in the pool. This procedure was repeated five times. The mouse were given five days of training with five 60 s training trials per day. After that training, the learning and memory abilities of mice in different groups were assessed by Morris water maze test. A Harvard Bioscience video camera was mounted above the center of the pool and all experiments were recorded. A video camera is based Smart 3.0 system. Harvard Bioscience tracking software is sensitive to light reflections from the water surface and these are minimized by indirect lighting.

Fig. 9. Morris water maze.

10. Data analysis

Antioxidant activity was expressed as 50% inhibition concentration (IC_{50}) of the compound that is required to cause a 50% DPPH inhibition. IC_{50} values of the test compounds were calculated using Prism 5 software program (GraphPad Software, La Jolla, CA, USA). The IC_{50} values were considered to be significantly different from one another when their 95% confidence limits did not overlap. The DPPH radical scavenging activity was calculated according to the following fomula: DPPH scavenging activity (%) = $\frac{(A0-A1)}{A0} \times 100$, where A0 is the absorbance of the control and A1 is the absorbance in the presence of the sample. The inhibition was obtained by the following formula: inhibition rate (%) = $[1 - (S - S_0)/(C - C_0)] \times 100$, where C was the absorbance of the control after 30 min of incubation, C_0 was the absorbance of the control at zero time, S was the absorbance of each sample after 30 min of incubation, and S_0 was the absorbance of each samples at zero time. Results were expressed as mean ± standard error (SE) of triplicate samples of three independent experiments. The Bonferroni multiple-comparison method was used to test for significant differences among the treatments (SAS Institute 2004).

RESULTS

1. Isolation and identification of active principles from *Nelumbo nucifera* root

The fractions obtained from the solvent hydrolysable of the ethanol extract of *N. nucifera* root were tested with DPPH assay (Table 2). Significant differences in antioxidant activity in fractions of the extract were observed. The ethyl acetate- and butanol-soluble fractions showed potent antioxidant activity. Therefore, the ethyl acetate- and butanol-soluble fractions were used to identify peak activity fractions for the next step in the purification.

Table 2. DPPH radical scavenging activity of fractions obtained from the solvent hydrolysable of the ethanol extract of the root from *Nelumbo nucifera*

Material	IC_{50}, mg/mL (95% CL[a])	Slope ± SE	χ^{2b}	*P*-value
Ethanol extract	0.805 (0.696–0.929)	1.8 ± 0.29	3.70	0.987
Hexane-soluble fr.	1.287 (1.186–1.396)	0.4 ± 0.22	1.89	0.997
Chloroform-soluble fr.	0.805 (0.670–0.960)	2.1 ± 0.45	3.92	0.983
Ethyl acetate-soluble fr.	0.053 (0.037–0.049)	1.5 ± 0.48	2.60	0.993
Butanol-soluble fr.	0.170 (0.130–0.224)	1.6 ± 0.29	3.25	0.995
Water-soluble fr.	2.644 (2.112–3.309)	2.1± 0.44	3.27	0.988
Ascorbic acid	0.049 (0.036–0.066)	2.0 ± 0.47	4.14	0.984

[a] CL denotes confidence limit.

[b] Pearson χ^2, goodness-of-fit test.

The antioxidant activity of each subfraction derived from the ethyl acetate-soluble fraction is given in Table 3.

Table 3. DPPH radical scavenging activity of each subfraction from ethyl acetate-soluble fraction derived from the root of *Nelumbo nucifera*

No.	Fraction	Antioxidant activity (%)	
		50 μg of sample	100 μg of sample
1	E1	–	0.35
2	E2	28.12	58.46
3	E3	51.20	89.12
4	E4	29.53	66.10
No.	Fraction	100 μg of sample	
5	E31	–	
6	E32	47.07	
7	E33	77.12	
8	E34	89.43	
9	E35	62.27	
10	E341	–	
11	E342	90.37	
12	E343	70.30	
13	E344	37.80	
14	E3421	–	
15	E3422	44.09	
16	E3423	82.74	
17	E3424	90.16	

The DPPH assay-guided fractionation of the ethanol extract from *N. nucifera* root afforded two active principles (compound **1** and **2**). Compound **1** was obtained as a clear liquid and identified by spectroscopic analysis, including EI-MS (Fig. 10), ^{1}H NMR (Fig. 11), and ^{13}C NMR (Fig. 12). EI-MS revealed a molecular ion at *m/z* 154 $[M]^{+}$ and its ^{1}H NMR spectra showed 6 protons. Its ^{13}C NMR spectra showed 7 carbons in the molecule comprising three chain and one carboxyl group, 2 hydroxyl carbons as indicated in DEPT (Fig. 13), suggesting the molecular formula $C_7H_6O_4$. This compound was characterized as 3,4-dihydroxybenzoic acid (**1**) (CAS No. 99-50-3) (Fig. 14). The interpretations of proton and carbon signals were largely consistent with those of Nova et al (2012). 3.4-Dihydroxy benzoic acid (**1**) was identified on the basis of the following evidence: white powder. EI-MS (70 eV), *m/z* (% relative intensity): 154 $[M]^{+}$ (100), 13 (10), 109 (10), 81 (10), 63 (15). ^{1}H NMR and ^{13}C NMR spectral data of 3.4-dihydroxy benzoic acid is given in Table 4

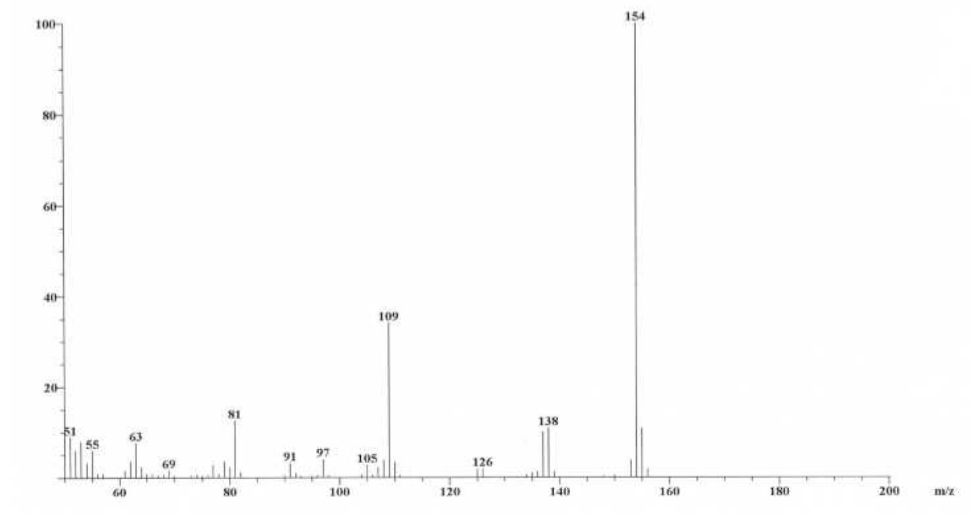


Fig. 10. Mass spectrum of compound 1.

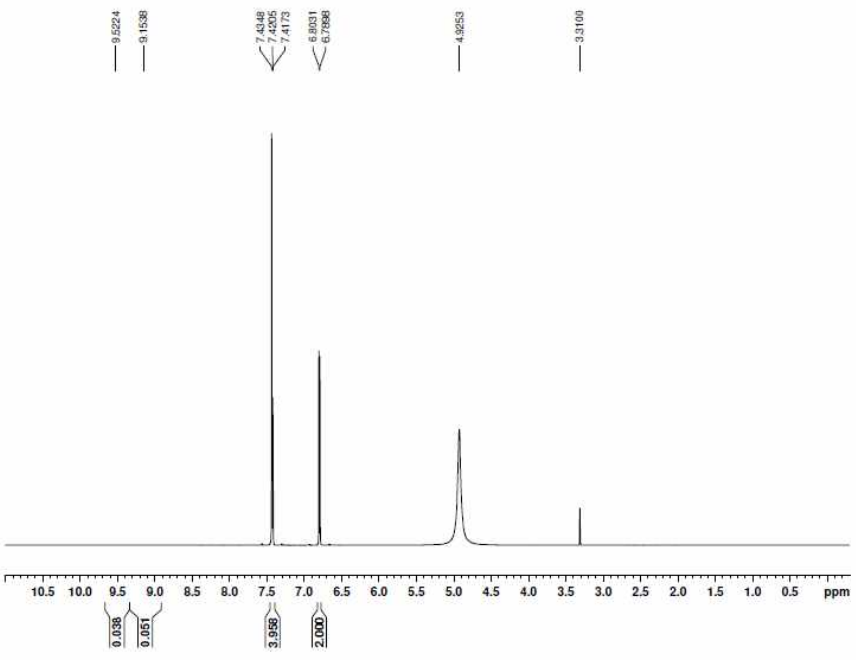


Fig. 11. ^{1}H NMR spectrum of compound 1.

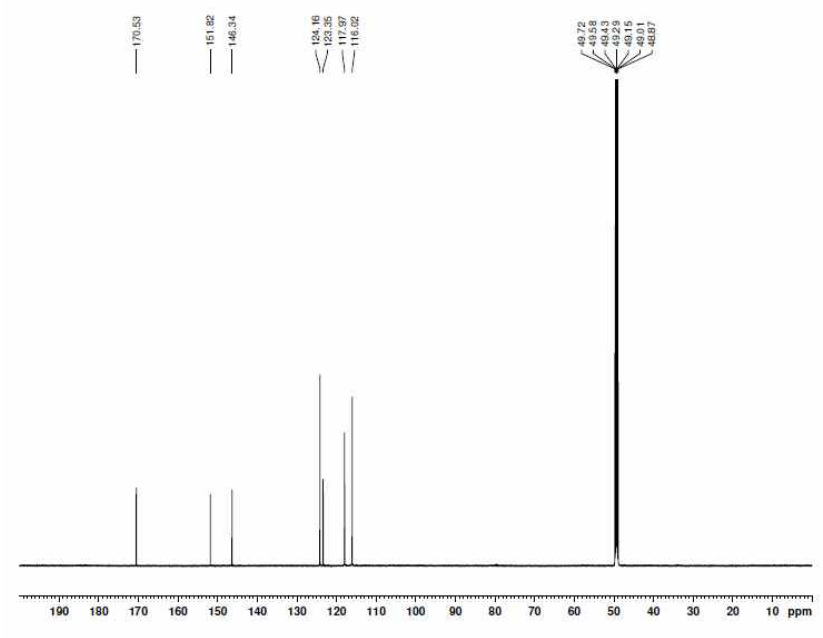


Fig. 12. ^{13}C NMR spectrum of compound 1.

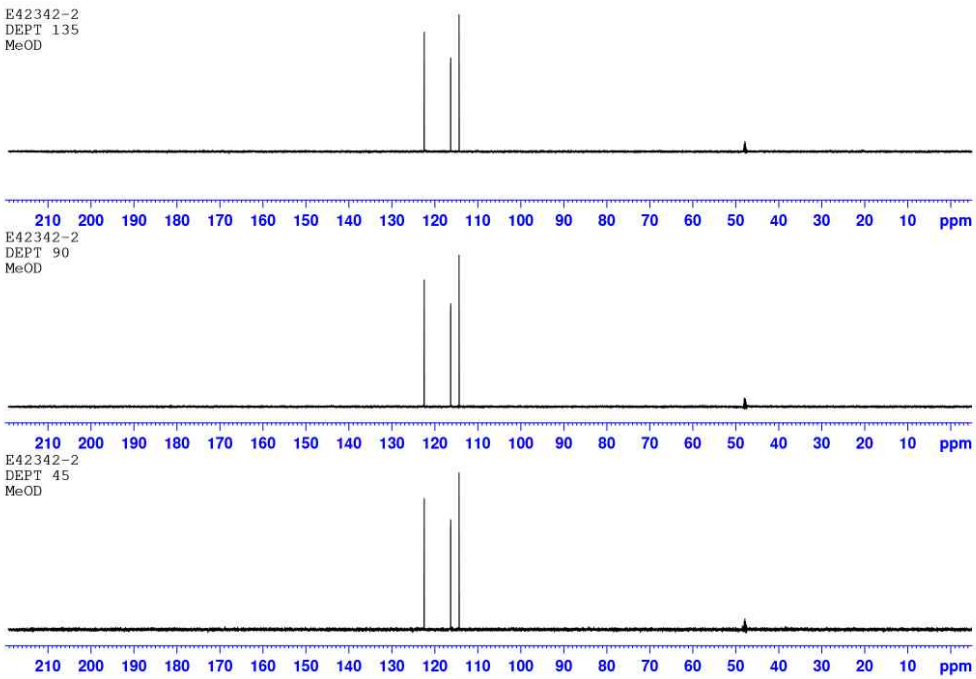


Fig. 13. DEPT spectrum of compound 1.

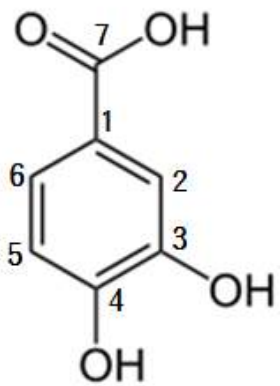


Fig. 14. Structure of 3,4-dihydroxy benzoic acid.

Table 4 . ^{1}H and ^{13}C NMR spectral data for compound 1

Position	Partial Structure	δ_C, ppm (MeOD, 150 MHz)	δ_H, ppm (MeOD, 600 MHz)	δ_C (ppm) Nova et al. (2012)	δ_H (ppm) Nova et al. (2012)
1	C	123.5		123.28	
2	CH	117.97	7.43 d (J = 2.0 Hz)	117.85	7.44 d (J = 2.0 Hz)
3	C	146.34		146.18	
4	C	151.82		151.66	
5	CH	116.02	6.80 d (J = 6.0 Hz)	124.03	6.79 d (J = 6.0 Hz)
6	CH	124.16	7.42 dd (J = 2.0, 6.0 Hz)	115.89	7.42 dd (J = 2.0, 6.0 Hz)
7	C=O	170.53		170.38	
	OH		11.0		11.0
	OH		5.0		5.01
	OH		5.0		5.01

Compound **2** was obtained as yellow powder. EI-MS revealed a molecular ion at m/z 286 $[M]^+$ (Fig. 15) and its ^1H NMR spectra (Fig. 16) showed 9 protons. Its ^{13}C NMR spectra (Fig. 17) showed 2 benzene rings, oxygen-containing ring, a 2-3 carbon double bond, and hydroxyl groups at carbons 5,7,3',4' position. It showed 15 carbons in the molecule comprising one ketone group, 4 hydroxyl carbons as indicated in DEPT (Fig. 18), suggesting the molecular formula $C_{15}H_{10}O6$. This compound was characterized as luteolin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-chromenone) (CAS No. 99-50-3) (Fig. 19). The interpretations of proton and carbon signals were largely consistent with those of Ozgen et al. (2011). Luteolin was identified on the basis of the following evidence: yellow powder. EI-MS (70 eV), *m/z* (% relative intensity): 286 [M+] (100), 258 (15), 153 (20), 129 (9). ^1H NMR and ^{13}C NMR spectral data of luteolin is given in Table 5.

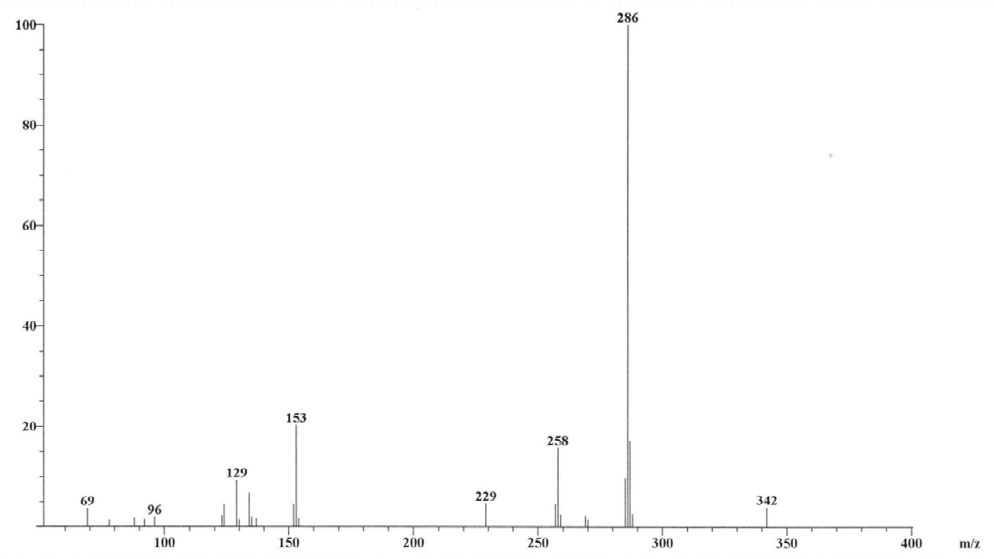


Fig. 15. Mass spectrum of compound 2.

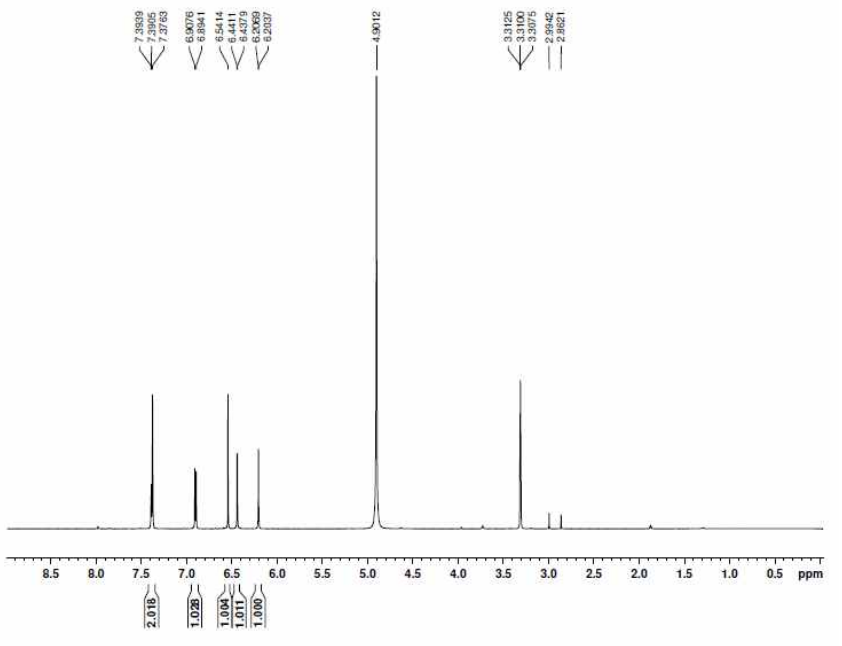


Fig. 16. ^{1}H NMR spectrum of compound

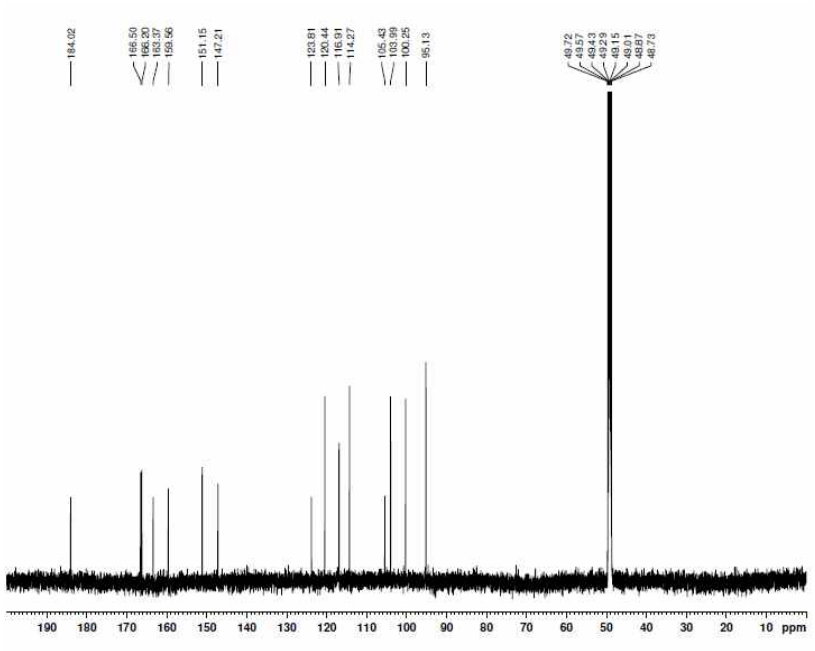


Fig. 17. ^{13}C NMR spectrum of compound 2.

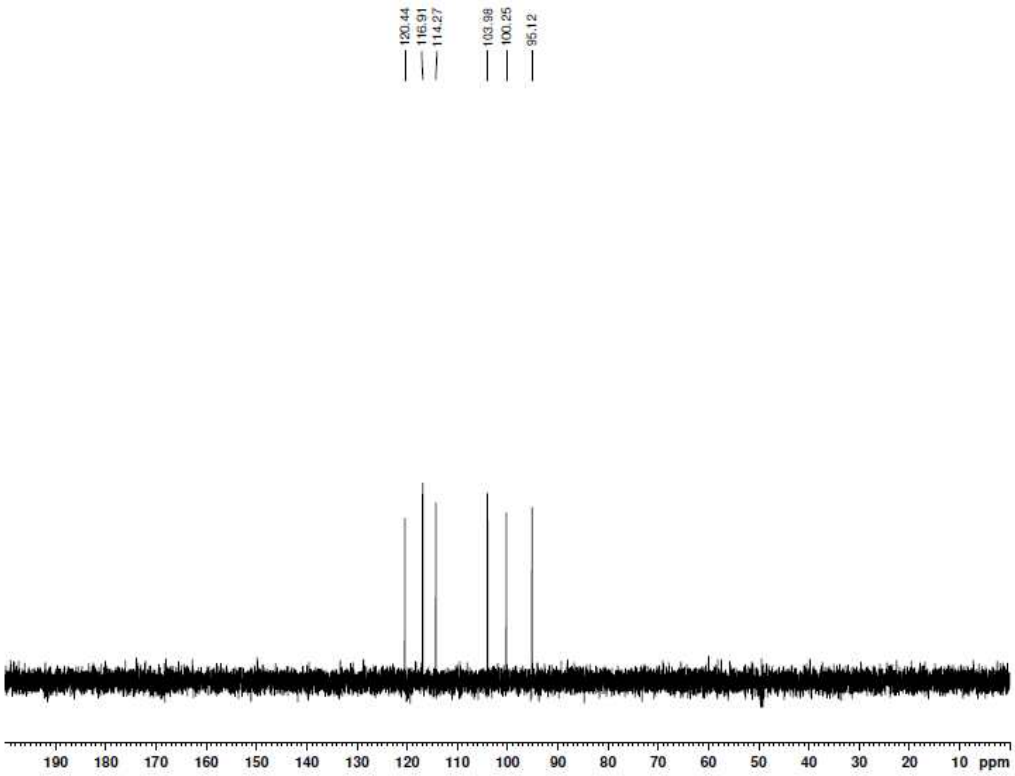


Fig. 18. DEPT spectrum of compound 2.

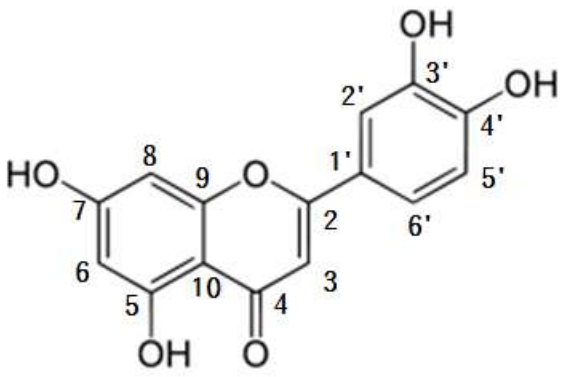


Fig. 19. Structure of luteolin.

Table 5. ^{1}H and ^{13}C NMR spectral data for compound 2

Position	Partial structure	δ_C, ppm (MeOD, 150 MHz)	δ_H, ppm (MeOD, 600 MHz)	δ_C (ppm) Ozgen et al. (2003)	δ_H (ppm) Ozgen et al. (2003)
1’	C	123.81		123.1	
2’	CH	114.27	7.40 d (J = 2.2 Hz)	113.5	7.51 d (J = 2.2 Hz)
3’	C	147.21		145.8	
4’	C	151.15		149.4	
5’	CH	116.91	6.91 d (J = 9.0 Hz)	116.0	7.00 d (J = 8.2 Hz)
6’	CH	120.44	7.39 dd (J = 8.2, 2.2 Hz)	119.5	7.48 dd (J = 8.2, 2.0 Hz)
2	C	166.50		164.2	
3	CH	103.99	6.54 s	103.6	6.59 s
4	C=O	184.02		182.4	
5	C=O	163.37		162.7	
6	CH	100.25	6.21 d (J = 2.0 Hz)	99.0	6.25 d (J = 2.0 Hz)
7	C	166.20		164.5	
8	CH	95.13	6.44 d (J = 2.0 Hz)	94.0	6.52 d (J = 2.0 Hz)
9	C	159.56		158.1	
10	C	105.43		104.7	
	OH		5.01		5.01
	OH		5.01		5.01
	OH		5.01		5.01
	OH		5.01		5.01

2. Isolation and identification of active principles from *Nelumbo nucifera* leaves

The fractions obtained from the solvent hydrolysable of the ethanol extract of *N. nucifera* leaves were tested with DPPH assay (Table 6). Significant differences in antioxidant activity in fractions of the extract were observed. The ethyl acetate-soluble fractions showed potent antioxidant activity. The hexane-, chloroform-, and water-soluble fractions were low active. Therefore, the ethyl acetate-soluble fractions were used to identify peak activate fractions for the next step in the purification.

Table 6. DPPH radical scavenging activity of fractions obtained from the solvent hydrolysable of the ethanol extract of the leaves from *Nelumbo nucifera*

Material	IC_{50}, mg/mL (95% CL[a])	Slope ± SE	χ^{2b}	*P*-value
Ethanol extract	0.943 (0.793–1.274)	1.4 ± 0.20	2.68	0.988
Hexane-soluble fr.	0.513(0.428−0.614)	2.6 ± 0.76	2.35	0.982
Chloroform-soluble fr.	0.653(0.538−0.793)	1.6 ± 0.34	2.56	0.972
Ethyl acetate-soluble fr.	0.071(0.065−0.077)	1.4 ± 0.12	2.01	0.997
Butanol-soluble fr.	0.173(0.153−0.196)	2.1 ± 0.20	2.29	0.997
Water-soluble fr.	1.291(0.766−2.173)	1. 1 ± 0.29	2.94	0.993
Ascorbic acid	0.049 (0.036–0.066)	2.0 ± 0.47	4.14	0.984

[a] CL denotes confidence limit.

[b] Pearson χ^2, goodness-of-fit test.

The antioxidant activities of each subfraction derived from the ethyl acetate-soluble fraction are given in Table 7.

Table 7. DPPH radical scavenging activity of each subfraction from ethyl acetate-soluble fraction derived from the leaves of *Nelumbo nucifera*

No.	Fraction	Antioxidant activity (%)	
		50 μg of sample	100 μg of sample
1	E1	–	–
2	E2	–	2.80
3	E3	2.63	20.56
4	E4	62.74	88.93
5	E5	47.93	86.26
6	E6	46.50	86.00
7	E7	39.50	80.00
No.	Fraction	100 μg of sample	
8	E41	49.22	
9	E42	85.33	
10	E43	30.13	
11	E421	5.06	
12	E422	29.11	
13	E423	81.43	
14	E424	48.80	
15	E425	41.00	
16	E4231	3.60	
17	E4232	87.55	
18	E4233	47.05	
19	E4234	46.53	
20	E4235	70.7	

Compound **3** was obtained as yellow powder. EI-MS revealed a molecular ion at *m/z* 69 $[M]^+$ (Fig. 20) and its ^1H NMR spectra (Fig. 21) showed 9 protons. Its ^{13}C NMR spectra (Fig. 22) showed 21 carbons in the molecule one ketone group, 8 hydroxyl carbons as indicated in DEPT (Fig. 23), suggesting the molecular formula $C_{21}H_{20}O_{12}$. The doublet at δ5.25 (diaxial coupling *J*=7.5Hz) was assigned to the anomeric proton of hexose and suggested a glycosidic β-linkage. This compound was characterized as isoquercetin (2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-3-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxyl-methyl)oxan-2-yl]oxychromen-4-one) (CAS No. 482-35-9) (Fig. 24). The interpretations of proton and carbon signals were largely consistent with those of Monirul et al. (2010). Isoquercetin (**3**) was identified on the basis of the following evidence: yellow powder. EI-MS (70 eV), *m/z* (% relative intensity): 69 $[M]^+$ (100), 55 (30), 57 (42), 60 (40), 71 (27), 73 (21), 81 (62), 83 (23), 95 (26). ^1H NMR and ^{13}C NMR spectral data of isoquercetin is given in Table 8.

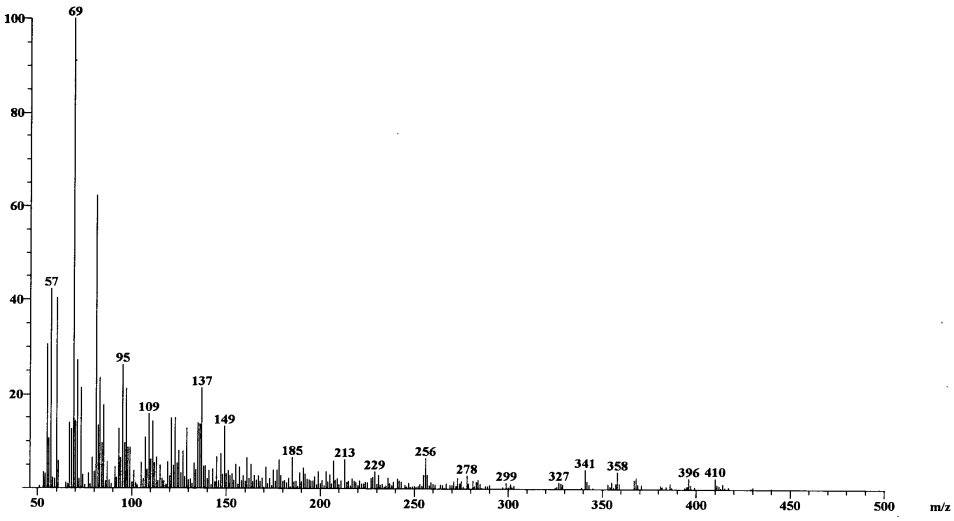


Fig. 20. EI-MS spectrum of compound 3.

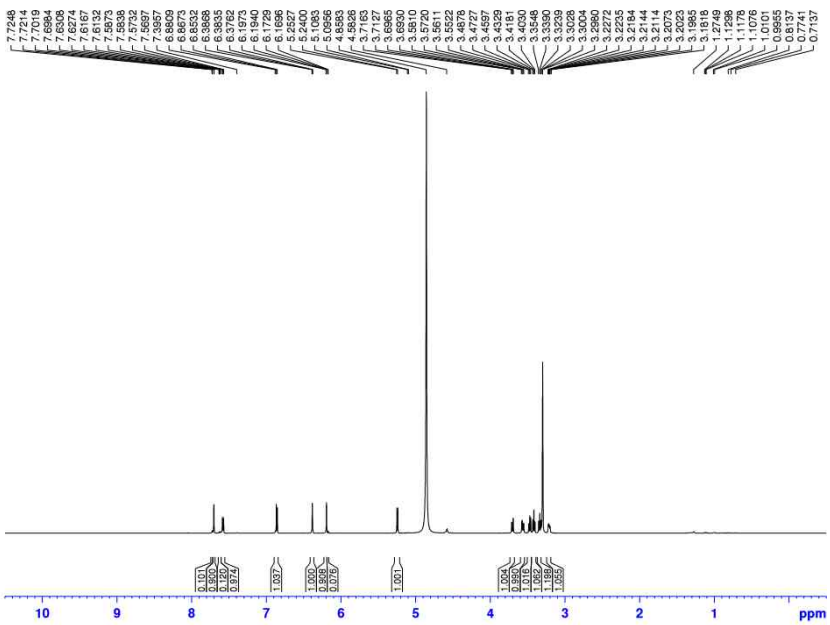


Fig. 21. ^{1}H NMR spectrum of compound 3.

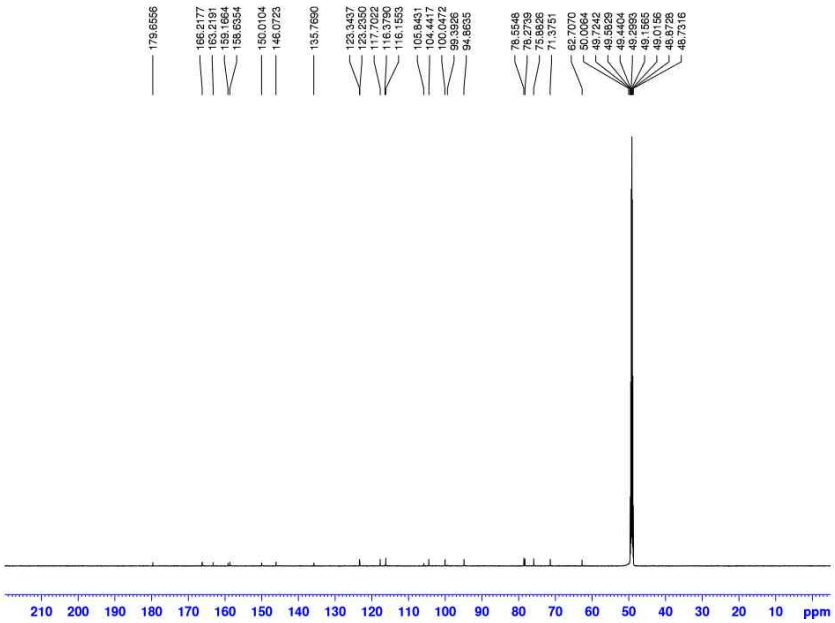


Fig. 22. ^{13}C NMR spectrum of compound 3.

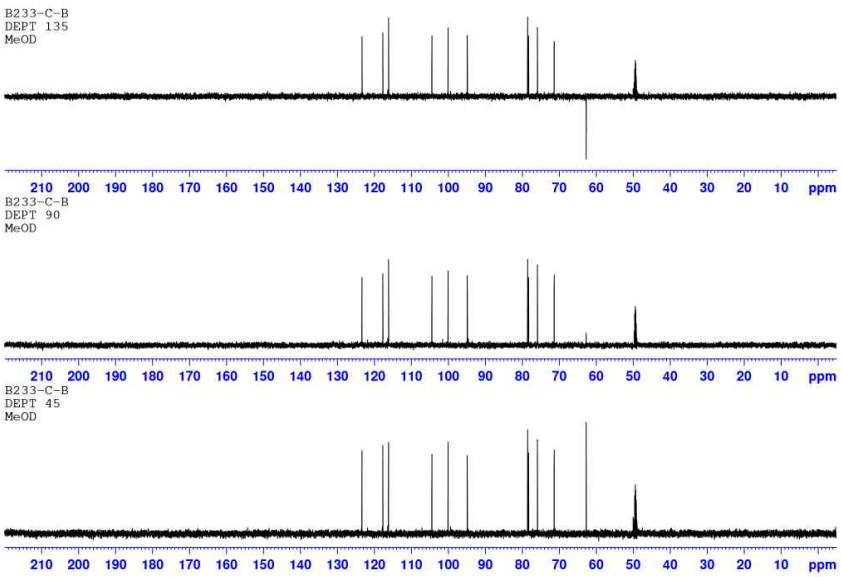


Fig. 23. DEPT spectrum of compound 3.

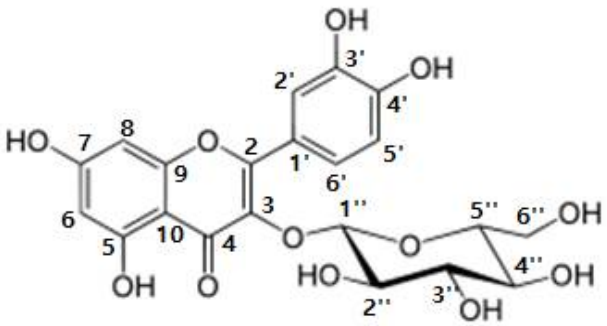


Fig. 24. Sturcture of isoquercetin.

Table 8. ^{1}H and ^{13}C NMR spectral data for compound 3

Position	Partial structure	δ_C, ppm (MeOD, 150 MHz)	δ_H, ppm (MeOD, 600 MHz)	δ_C (ppm)	δ_H (ppm) Monirul et al. (2010)
2	C	158.63		158.44	
3	C	135.76		135.64	
4	C=O	179.65		179.48	
5	C	163.21		162.99	
6	CH	99.39	6.19 d (J = 2.0 Hz)	99.89	6.19 d (J = 2.0 Hz)
7	C	166.21		165.97	
8	CH	94.86	6.38 d (J = 8.0 Hz)	94.73	6.38 d (J =2.0 Hz)
9	C	159.16		158.44	
10	C	105.84		105.68	
1’	C	123.34		123.08	
2’	CH	117.70	7.72 d (J = 2.5 Hz)	117.59	7.71 d (J = 2.0 Hz)
3’	C	146.07		145.87	
4’	C	150.01		149.83	
5’	CH	116.37	6.88 d (J = 8.5 Hz)	116.01	6.87 d (J = 8.4 Hz)
6’	CH	123.23	7.56 dd (J = 8,5, 2.5 Hz)	123.20	7.58 dd (J = 8.5, 2.0 Hz)
1’’	CH	104.44	5.25 d (J = 7.5 Hz)	104.39	5.23 d (J = 7.6 Hz)

Table 8.
(Contiruned)

2''	CH	75.88	3.48 m	75.73	3.48 t (J = 9.2 Hz)
3''	CH	78.27	3.35 m	78.11	3.35 t (J = 8.8 Hz)
4''	CH	71.37	3.45 m	71.22	3.43 t (J = 9.6 Hz)
5''	CH	78.55	3.22 m	78.35	3.24 m
6''	CH_2	62.70	3.71 m, 3.57 m	62.58	3.73 dd (J = 2, 11.6 Hz), 3.56 dd (J =2, 11.6 Hz)
	OH		2.02		2.04
	OH		2.02		2.04
	OH		2.02		2.04
	OH		2.02		2.04
	OH		5.01		5.02
	OH	5.01			5.02
	OH	5.01			5.02
	OH	5.01			5.02

3. DPPH radical scavenging activity of test compounds

The antioxidant activities of three isolated compounds, six related compounds, and antioxidant agent ascorbic acid were evaluated using a DPPH assay (Table 9). Based on IC_{50} values, 3,4-dihydroxy benzoic acid (10.90 μM) was the most antioxidant compound and was ≈5 times more active than ascorbic acid. The antioxidant activity of luteolin and ascorbic acid did not differ significantly. IC_{50} of fisetin, isoquercetin, and quercetin was between 96.10 and 123.47 μM. IC_{50} of kaemfperol was 203.81 μM. Low antioxidant activity was observed with galangin. Acacetin and chrysin were ineffective.

4. AChE inhibitory activity of test compounds

Because AChE is one of the major targets of Alzheimer's disease, the inhibitory activity of 3,4-dihydroxy benzoic acid, luteolin, and isoquercetin was compared with that of tacrine (Table 10). As judged by IC_{50} values, luteolin (9.35 μM) was the most AChE inhibitory compound and was ≈12 times less active than tacrine. IC_{50} of isoquercetin was 89.12 μM. 3,4-Dihydroxy benzoic acid was ineffective.

Table 9. DPPH radical scavenging activity of 3,4-dihydroxy benzoic acid, luteolin, isoquercetin, and antioxidant agent ascorbic acid

Compound	IC_{50}, μM (95% CL[a])	Slope ± SE	χ^{2}[b]	*P*-value	RT[c]
3,4-DBA[d*]	10.90 (8.76–12.40)	1.9 ± 0.48	2.60	0.989	5.34
Luteolin[*]	67.14 (63.89–70.53)	3.1 ± 0.04	3.15	0.982	0.87
Fisetin	96.10 (55.96–165.10)	1.2 ± 0.26	4.06	0.980	0.61
Isoquercetin[*]	123.23 (93.15–153.45)	2.1 ± 0.44	3.27	0.982	0.47
Quercetin	123.47 (103.06–147.96)	1.6 ± 0.21	3.20	0.987	0.47
Kaemfperol	203.81 (162.06–256.25)	1.2 ± 0.23	4.35	0.984	0.29
Galangin	855.90 (681.77–1060.20)	1.1 ± 0.46	3.95	0.985	0.07
Acacetin	>1000				
Chrysin	>1000				
Ascorbic acid	58.19 (46.96–68.53)	2.0 ± 0.47	4.14	0.984	1.00

[a] CL denotes confidence limit.

[b] Pearson χ^{2}, goodness-of-fit test.

[c] Relative toxicity, IC_{50} of ascorbic acid/IC_{50} of test compound.

[d] 3,4-Dihydroxy benzoic acid.

[*] Compound isolated in this study.

Table 10. *In vitro* inhibition of electric eel acetylcholinesterase (AChE) by test compounds and AChE inhibitor tacrine

Compound	IC_{50}, μM (95% CL[a])	Slope ± SE	χ^{2b}	*P*-value
Luteolin	9.35 (6.37–12.27)	2.1 ± 0.54	2.55	0.988
Isoquercetin	89.12(75.26–94.83)	3.0 ± 0.09	3.14	0.988
3,4-DBA[c]	>500			
Tacrine	0.76 (0.52–0.93)	2.3 ± 0.39	2.61	0.990

[a] CL denotes confidence limit.

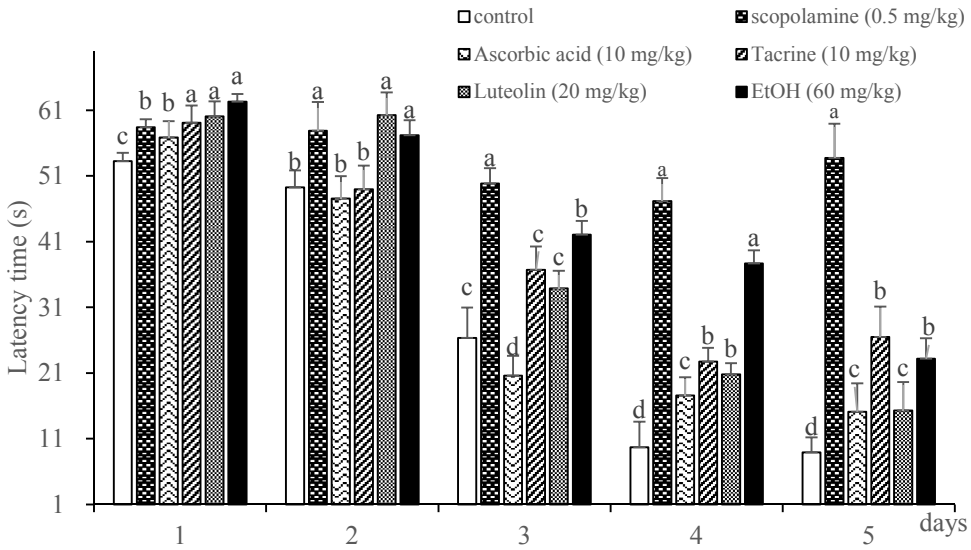
[b] Pearson χ^2, goodness-of-fit test.

[c] 3,4-Dihydroxy benzoic acid.

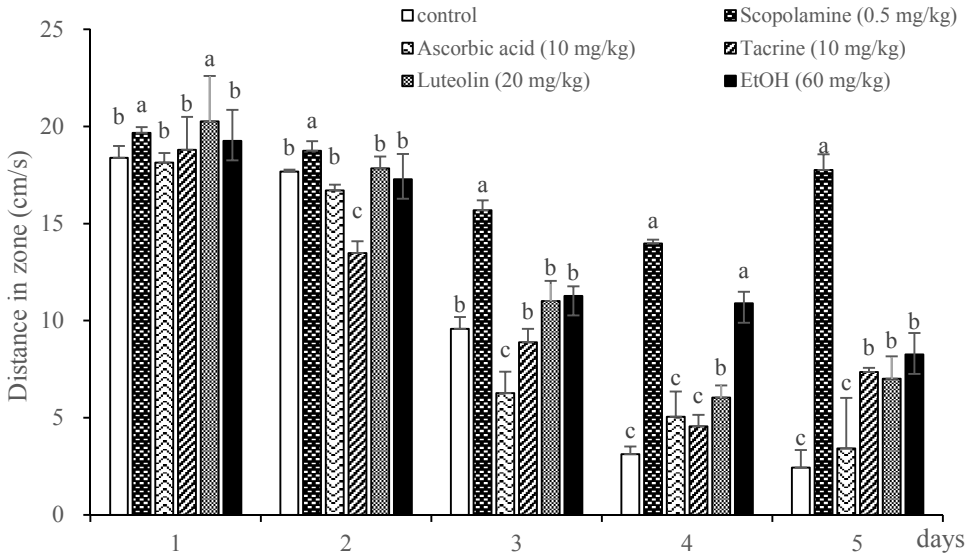
5. Effect of luteolin on learning and memory of scopolamine-induced mouse

The effect of luteolin (20 mg/kg, i.p.) on spatial learning of scopolamine-induced cognitive impairment mouse model was evaluated using the Morris water maze. As shown Fig. 25A, scopolamine-treated group (0.5 mg/kg, i.p.) exhibited longer latency time than saline-treated control group during the 5 days. However, the luteolin (20 mg/kg) or tacrine (10 mg/kg) plus scopolamine-treated groups showed significantly shorter latency time than the scopolamine-treated group during the 5 days. The scopolamine-treated group exhibited longer distance to platform than saline-treated control group during the 5 days (Fig. 25B). However, the luteolin or tacrine plus scopolamine-treated groups showed significantly shorter distance to platform than the scopolamine-treated group during the 5 days. The scopolamine-treated group exhibited slower in speed in zone than saline-treated control group (Fig. 25C). However, luteolin or tacrine plus scopolamine-treated groups showed faster in speed in zone than scopolamine-treated group during the 5 days. Examples of representative swimming paths of mouse in different groups in a probe trial are shown in Fig. 26.

A


control
scopolamine (0.5 mg/kg)
Ascorbic acid (10 mg/kg)
Tacrine (10 mg/kg)
Luteolin (20 mg/kg)
EtOH (60 mg/kg)
Latency time (s)
61
51
41
31
21
11
1
1
2
3
4
5
days

B


control
Scopolamine (0.5 mg/kg)
Ascorbic acid (10 mg/kg)
Tacrine (10 mg/kg)
Luteolin (20 mg/kg)
EtOH (60 mg/kg)
Distance in zone (cm/s)
25
20
15
10
5
0
1
2
3
4
5
days

C

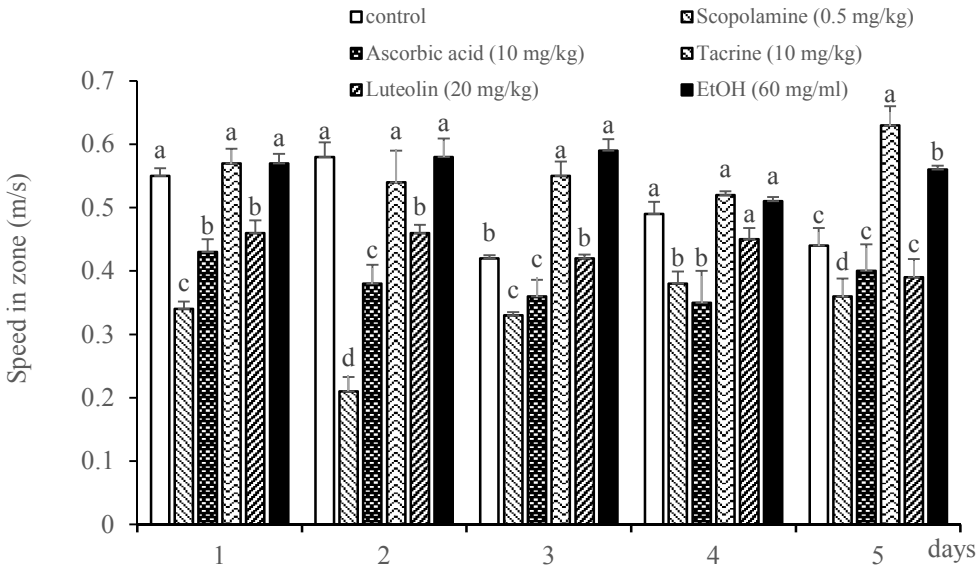


Fig. 25. Latency time and distance to target platform. The effect of *Nelumbo nucifera* ethanol extract and luteolin on learning and memory of scopolamine-induced cognitive impairment mouse model in Morris water maze. To investigate latency time (A), distance (B), and speed in zone (C), ascorbic acid (10 mg/kg, i.p.), tacrine (10 mg/kg, i.p.), luteolin (10 mg/kg, i.p.), *Nelumbo nucifera* ethanol extract (60 mg/kg, i.p.), or the same volume of control (0.9% saline solution) were administered to mice 30 min after a single scopolamine treatment. Significantly different at $P = 0.05$ (Bonferroni method).

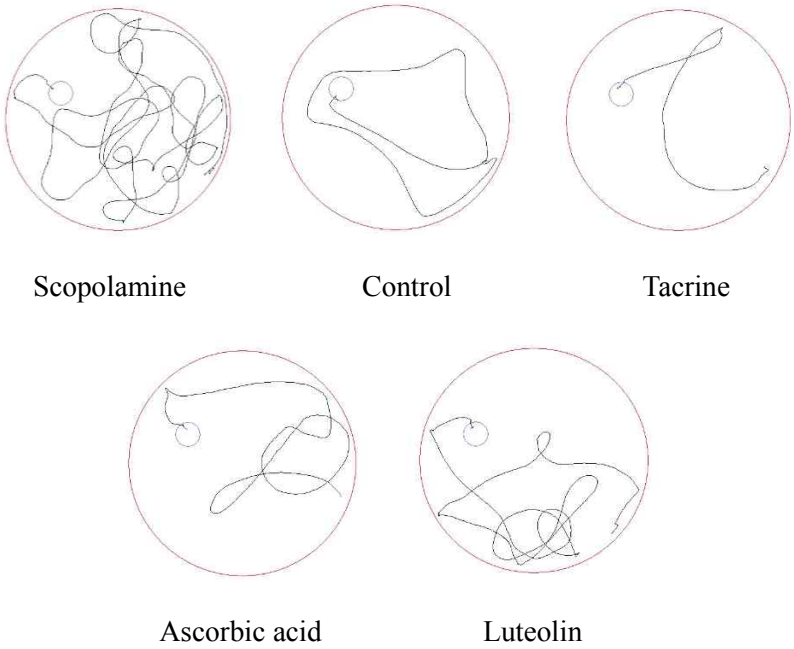


Fig. 26. Representative path tracings of the each group. Ascorbic acid (10 mg/kg, i.p.), tacrine (10 mg/kg, i.p.), luteolin (10 mg/kg, i.p.), *Nelumbo nucifera* ethanol extract (60 mg/kg, i.p.), or the same volume of control (0.9% saline solution) were administered to mice 30 min after a single scopolamine treatment.

DISCUSSION

ROS produced by from mitochondria and other cellular organelle have been traditionally regarded as toxic byproducts of metabolism leading to damage lipids, proteins, and DNA (Freeman, 1982; Thannickal and Fanburg, 2000). This phenomenon has been implicated in a variety of human degenerative phenomena, including aging and diabetes (Srivastava et al., 2005) and inflammation (Azad et al., 2008). Oxidative stress may be broadly defined as an imbalance between oxidant production and the antioxidant capacity of the cell to prevent oxidative injury. Oxidative stress is recognized as a common factor in many neurodegenerative diseases and is a proposed mechanism for age-related degenerative processes as a whole (Lin and Bear, 2006). Numerous studies have provided compelling evidence linking neuronal oxidative stress to Parkinson's disease(PD) (Jenner and Olanow, 1996; Mandel et al., 2003; Tatton et al., 2003) and Alzheimer's disease (AD) (Nunomura et al., 2001) to highlight but a few.

The antioxidants may be natural or synthetic ones. Polyhydroxy flavones, flavanones, flavanols, isoflavones, chalcones, and many members are of these groups of natural substances which proved to have a high degree of antioxidant activity, and they are found to be widely spread in plant material (Rajani, 2004). Natural antioxidants include ascorbic acid, tocopherol, lipoic acid, uric acid, glutathione, and polyphenol metabolites. Synthetic antioxidant such as BHA and BHT have been used. However, the safety of synthetic additives has been questioned stimulating the evaluation of naturally occurring compounds with antioxidant properties. Although there is no assurance of the safety of

natural antioxidants, there is some comfort knowing that such antioxidants are purified from natural products that have been consumed. Antioxidant constituents derived from natural products include that scavenge the free radicals and detoxify the organism. Natural antioxidant has been of great interest for their potential antioxidant effects for the preservation of the foods from the toxic effects of the oxidants (Zygadlo et al., 1995; Maestri et al., 1996; Maestri et al., 1998; Tepe et al., 2004).

N. nucifera root has been used both as vegetables and medicine in Eastern Asia, particularly in China. It is regarded to be one of the many vegetables rich in antioxidant components (Kaur et al., 2002), and many of its functional effects were related to the antioxidants that it contains (Shui et al., 2004). Despite this, *N. nucifera* root has not been explored extensively for its phytochemical attributes. Several researches using different methods have proved that *N. nucifera* exhibited effective antioxidant capacity (Hu and Skibsted, 2002). Pharmacological studies of the plant revealed that the whole plant possesses antidiabetic, antipyretic, antiinflammatory, anticancerous, antimicrobial, antiviral, and antiobesity properties (Kashiwada et al., 2005).

Antioxidant constituents derived from plants include the alkaloids [e.g., jateorrhizine and groenlandicine, IC_{50} 0.78 and 0.84 μM (Jung et al., 2009)], the terpenoids [e.g., vitamin E, IC_{50} 14.12 μM (Yousefbeyk et al., 2014); paeoninol and paeonin C, IC_{50} 147 and 498 μM (Naheed et al., 2004)], the phenylpropanoids [e.g., eugenol, IC_{50} 6.89 μM (Owena et al., 2003)], the flavonoids (e.g., quercetin, IC_{50} 0.83 μM (Azeez et al., 2012); lespedin and tiliroside, IC_{50} 10.19 and 2.19 μM (Oliveira et al., 2012); orientin, IC_{50} 316 μM (Nan et al., 2009); kaempferol, IC_{50} 15.4 μM (Keunha et al., 2011); baicalein,

IC_{50} 3.12 μM (Shieh et al., 2000); pyrogallol, gallic acid and chlorogenic acid, IC_{50} 5.28, 4.33, and 17.28 μM (Biskup et al., 2013)], and the tannins [e.g., epigallocatechin, catechin, and gallocatechin, IC_{50} 8.56, 9.93, and 15.26 μM (Sasak et al., 2007)].

In this study, the ethanol extracts of *N. nucifera* roots and leaves was proved to have antioxidant activity. The antioxidant principles were determined to be the simple benzoic acid 3,4-dihydroxy benzoic acid and the flavone luteolin from the roots and the flavonol isoquercetin from the leaves using DPPH assay. IC_{50} of these constituents was between 10.90 and 123.23 μM, although IC_{50} of the natural compounds stated previously is between 0.78 and 498 μM. 3,4-Dihydroxy benzoic acid was more active than either luteolin or isoquercetin and was more effective than ascorbic acid. This original finding indicates that materials derived from *N. nucifera* can hold promise for the development of novel and effective naturally occurring antioxidant agent. 3,4-Dihydroxy benzoic acid was reported to possess antibacterial (Chao and Yin., 2009), antioxidant (Wang et al., 2011), antidiabetic (Scazzocchio et al., 2011), anticancer (Tanaka et al., 2011), antiulcer (Kore et al., 2011), antiageing (Shi et al., 2006), antifibrotic (Jiang et al., 2012), antiviral (Zhou et al., 2007), antiinflammatory (Lende et al., 2011), antiatherosclerotic (Lende et al., 2011), hyperlipidemic (Borate et al., 2011), hepatoprotective (Liu et al., 2002), nephroprotective (Lee et al., 2009), and neurological (Guan et al., 2006) activities. Luteolin was reported to possess antioxidant (Miguel, 2009), antiinflammatory (Serhan et al., 2008), antimicrobial (Cushnie and Lamb, 2005), and anticancer (Elangovan et al., 1994) activities. Isoquercetin was reported to possess antiviral (Rajani, 2004), antibacterial (Maestri et al., 1998), anticarcinogenic (Tatton et al., 2003) activities.

Oxidative damage is associated with aging and is widespread in the brain in AD (Lovell and Markesbery, 2007). Free radical species mediate damage to proteins, lipids, mitochondria, and DNA and may activate the cell cycle; overwhelm endogenous antioxidant defenses in the brain; and contribute to neuronal damage(Lovell and Markesbery, 2007; Montine et al., 2002). Observational studies suggest that an antioxidant-rich diet may reduce the risk of AD (Morris, 2009). Cholinergic abnormalities, neurofibrillary tangles, and extensive neuronal loss are the major characteristics in AD (Kihara and Shimogama, 2004). Both nicotinic and muscarinic acetylcholine receptors are decreased in AD, and reduction in the number of acetylcholine receptors precedes other pathologic changes (Bymaster et al., 1993). Inhibition of the down-regulation of acetylcholine is a strategy for the treatment of AD (Bymaster et al., 1993). In this study, luteolin extracted from lotus was proved to have most potent AChE inhibition activity, although it was less inhibitory than the widely used AD drug tacrine.

The Morris water maze is commonly used to assess hippocampal-dependent spatial memory in rodents (Morris, 1984). It is also used to assess hippocampal-dependent spatial learning ability (Morris, 1984; Barnes et al., 1996). Furthermore, it has been reported that escape latencies observed on a day-to-day basis reflect long-term memory (Morris, 1984). In this study, scopolamine increased latency time during the training-trial sessions. Luteolin (20 mg/kg) ameliorated scopolamine-induced memory impairment, and recovered latency time to the control group level. In addition, the scopolamine-induced reduction in distance to platform within the platform was significantly ameliorated by luteolin, indicating a positive effect on spatial learning and memory. Collectively, these

behavioral results suggest that lutoelin ameliorates long-term memory in the scopolamine-induced amnesic mouse model.

In conclusion, *N. nucifera*-derived preparations containing 3,4-dihydroxy benzoic acid, luteolin, and isoquercetin could be useful as sources of potential antioxidants or lead molecules for treatment of several diseases in which there is an increase in free radical production. In particular, luteolin possessed antioxidant properties and cognitive enhancing effect in mice. The antioxidant action of these constituents may be an indication of at least one of the pharmacological actions of lotus. For practical use of the preparations as novel antioxidant products to proceed, further research is needed to establish their safety with respect to humans and whether this activity could be exerted *in vivo* after consumption of *N. nucifera*-derived product by humans. Historically, it has been used as a wild vegetable and herbal drug in traditional therapies (Qichao, 2005). 3,4-Dihydroxy benzoic acid and luteolin have no acute oral toxicity on mouse at 800 mg/kg (Shahil et al., 2014) and 180 mg/kg (Peng, 1981), respectively, although toxicity of isoquercetin is not reported. In addition, detailed tests are needed to understand how to improve antioxidant potency and stability for eventual commercial development.

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Scopolamine 처리 생쥐에 대한 연 유래 화합물의 기억력 증진효과

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장 명 진

초 록

다양한 활성산소는 호기성 메커니즘에서 생기는 부산물로, 만약 활성산소의 양이 증가하여 항산화 물질이 처리할 수 있는 범위를 벗어나게 되면 효소, DNA, 단백질, 등이 파괴된다. 이로 인해 심장질병, 알츠하이머, 뇌질환을 초래하게 된다. 항산화 물질은 이러한 산화적 스트레스를 줄임으로써 다양한 질병을 예방할 수 있다.

본 연구에서는 항산화 활성을 가지며 기억력 증진 효과가 있을 것으로 예측되는 물질에 대한 효능검증을 실시하였다. 실험결과는 항산화제로 널리 사용되고 있는 ascorbic acid을 양성 대조군으로 하여 비교하였다. 연근 18 kg에서 324 g의 에탄올 추출물을 얻었으며 층 분리, Open column, MPLC,

HPLC를 통해 2개의 물질을 분리하였다. 분리한 물질 3,4-Dihydroxy benzoic acid과 Luteolin은 항산화 활성(IC50, 10.90μM, 67.14 μM)을 보였다. 또한 연잎 1.72kg에서 51.90g의 에탄올 추출물을 얻었으며 단일물질로 분리하여 동정한 결과 얻은 Isoquercetin도 항산화 활성 (IC50, 123.23 μM)을 보였다. 또한 scopolamine을 주입하여 치매에 걸린 쥐에 EtOH extract과 Luteolin을 주입한 후 물 속 미로 찾기 테스트를 실시한 결과, 양성대조군으로 사용한 Tacrine과 비슷한 기억력 증진효과를 나타내었다.

이상의 결과를 바탕으로 본 논문의 연구는 연에 함유된 활성본체들을 분리 동정 하였고, 활성 물질들의 작용기구를 연구했다는데 그 의의가 있고, 구성 물질에 대한 생물검정을 통해 항산화제 및 기억력증진제로써의 가능성을 탐색하고 연의 새로운 생리활성을 밝혀내어 농업적, 산업적으로 그 활용 가능성이 높다고 판단되며 이에 더하여 추가적 연구가 요구된다.

검색어: 천연항산화제, 노인성치매, 연근, 3,4-dihydroxy benzoic acid, luteolin, isoquercetin

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