The Interaction of Urolithins and its Metabolites with Cholinesterase and the Oxidative Stress Mechanisms

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ABSTRACT

The beneficial effects of ellagitannin rich diet has been known for the treatment of Alzheimer Disease. Regarding the lack of bioavailability of ellagitannins and their in gastrointestinal tract to urolithins, these biological effects are attributed to the bioavailable urolithin derivatives.

Urolithins are benzo[c]chromen-6-one derivatives, with hydroxyl substitutions. Within this study, we have synthesized the major urolithins, their methyl ether metabolites, concomitant to some synthetic analogues. The potential of compounds to inhibit cholinesterases, monoamine oxidase-B, cyclooxygenase enzymes were investigated. Besides, their antioxidant activities were also evaluated in DPPH antioxidant assay system.

The results for the first time described a structure activity relationship in some of the biological activities of urolithins. Furthermore, a mechanistic approach for the action of ellagitannin rich food for the treatment and preventation from Alzheimer Disease was also provided.

Keywords: alzheimer disease, urolithin, benzo[c]chromen-6-one, cholinesterases, cyclooxygenase enzymes, monoamine oxidase-B, DPPH antioxidant, urolithin.

ÖZ

Alzheimer Hastalığı tedavisinde ellagitannin yönünden zengin besinlerin faydası uzun zamandır bilinmektedir. Ellagitanninlerin biyoyararlanımlarının olmaması ve mide-barsak kanalında ürolitinlere dönüşümü sebebiyle ellagitanninlerin biyolojik aktiviteleri ürolitinlerle ilişkilendirilmektedir.

Ürolitinler hidroksil sübstitüe benzao[c]kromen-6-on türevleridir. Bu çalışmada major ürolitinleri, metil eter metabolitleri ile bazı sentezledik analoglarını sentez ettik. Bu bileşiklerin kolinesteraz, MAO-B, siklooksijenaz enzimlerini inhibe etme potansiyellerini araştırdık. Bunun yanında, DPPH antioksidan deney sisteminde antioksidan etkilerini de inceledik.

Sonuçlar, ilk kez ürolitinlerin bazı biyolojik etkilerinin yapı-aktivite ilişkisini açıklamıştır. Ayrıca, ellagitannin yönünden zengin besinlerin alzheimer hastalığı tedavisindeki ve önlenmesindeki etkilerine yönelik mekanistik bir yaklaşım da ortaya konmuştur.

Anahtar Kelimeler: alzheimer hastalığı, ellagitannin, benzao[c]kromen-6-on, kolinesteraz, siklooksijenaz, DPPH antioksidan, MAO-B, urolithin.

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LIST OF SYMBOLS AND ABBREVIATIONS

AChE	Acetylcholinesterase
Ach	Acetylcholine
AD	Alzheimer's disease
ApoE	Apolipoprotein E
APP	Amyloid precursor protein
ASP	Aspartyl protease
Aβ	Amyloid-β
BuChE	Butyrylcholinesterase
C	Concentration
CNS	Central nervous system
COMT	Catechol-O-Methyl Transferase
COX	Cyclooxygenase Enzyme
CSF	Cerebrospinal fluid
DMF	Dimethylformamide
EA	Ellagic acid
ETs	Ellagitannins
FT–IR	Fourier transform infrared spectroscopy
HPLC	High-performance liquid chromatography
IR	Infrared spectrum/ spectroscopy
MAO	Monoamine Oxidase Enzyme
MP	Melting Point
NFT	Neurofibrillary tangle
NMDA	N-Methyl-D-aspartate

NMR	Nuclear Magnetic Resonance Spectroscopy
R_f	Retention factor
TLC	Thin layer Chromatography

Chapter 1

INTRODUCTION

1.1 Alzheimer's Disease

This disease was first diagnosed by Dr. Alois Alzheimer in 1906 and since then, the disease has been so-called Alzheimer's Disease (AD). AD is the most prevalent form of dementia among older people, which causes problems with memory accounts for 60%-80% of cases. In the year 2019, approximately 5,800,000 American population suffered from AD, and the majority of them are over 65 years old (Langa & Burke, 2019). AD is a neurodegenerative disease that is getting worse over time and leads to neuron loss, particularly in the hippocampus and cortex. Memory erosion, decreasing of decision-making capacity and cognitive ability are the clinical symptoms of the disease (Okonkwo et al., 2008). AD is the 6th important cause of death in industrialized countries. It influences all ethnic groups and happens in women slightly more than in men. The rate of death owing to AD has been rising by 55% from 1999 to 2014 and only in 2014, 93500 people died because of this disease in USA. Actually, no drugs have been designed to stop or even stabilize the progression of the AD, but currently, several potential therapies are undergoing both in pre-clinical and clinical studies ("Deaths from Alzheimer's Disease | Features | CDC," n.d.).

1.2 Progression of Alzheimer's Disease

AD is often mistaken with dementia of ordinary aging. Losing memory which is a symptom of AD is not a normal sign of ordinary aging. Healthy aging can result in hair loss, body fat increasing, and height decreasing and losing muscle mass skin,

strength and bone density (Kalyani, Corriere, & Ferrucci, 2014). There may be a decrease in some sense like hearing ability and also vision, and also a reduction in metabolic rate. A very small decline in memory, such as the slowdown in material remembering, is usual, but actually the cognitive decline that has very big effects on daily activities is not really normal in ordinary aging. Dementia is described as a serious decrease in cognitive ability that affects on the social function of people. This inability can be caused by different illnesses that gives damage to brain tissue (Rivera et al., 2005). There are too many types of dementia, with their special causes and symptoms. For instance, a decrease in brain blood supply causes stroke, and as a result, vascular dementia. In patients with Parkinson's disease and hydrocephalus, dementia may also be present. However, AD related dementia is the most popular form of dementia resulting from the formation of the beta-amyloid plaques in brain tissue. AD gradually develops and there are 3 main phases of the disease, and each has its own problems and symptoms. By recognizing each phase of the disease, physicians may predict the upcoming symptoms and possible treatment (McGeer & McGeer, 2002).

1.2.1 The Preclinical and Early-Stage of Alzheimer's Disease

In the first phase, valid up to 4 years, the disease is first diagnosed and relatives may start to realize at this point that the cognitive ability of the patient getting lower. At this point, typical signs include, trouble to memorize new information, memory loss disrupting life, trouble in solving problems or making decisions, difficulty in handling finances and other daily life activities, changes in character, and thought expression difficulties (Mortimer, Ebbitt, Jun, & Finch, 1992).

1.2.2 Moderate Stage of Alzheimer's Disease

In most of the patients, this stage is the longest one and it lasts up to 10 years. Memory difficulty is increased and patients need help for daily activities. Common symptoms

of this stage include significant changes in personality, decrease in the ability of judgment and increase in distraction, the lack of ability to complete complex issues, including many of the daily life activities like financial management, shopping, making plans, and organization (Vellas et al., 2012). Also in this stage, patients suffer from main memory loss, for example, patients cannot remember their personal details of history, and finally, the patient may be eliminated from relationships, and they indeed, need professional healthcare (K. Ito et al., 2010).

1.2.3 Severe and Clinical Stage of Alzheimer's Disease

Cognitive capacity in the last or final stage continuously gets worse and physical ability is severely affected. The period of this stage is around three years. Due to the relatives' inability to look after the patient, the last stage usually needs caregiving at home and also other care facilities (Schneider, Insel, & Weiner, 2011). Common symptoms in the last stage are lack of communication skills, short speaking ability, simple phrases, inability or lack of ability in their physical function, such as feeding, getting dressed, and restroom employments, walking problem, and finally impaired swallowing (Schmidt et al., 2011).

1.3 Changes in the Brain

In AD, two brain deformities happen; neurofibrillary tangles and senile plaques deformity. The neurofibrillary tangles deformity, present in the entorhinal cortex, can be seen in the neuron cytoplasm. The structure of neurotic plaques is spherical and is surrounded by an abnormal amyloid protein. The appearance of plaques is amorphous and lack neurites. In fact, both types of plaques are noticeable in the brain's neocortex tissue. When the amount of plaques and tangles rises, healthy neurons start to work less efficiently(Hebert, Weuve, Scherr, & Evans, 2013). The neurons slowly lose their capacity to transmit and neuron loss leads to general brain tissue shrinkage. The death

of neurons, especially in the hippocampus, limits the capacity of the patient to create new memories (Zhang et al., 2010).

1.4 Death from Alzheimer's Disease

Deaths from Alzheimer's disease have grown dramatically since 1991 related to increase in median age of people worldwide (Isaac, Gilboa-Fried, Salmerón Ríos, & Strutt, n.d.). Generally, the main cause of death is not the brain changes, caused by AD. AD frequently makes troubles like immobility and difficulty in swallowing. These troubles can trigger malnourishment and also enhanced pneumonia risk, even leading to death in these people. Beside these factors, there is also a risk in the development of infection, regarding the decrease in the immunological status of the patient (Williams & Murray, 2018).

1.5 Risk Factors

1.5.1 Age

Age is accepted as the biggest factor for the growing risk of Alzheimer's disease. Most instances of AD occur in older adults, 65 years of age, or elderly. Approximately 5% of individuals develop AD between the ages of 65 and 74. The risk rises to 50% for people over 85 years old (Gaugler, James, Johnson, Scholz, & Weuve, 2016).

1.5.2 Genetics

There is a relationship between AD and (ApoE) gene. This gene is very important for instructing a protein called apolipoprotein E. Apolipoprotein E is very important for cholesterol packaging and carrying in the systemic circulation. This gene has different forms and a linkage has been found between formation of senile plaques and apoE4 gene. It has been shown that ApoE increases the possibility of accumulation of amyloid beta protein and disease development (Minati, Edginton, Grazia Bruzzone, & Giaccone, 2009). 10% of AD cases are familial and linked to genetics. In this case, the

disease appears before age 65 and it is caused by a genetic mutation. This mutation happens on the chromosomes (Mayeux & Stern, 2006).

1.5.3 Education

There is a relation between the risk of AD development and the level of education. The risk is more for people with fewer education years (Buchman et al., 2012). The exact reason is unknown but the creation of further synaptic connections between neurons in the brain due to many years of studying might be critical and that may lead to forming "synaptic reserves" in the brain, and these synaptic reserves may be available to compensate the loss (Stern, 2012).

1.5.4 Health Problems

Cerebrovascular diseases, a history of type-2 diabetes, sleep disorders, hypertension, alcohol, smoking, depression, obesity, traumatic brain injury, and higher content of blood cholesterol may boost the risk of AD (Broce et al., 2019). The high blood pressure and blood cholesterol damage brain blood vessels and as a result, low blood flow can lead to neurodegeneration (Gutierrez et al., 2019).

1.6 The Targets Offered for the Treatment of Alzheimer's Disease

Although, the exact reason and mechanism of the AD is not perfectly understood, AD is described by general atrophy of the brain because of major loss of synaptic neurons (Rabeh, Benzarti, & Amiri, 2018). The most important theories and potential causes considered to be involved in degeneration of the brain as cause of AD are discussed in the following subtitles.

1.6.1 Cholinergic Hypothesis

The cholinergic hypothesis of AD indicates that in basal forebrain the cholinergic system is destructed and leads to reduction in cholinergic neurons that result in decreased acetylcholine (Ach) neurotransmitter activity (Craig, Hong, & McDonald,

2011). ACh is hydrolyzed by cholinesterase enzymes (i.e. Acetylcholinesterase and Butyrylcholinesterase). The amount of ACh in central nervous system (CNS) is reduced in AD patients and cholinesterase inhibitors such as rivastigmine, donepezil, and tacrine, etc. increase the amount of available ACh in CNS (Fotiou, Kaltsatou, Tsiptsios, & Nakou, 2015). This theory is based on the involvement of the cholinergic system for the maintenance of cognitive abilities. Indeed, through the action of acetylcholine on varies muscarinic and nicotinic receptors, cognitive skills are retained (Contestabile, 2011).

1.6.2 Amyloid-β Theory

Without considering Amyloid-β plaques (Aβ plaques), it's impossible to explain AD causes, and it's the most important and accepted hypothesis for AD. β-amyloid precursor protein commonly called AβPP, leads to the formation of amyloid plaques. In a normal brain, an enzyme called α-secretase, acts on amyloid precursor protein (APP), cleaves it into secreted APP-α, also called sAPP-α, a 83 amino acid polypeptide (Nasica-Labouze et al., 2015). Alternatively, in AD, an enzyme called β-secretase acts on APP and cleave it into APP-β, also called sAPP-β, a 99 amino acid polypeptide. In normal signaling, a gamma-secretase enzyme also has function to act on amyloid precursor protein. It's also important to note that APP-α gets secreted from the neurons and drives normal synaptic signaling leading to synaptic plasticity, learning, memory, neuronal survival, and emotional behaviors (Castellani, Plascencia-Villa, & Perry, 2019). In AD, gamma-secretase complex is assembled, β 40-42 peptides. The Aβ 40-42 peptides, however, are involved in several downstream pathways related to AD. Aβ 40-42 initially interacts with ApoE which results an aggregation of Aβ oligomers to generate amyloid plaque. These can be detected via immunohistochemistry staining using Aβ 42 specific antibodies. In the downstream, Aβ 40-42 also interacts with

mGluR5 (Metabotropic Glutamate Receptor 5), NMDAR (NMDA receptors), and α-7 NACHR (α-7 Nicotinic Acetylcholine Receptor), and leads to reactive oxygen species and oxidative damage (Fodero-Tavoletti et al., 2011). Overall, the outcomes are in blocked ion channels, disrupted calcium ion homeostasis, dysregulated energy and glucose metabolism, mitochondrial oxidative stress, and neuronal apoptosis which ultimately results in dementia or memory loss. β-secretase inhibitor and γ-secretase inhibitor compounds are designed for lowering the production of Aβ-plaques (Arispe, Pollard, & Rojas, 1993).

1.6.3 Tau Theory and Alzheimer's Disease

The other causal theory of AD is called 'tau hypothesis'. Neurofibrillary tangles (NFTs) are insoluble fibers that can be aggregated. These tangles are made of hyperphosphorylated tau protein and present within the cells of the brain in AD. This hypothesis indicates that tau hyperphosphorylation may result in neurodegeneration. Tau protein is found plentifully in CNS and especially in neurons (Wischik, Harrington, & Storey, 2014). This protein interacts with tubulin to increase flexibility and also stabilize microtubules. Tau protein employs different grafting and phosphorylation isoforms for its special functions (Rafii & Aisen, 2015). In a healthy body, tau is phosphorylated and de-phosphorylated, but in AD, abnormal phosphorylation leads to hyperphosphorylation of tau (HP-tau), and hyperphosphorylated tau disassembles microtubule and finally leads to neurodegeneration via synaptic dysfunction. Furthermore when HP-tau dissociates from microtubules, dissociated protein aggregates in the brain cell and makes paired helical filaments (PHF) and eventually can lead to the death of neurons (Šimić et al., 2016).

1.6.4 Inflammation

It has been known that AD involves neuroinflammation. In fact, an excessive immune system activity destroys neurons. Microglia cells are part of CNS immune system. When the microglia detects any infection caused by toxic proteins such as beta-amyloid in CNS, it releases cytokines to activate other microglia. In the normal brain, after clearing the brain from the toxic protein, microglia move back to the "resting" state (Y. J. Lee, Han, Nam, Oh, & Hong, 2010). Researchers suggest that in AD, overactiviton of microglia and increasing in the production of cytokines could cause neuroinflammation. Most researches in the 1990s suggested a decrease in AD symptoms after using non-steroidal anti-inflammatory medicines (NSAIDs). Several inflammatory markers have been found in AD (Christensen, Beach, Serrano, & Kanaan, 2019). Many studies indicate that inflammatory marker levels in AD raise up and, COX enzymes are also involved in inflammatory responses (Shivanand, n.d.). These enzymes convert arachidonic acid to prostaglandins and they are related to inflammation. Through increasing prostaglandin E2 production, the production of Aβ peptide increases. Prostaglandin E2 raises γ-secretase expressed Aβ and generate Aβ 40-42. This progress is inhibited by COX inhibitor drugs ("2010 Alzheimer's disease facts and figures," 2010).

1.6.5 Oxidative Stress and Mitochondrial Dysfunction

Mitochondria utilizes oxygen to produce energy, and any fault in the system may cause serious problems, so that mitochondria is one of the main sources of oxidative stress (Chrysostomou, Rezania, Trounce, & Crowston, 2013). ROS (reactive oxygen species) directly oxidize and damage proteins, lipids, and DNA, which lead to nerve cell damage and neuronal loss. As the body gets older, oxidative stress markers rise, and also the ability of the molecules to clean up and treatment of these problems often

deteriorate. This situation causes more oxidative stress (X. Wang et al., 2014). This procedure also leads to inflammation. The balance of oxidative stress is connected to AD, so some physicians suggest anti-oxidant therapies. For example, using vitamin E is suggested for improvement in patients for late stages of AD. Treatments including methods like using coenzyme Q10, manganese superoxide dismutase mimetics, creatine, and mitochondrial uncoupling agents to increase the efficiency of mitochondrial function are alternatives ("Handbook of Mitochondrial Dysfunction - Google Books," n.d.).

1.6.6 Metal Imbalance

Scientific studies indicate that accumulation of excess metals, for instance aluminum, in the brain (from food and environment) may trigger the development of AD (G. Liu et al., 2006). Also studies on animal samples suggested that copper metal existing in drinking water and a diet with high level of cholesterol initiated amyloid β-peptide accumulation. Furthermore exposure to lead during growth can raise Aβ peptide level in CNS later in aging. In healthy body, metals are essential for redox reactions and also they are part of some enzymes (Budimir, 2011). Especially metals are very important for the brain cell function. Neurodegeneration and oxidative stress can occur due to an imbalance in metals. Metal chelators are good choice to disrupt senile plaques and other amyloid sediment, but they can be toxic and therefore a treatment method on them is limited. In both animal and human studies, using clioquinol, the metal chelator, (as a drug to treat malaria) is partially found successful (Iwatsubo et al., 2018).

1.7 Diagnosis

1.7.1 Diagnostic Criteria

Dementia patient is commonly referred to the general physician with memory problem. However, without specialist advice just 50% diseases are diagnosed right. For those cases with signs of mild cognitive impairment, patient should visit memory specialist (Dubois et al., 2016). Within such specialized examination, when controlled longitudinally, the detection of dementia from AD will rise to 95% accuracy. A detailed examination should be performed, involving history from the suspected AD, history from family members, cognitive and mental health condition assessment, physical examination, analysis of taking drugs to avoid cognitive decline side effects (Morris et al., 2014). Cognitive disorder testing usually includes a test called, Mini Mental State Examination (MMSE). This examination tests focuses on attention ability, orientation, awareness, short term memory ability, long term memory strength, learning quality, speech ability, and executive function state. Moreover, there are alternative methods to detect AD, such as the General Practitioner assessment of Cognition (GPCOG), 6-item Cognitive Impairment Test (6-CIT), and the 7-minute screen. To rule out any other types of dementia, baseline tests must be also taken out. According to the history of the patient, it may be necessary to do routine biochemistry tests. After these tests and considering the results, the patients may be classified as AD category or unlikely AD category (Reitz & Mayeux, 2014).

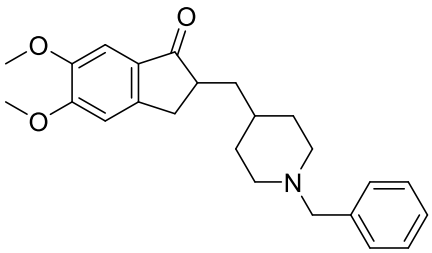
1.8 Current Drugs Used in the Treatment of Alzheimer's Disease.

Actually there is no complete treatment for AD, but limited number of drugs has been shown to cure symptoms. These drugs are also categorized as cognitive enhancer. Cognitive disorder medication includes changing the brain's impact on chemical messengers. Two kinds of therapy have been authorized for this aim by FDA,

cholinesterase inhibition and NMDA receptor antagonism (Sivaraman et al., n.d.). Cholinesterase inhibitors inhibit cholinesterase enzymes and result in more available acetylcholine amount in CNS ("US10233222B2 - Methods, compositions, diagnostics and assays for the treatment of Alzheimer's disease - Google Patents," n.d.). Acetylcholine, a neurotransmitter in CNS, is essential for remembering and learning the skills. The aging process in people makes a small reduction in the level of acetylcholine, resulting in forgetfulness (Hampel et al., 2018). But in AD, the concentration of acetylcholine can be reduced by 90%, leading to impaired cortical cholinergic function. These drugs raise the level of acetylcholine to promote the connection between neurons. Three acetylcholinesterase inhibitor drugs are prescribed frequently; donepezil hydrochloride, galantamine hydrobromide, and rivastigmine. Memantine is a N-methyl-D-aspartate receptor antagonist ("US10004703B2 - Treatment of alzheimer's disease using compounds that reduce the activity of non-selective CA++ activated ATP-sensitive cation channels regulated by SUR1 channels - Google Patents," n.d.).

1.8.1 Donepezil Hydrochloride

Donepezil is one of the commonly prescribed cholinesterase inhibitors. Donepezil reversibly and non-competitively inhibit central active acetylcholinesterase, the enzyme responsible for the hydrolysis or the degradation of acetylcholine. This results in an increased concentration of acetylcholine available for synaptic transmission within the CNS. Pharmacological half-life of donepezil is very long so that single dose in a day is adequate (Arai, Hashimoto, Sumitomo, Takase, & Ishii, 2018). It is employed to treat very severe AD cases and it is effective at slowing memory decrease ("US20190247320A1 - Methods for treating alzheimer's disease with donepezil transdermal system - Google Patents," n.d.).

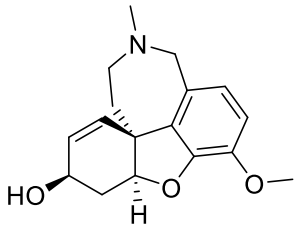


2-((1-benzylpiperidin-4-yl)methyl)-5,6-dimethoxy-2,3-dihydro-1H-inden-1-one

Figure 1: Structure of Donepezil and its IUPAC name

1.8.2 Galantamine Hydrobromide

Galantamine is another type of cholinesterase inhibitor, used for mild to moderate stages of AD. It prevents the hydrolysis of ACh, so as result can increase acetylcholine levels in CNS. The half-life for galantamine is shorter than donepezil. (Saito et al., 2019). The effect of galantamine on AD symptoms at 90 days and 180 days are investigated and it was found a considerable recovery at both period. But after 180 days the treatment was more efficient. Another study found that galantamine can slow down the decline of symptoms with adverse effects occurring in a small number of voluntary patient (Davis, 2016).



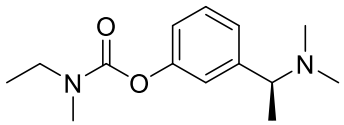
(4a*S*,6*R*,8a*S*)-3-methoxy-11-methyl-4a,5,9,10,11,12-hexahydro-6*H*-benzo[2,3]benzofuro[4,3-*cd*]azepin-6-ol hydrobromide

Figure 2: Structure of Galantamine and its IUPAC name

1.8.3 Rivastigmine

Another prescribed cholinesterase inhibitor is Rivastigmine. It is used to treat the symptoms of mild to moderate AD (Y. Lee, Seol, Park, Lee, & Lee, 2019). It's

important to note here that rivastigmine is the only butyrylcholinesterase selective inhibitor. It is known that BuChE amount becomes higher in CNS throughout the development of AD (Gulcan et al., 2014a).

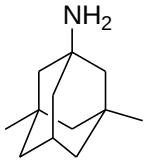


(*S*)-3-(1-(dimethylamino)ethyl)phenyl ethyl(methyl)carbamate

Figure 3: Structure of Rivastigmine and its IUPAC name

1.8.4 Memantine

Memantine is employed for the treatment of patient with moderate to severe symptoms of AD. Memantine acts on regulation of N-methyl-D-aspartate receptors, so it can alleviate AD symptoms. NMDA receptors belong to the family of ionotropic glutamate receptors, which mediate most of the excitatory synaptic transmission in the brain. They are thought to play a role in learning and memory (Xia, Chen, Zhang, & Lipton, 2010). Research studies suggest that beta-amyloid protein, which accumulate in the brain of Alzheimer patients may cause abnormal rise in extra-synaptic glutamate level by inhibiting glutamate uptake or triggering glutamate release from glial cells. The binding of glutamate to NMDA receptors results in an influx of extracellular calcium, which control membrane excitability and transmission. So when the glutamate level become abnormally elevated, overstimulation of NMDA receptors, can result leading to excessive influx of calcium, ultimately causing cell to rapture and loss (McShane et al., 2019). To address this potential problem, scientists developed NMDA receptor antagonists, Memantine, which works through blocking NMDA receptors and thus limiting calcium influx into the neuron (Kanasty et al., 2019).



3,5-dimethyladamantan-1-amine

Figure 4: Structure of Memantine and its IUPAC name

1.8.5 Behavioral and Psychological Therapy

Alzheimer's patients often have behavioral and psychological symptoms and behavioral therapy may be more efficient in treating signs and symptoms. Neuropsychiatric symptoms are stress, insomnia, anger, and illusion. Possible forms of therapy include non-drug treatments and medicinal treatment to relieve the current symptoms (Carrion, Folkvord, Anastasiadou, & Aymerich, 2018). One of the ways to minimize obstacle is changing the environment and increase safety. Also based on the signs and symptoms, several drugs can be used. For instance, antidepressants might be alternate if the patient is suffering from depression. Anti-psychotics and anxiolytics can be used to reduce anxiety (Corbett, Smith, Creese, & Ballard, 2012).

1.9 The Economic Effect of Alzheimer's Disease

Each year approximately 172 billion $ has been spent to deal with AD problems. Patients who are suffering from AD use a huge number of services for medical care, nursing home, and long term care. From this point of view, researcher studies on the discovery of novel mechanisms and drugs for the treatment of AD have been founded by developed countries (Getsios, Blume, Ishak, MacLaine, & Hernández, 2012).

Chapter 2

HYPOTHESIS AND OBJECTIVE OF THE STUDY

As a progressive neurodegenerative disease, AD has a very complex pathology. As the main outcome of the disease, the development of dementia has been evaluated as the key part to be treated. This is rightful in one way considering the worsening of dementia symptoms ending up with total character loss and the requirement for 24h medical care. Therefore, it is logical to observe cholinesterase inhibitors reaching to the market for the treatment of AD within the last quarter of 20^{th} century. Indeed, following the failure of tacrine, several cholinesterase inhibitors have reached to the market, and they are still employed for the mild to moderate stages of AD.

The last 30 years have witnessed to those research studies focusing on the mechanisms of the pathophysiology of AD. The sophisticated CNS monitoring techniques clearly demonstrated the excessive beta-amyloid plaque formations concomitant to aggregated hyperphosphorylated tau-proteins in AD patients. This resulted in a huge effort to identify mechanisms responsible for protein aggregation in CNS. Beta,-Gamma-secretase inhibitors concomitant to some specific kinase inhibitors have been tried in clinical assays (Macleod, Hillert, Cameron, & Baillie, 2015). Even, vaccines against beta-amyloid have been generated (Lambracht-Washington & Rosenberg, 2013). Although these studies demonstrated that some of those compounds were found to be potentially active in terms of prevention of protein aggregation, these studies

were lacking to display any improvement in cognitional status of patients (Roberts, 2014).

Beside these, alternative systems have also been assessed. One of them is the neurodegenerative characteristics of the disease. The systems that might have key function in neuronal cell loss have been questioned. The high capacity for oxidation reactions was investigated. Antioxidants activity on the prevention of the progress of disease is still a topic under scientific research studies.

Moreover, it is known that it is not just the cholinergic system to be getting inactive in time with the development of the disease, but other systems are also affected (Gülcan & Orhan, 2020). Indeed, serotonin and dopamine levels were found to be lower as well. Beside, majority of the AD patients develop depression in time. This is partially relevant to the stress that patients suffer with the decrease in cognitive abilities, however, lower dopamine and serotonin levels can also induce this state. Furthermore, MAO catalyzed reactions involve the biological degradation of dopamine as well. In one way, MAO inhibitors can be evaluated as enhancers of these catecholamine neurotransmitters (Bortolato, Chen, & Shih, 2008). On the other hand, MAO catalyzed reactions generate aldehyde and hydrogen peroxide. Particularly for catecholamine biotransformation, aldehydes can be evaluated as electrophiles. Besides, these compounds also tend to form quinone and semiquinone metabolites, open to Michael additions to yield out protein-, lipid-, or DNA-adducts. Considering these, MAO inhibitors, nowadays, are also assessed as neuroprotective compounds both in prevention of aldehyde metabolites as well as hydrogen peroxide, an important source of oxygen radicals (Bolasco, Carradori, & Fioravanti, 2010).

There have been quite accumulated data on the neuroprotective and cognitive enhancer effects of ellagitannin rich dietaries. This is particularly involving pomegranate. However, the possible mechanisms for these studies are quite limited and generally a single substance dependent. As known, ellagitannins are converted to urolithin compounds (i.e., hydroxylated benzo[c]chromen-6-ones) in gastrointestinal tract after exposure (González-Barrio, Edwards, & Crozier, 2011). Since urolithins are the bioavailable components of ellagitannin rich diet, the possible positive effects of ellagitannin exposure for the treatment of AD can be attributed to the physiological activities of urolithins (H. Liu et al., 2018).

Starting from this point, this study first aimed to synthesize main urolithin derivatives (i.e., Urolithin A and B, as the 3,8-dihydroxyl substituted, and 3-hydroxyl substituted urolithins, respectively) concomitant to their methyl ether analogues, since these compounds can generate methyl ether metabolites through the function of catechol-O-methyl transferases (Ludwig et al., 2015). Beside, some other synthetic urolithin analogues were also aimed to be synthesized.

Following structure identification studies, the enzyme inhibitory characteristics of the title molecules were also questioned. From this perspective, their potential to inhibit cholinesterases, MAO-B, and cyclooxygenase enzymes were aimed to be determined. Activities on cyclooxygenase enzymes are also found to be critical to be evaluated, since the functions of these enzymes are important to produce prostaglandins, as important inflammatory agents. Indeed, there is also accumulated data on the preventive role of cyclooxygenase inhibitors in the development of AD (Broussard, Mytar, Li, & Klapstein, 2012).

Beside, regarding the increasing number of phenolic hydroxyl groups, the screenings of the antioxidant activity of the compounds were also found to be critical to be investigated. In fact, in studies Urolithin A and B were shown to be antioxidant compounds (Qiu et al., 2013).

Regarding the limited information within this concept, this study therefore, first aimed to point out possible mechanism of activity of these urolithins in the treatment of AD. Additionally, since the study designed to cover many natural and synthetic urolithin analogues, a first time structure-activity relationship study was taken under consideration.

Chapter 3

LITERATURE REVIEW

3.1 Acetylcholine (ACh)

Acetylcholine is the first neurotransmitter discovered, and it is the acetate ester of choline.

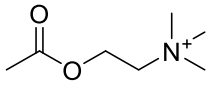


Figure 5: Acetylcholine structure

There are several clusters of cholinergic neurons throughout the brain, stimulated by acetylcholine. Acetylcholine acts on two types of receptors, nicotinic and muscarinic receptors and each receptor family has several subtypes. Nicotine has high binding affinity for nicotinic receptors; therefore, the name comes from this alkaloid. Nicotinic receptor activation generally results in the excitation of neurons. Nicotinic receptors present in the neuromuscular junctions called the motor endplate (Ferraro, Molinari, & Berghella, n.d.). The response of nicotinic receptors can stimulate cognitive and memory processes, although in muscles it trigger contraction. The second type of cholinergic receptors is muscarinic receptors. Muscarine, an alkaloid, is able to selectively bind to these receptors (Rudolf, Khan, Labeit, & Deschenes, 2014). Muscarinic receptors are especially related to cardiac and smooth muscles and also to the peripheral nervous system to stimulate the parasympathetic nervous system. They play a role in the compromise of the eyes for near vision, decrease in heart rate,

decreasing in blood pressure, and contraction of bronchial smooth muscles. Also muscarinic receptors have important function in cognition (Witzemann, Chevessier, Pacifici, & Yampolsky, 2013).

3.2 Cholinesterase Enzyme

The action of acetylcholine in the synapse is terminated by the enzymes called cholinesterase, which break acetylcholine down into acetate and choline. The choline is then transported back into neurons to synthesize acetylcholine if needed. There are two cholinesterase enzymes: Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE). Under normal condition, AChE is mainly responsible in CNS to hydrolyze ACh. BuChE, also referred to a pseudo-cholinesterase, is abundant in peripheral. However, studies indicate the increase in the expression of BuChE in CNS throughout the development of AD (Fadaeinasab, Hadi, Kia, Basiri, & Murugaiyah, 2013).

3.2.1 Acetylcholinesterase

Acetylcholinesterase (AChE) or acetylhydrolase enzyme is a primary protein to breakdown or hydrolysis of acetylcholine. The hydrolysis of ACh happens in its active site. “Esteratic site” or catalytic triad, the “oxyanion hole”, the “anionic subsite” and the “acyl pocket” are the parts of the grove ending up with the active site (Turkan, Cetin, Taslimi, & Gulçin, 2018). The catalytic triad has Ser200, His440, and Glu327 amino acids. The hydrogen bond between Histidine and Serine boosts the nucleophilicity of Serine. Glutamate has a stabilization role for histidinium cation of the intermediate. Like other hydrolases, acetylcholine esterase contains an “oxyanion hole” (OH) with Gly118 Gly119 and Ala201 (Jemec, Drobne, Tišler, & Sepčić, 2010). The hydrophobic “anionic subsite” is involved in three aromatic peptides, Trp84, Phe330, and Glu199, which bind to quaternary amine. The positive charge on the nitrogen group in ACh has an interaction with the electron-rich aromatic rings. The

"acyl pocket" is built from Phe288, and Phe290 residue, and it controls the dimension of substrate to be able to enter to the active site (Francis, Palmer, Snape, & Wilcock, 1999).

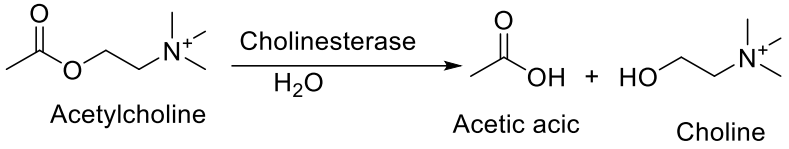


Figure 6: Acetylcholine hydrolysis reaction

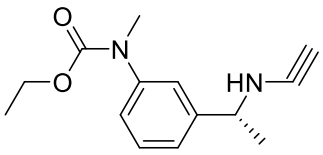
3.2.2 Butyrylcholinesterase Enzyme

The active sites of both enzymes are located at the bottom of a cavity and act as nucleophiles to attack the carbonyl groups of substrates or pseudo-substrate inhibitors (Bajda et al., 2013). The studies on animals have shown that BuChE can control the transmission of the cholinergic signals and take on the role of AChE. During AD, BuChE in different brain areas was found significantly increased. Additionally, BuChE inhibition may raise ACh levels in the brain. These findings indicate that in the treatment of AD symptoms, targeting BuChE may consider as a therapeutic strategy (Ibrahim et al., 2013).

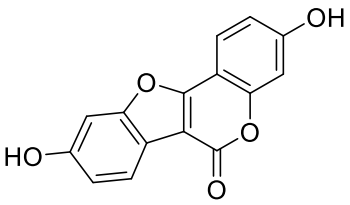
3.2.3 Cholinesterase Inhibitors

Cholinesterase inhibitors are type of drugs which bind to cholinesterase and prevent hydrolysis and inactivation of acetylcholine. Cholinesterase inhibitors increase both the availability and duration of actions of acetylcholine in both central and peripheral nervous system ("Enzymes of the Cholinesterase Family - Google Books," n.d.). This group of drugs is widely used clinically for treatment of neurogenerative diseases such as AD, Lewy body dementia, Parkinson disease, myasthenia gravis, and at the end of surgeries to counteract as muscle relaxants. Cholinesterase inhibitors can act either

reversible and irreversible (Rehman, Khan, Khan, & Roohullah, 2013). For therapeutic purposes, reversible cholinesterase inhibitors are used, and irreversible inhibitors are generally utilized in pesticides. Inhibitors can have two different mechanism of actions, some inhibitors can act on catalytic active site, some of them on the peripheral anionic site, and some of them acts as dual inhibitors (Monte-Millán et al., 2006).



ethyl (*R*)-(3-(1-(ethynylamino)ethyl)phenyl)(methyl)carbamate



3,9-dihydroxy-6*H*-benzofuro[3,2-*c*]chromen-6-one

Figure 7: Two examples of cholinesterase inhibitor molecules (L. Huang, Shi, He, & Li, 2010)

3.2.4 Alzheimer's Disease and Cholinesterase Inhibitors

In people with AD, cholinesterase inhibitor drugs increase communication between the nerve cells, due to increase in the level of acetylcholine, and relieve the symptoms of AD (Deardorff, Feen, & Grossberg, 2015). The improved functions through these drugs are: function in daily activities, memory, and ability to think clearly, behavioral and psychological symptoms. Utilizing cholinesterase inhibitor drugs is just one of the different available pharmaceutical treatment to relief the symptoms of AD (Anand & Singh, 2013).

3.3 Monoamine Oxidase Enzymes

Monoamine oxidase enzymes (MAO) are mitochondrial enzymes involved in the oxidative deamination of catecholamines. MAO exists in two subtypes (i.e. A and B) which are differently distributed in tissues, such as the brain, gut, and liver (Uehara, Uno, & Yamazaki, 2020). MAO-A exists inside the catecholaminergic neurons and preferentially metabolizes serotonin but will also metabolize norepinephrine and dopamine, while MAO-B exists in glia cells and preferentially metabolizes dopamine. (C. C. Wang, Billett, Borchert, Kuhn, & Ufer, 2013).

MAO enzymes are used as target for the treatment of various disease states. In particular, MAO-A inhibitors are employed for the treatment of depression and MAO-B inhibitors are preferred as alternative treatment to Parkinson Disease. This is related to their substrate specificities. Indeed, as a complex disease state, depression develops through the reduction of available important neurotransmitters, particularly serotonin (L. Huang et al., 2012).

Since serotonin metabolism involves MAO-A employment, MAO-A inhibitors are used in clinic for the treatment of depression. Through the development of Parkinson Disease, there is difference in the activity of dopamine receptors (Finberg & Rabey, 2016). From this point of view, MAO-B inhibitors are used for an alternative treatment of Parkinson Disease, since MAO-B is highly responsible in CNS metabolism of dopamine (Meiser, Weindl, & Hiller, 2013). It is important to note that there are selective MAO-A and MAO-B inhibitors, however their selectivity is dose dependent which means at high concentrations, these inhibitors in general, are able to inhibit both enzymes (Carradori, D'Ascenzio, Chimenti, Secci, & Bolasco, 2014).

3.3.1 Monoamine Oxidase-B as a Target for the Treatment of Alzheimer Disease

MAO-B is an enzyme that is released from the mitochondrial outer membrane that catalyzes the oxidative deamination of amines, particularly the neurotransmitter dopamine (Chajkowski-Scarry & Rimoldi, 2014). Dopamine is a neurotransmitter involved in several pathways such as motivation, reward, attention, and energy. MAO-B is a dimer of two identical polypeptide chains each consisting of 520 amino acids. Approximately the last 30 amino acids in each subunit form an alpha-helix is responsible for the immigration into the mitochondrial outer layer membrane. A typical MAO-B catalyzed dopamine metabolism is shown below (Ramsay, 2016):

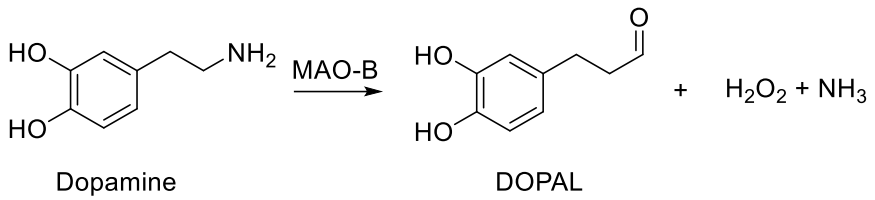


Figure 8: A typical MAO-B catalyzed dopamine metabolism

As seen in figure 8, dopamine metabolism yields out an aldehyde metabolite, hydrogen peroxide, and ammonia. The aldehyde metabolite, DOPAL, is further subjected to metabolism reactions either to form carboxylic acid or alcohol derivatives. None of these metabolites are active on dopaminergic receptors. Beside these metabolic pathways, there are studies indicating the quinone and semiquinone metabolite formations from dopamine metabolite (Cavalieri & Rogan, 2011).

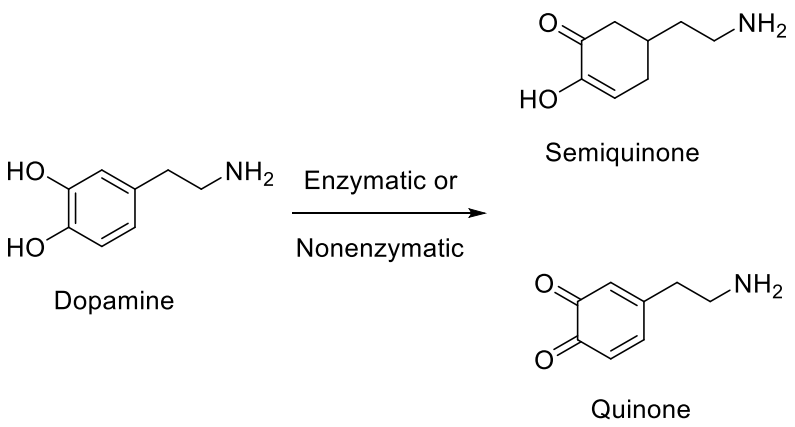


Figure 9: Quinone and semiquinone metabolite formations from dopamine

These metabolites are potential electrophiles, and they readily react with nucleophilic sites of cellular macromolecules (e.g. DNA, proteins) to results in adducts. On the other hand, H_2O_2 is also a source of oxygen radicals. Through Fenton-like reactions H_2O_2 can generate oxygen radicals available to oxidize DNA, lipid, and protein, components. Therefore, excessive MAO-B catalyzed reactions act as one of the pathways in neurodegeneration. For the last couple decades, MAO-B inhibitors are also assessed in research studies as neuroprotective compounds. Below is shown some representative MAO inhibitor structures.

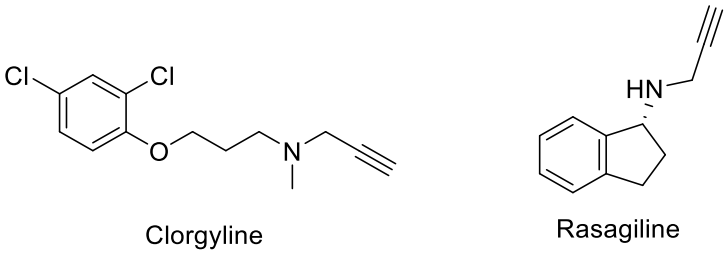


Figure 10: Drug molecules developed as MAO-B inhibitors

3.4 Cyclooxygenase Enzymes

There are two COX enzymes (i.e. COX-1 and COX-2). The first one COX-1 isoform is expressed constantly in body and it's primarily responsible for the production of thromboxane and prostaglandins which stimulate normal body function such as secretion of protective gastric mucus, regulation of gastric acid, promotion of platelet aggregation, and maintenance of renal blood flow ("Cyclooxygenase - an overview | ScienceDirect Topics," n.d.). On the other hand, COX-2 isoform is not expressed constantly in most tissue but instead, it is induced at sites of inflammation so, unlike COX-1, COX-2 driven prostaglandins, mediate mainly inflammation, pain, and fever. COX-1 and COX-2 are bi-functional enzymes that are responsible for catalyzing the formation of prostaglandins. In the active site of cyclooxygenase enzyme, arachidonic acid is converted to prostaglandins. They constitute a large family of inflammatory intermediates (Fitzpatrick, 2005).

3.4.1 Cyclooxygenase Enzyme Inhibitors

COX inhibitors reduce the production of prostaglandins, as a result they produce anti-inflammatory, antipyretic, and analgesic effects. Based on the selectivity for COX enzymes inhibitors, they can be divided into 3 categories; selective COX-1 inhibitors, non-selective COX inhibitors, and selective COX-2 inhibitors (Bunimov & Laneuville, 2008). Below is shown some representative COX inhibitor structures:

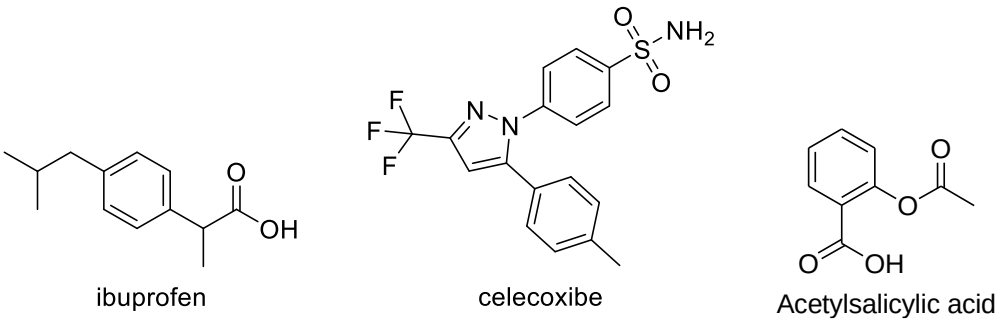


Figure 11: Drug molecules developed as COX inhibitors (Aspirin as COX-1 selective, celecoxibe as COX-2selective, and ibuprofen as non-selective COX inhibitor representative)

3.4.2 Alzheimer's Disease and Cyclooxygenase Enzymes

Non-steroidal anti-inflammatory drugs inhibit COX-1 and COX-2; reduce the risk of developing AD in normal ageing people (Fitzpatrick, 2005). In AD patients, the changes in the level of COX-1 and COX-2 enzymes clearly are related to the progress stage of the illness. COX-1 is found in the central nervous system in the frontal and temporal parts of the cortex. The amount of COX-1 is increased in AD and the formation of Aβ plaque is completely related to this raised level (Listì et al., 2010). Throughout the early phases of AD, the level of COX-2 in neuron cells is raised, and in the advanced stages of AD the COX-2-positive neurons are greatly reduced because of selective degradation of COX-2-expressing cells (Tietz, Marshall, Wuest, Wang, & Wuest, n.d.).

3.5 Oxidative Stress and Alzheimer's Disease

Oxidative stress is involved in cognitive impairment and AD. One of the emerging causative factors associated with AD pathology is oxidative stress (W. J. Huang, Zhang, & Chen, 2016a). This AD-related increasing oxidative stress has been attributed to a decrease in the levels of the brain antioxidant glutathione. The body uses anti-oxidants to limit the damage by oxidative stress, and glutathione is the body's

most effective self-made anti-oxidant. Glutathione is the part of the body's natural defense against free radicals damages (Chen & Zhong, 2014).

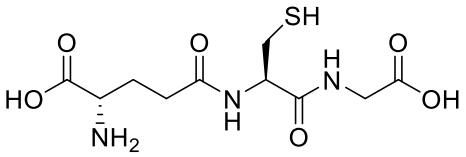


Figure 12: Structure of glutathione

Throughout the development of AD, there have been lots of mechanisms shown to be involved in the increase of oxidative stress. As it is shown previously for dopamine metabolism, the excess oxygen radical generation through various sources is one of them. On the other hand, beta amyloid plaque formation concomitant to tau protein fiber aggregations have also been shown to be component of oxidative stress. From this perspective, antioxidant molecule employment has been suggested to both preventation from and treatment of AD (Albrecht, Bogdanovic, Ghetti, Winblad, & LeBlanc, 2009). Various antioxidant molecules, including vitamin C and E, and herbal molecules have been shown to be neuroprotective with respect to their properties to balance excessive oxidative stress in many CNS diseases including AD. Majority of the data employs either in vivo or ex vivo experimental systems. Now, it is well-accepted that these compounds are able to act as neuroprotectors, however, none of them has been shown to display potential to act as cognitive enhancer (W. J. Huang, Zhang, & Chen, 2016b).

3.6 Urolithins

Urolithins are hydroxylated benzo[c]chromen analogues. These compounds are formed through the metabolism of ellagitannins (H. Ito, 2011). Ellagitannins are bioactive macromolecules, abundantly available in dietaries, such as nuts, some fruits

like berries, and pomegranate. Not just specific to human kind, it has been shown that ellagitannins are hydrolyzed and release ellagic acid within the gastrointestinal tract. The gastrointestinal tract micro flora transform ellagic acid into smaller molecules called urolithins, through several reactions such as reduction, decarboxylation, dihydroxylation including sequential reduction to different compounds involving, tetra-hydroxyl (or urolithin D), tri-hydroxyl (or urolithin C), di-hydroxyl (or urolithin A and isourolithin A), and mono-hydroxyl dibenzopyranones (or urolithin B) (Yuzugulen et al., 2019). Urolithin compounds are the bioavailable micro flora human metabolites of ellagitannins and they can be absorbed from the GI tract (Yuan et al., 2016). Urolithin A (i.e., 3,8-dihydroxy-6H-benzo[c]chromen-6-one) and urolithin B (i.e., 3-hydroxy-6H-benzo[c]chromen-6-one) are the major metabolites found in plasma, so they are considered as biomarkers of ellagitannins.

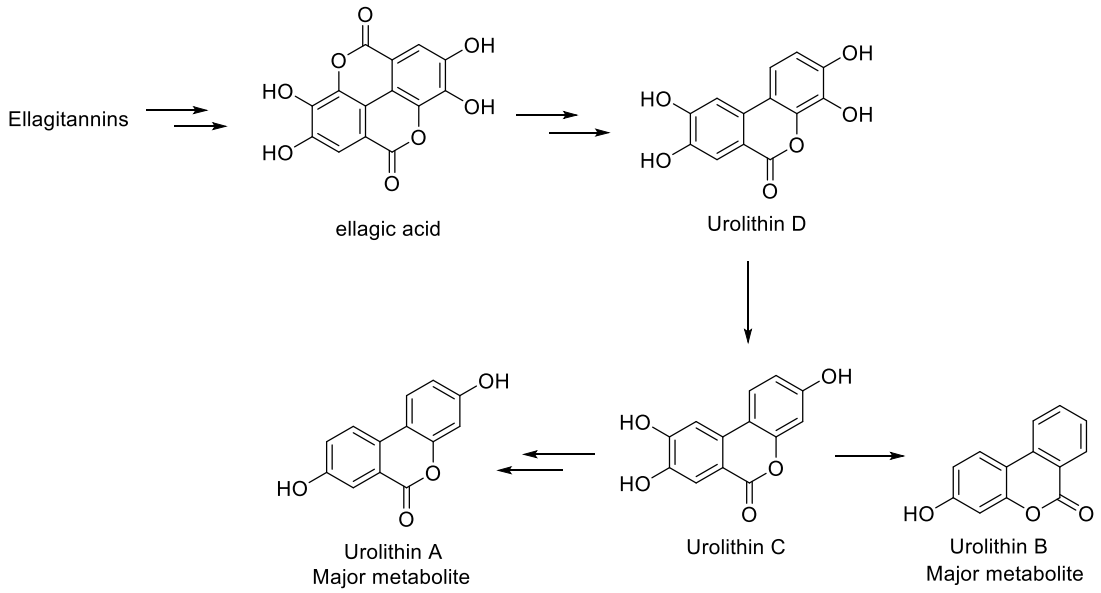
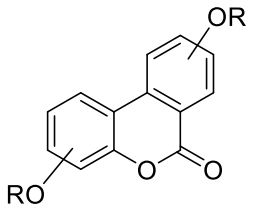


Figure 13: The formation of major urolithins through gastrointestinal tract metabolism reactions

Urolithins are subject to phase II conjugation reaction to form glucuronide, and sulfate metabolite (Piwowarski, Stanisławska, Granica, Stefanska, & Kiss, 2017).

Furthermore the reactions catalyzed by catechol-O-methyl transferase (COMT) are also possible since, methyl ether metabolites are also detected (NOSHADİ, ERCETİN, MAVİDENİZ, & GÜLCAN, 2020). The routine exposure to these dietaries has been reported to have several biological benefits including antioxidant, antiinflammatory, anticancer, and antimicrobial activities (Jing et al., 2019). These activities have been attributed to urolithins, since they are the bioavailable metabolites.



R: Sulfate, glucuronide, and methyl ether conjugates

Figure 14: The phase II conjugates of urolithins

3.6.1 Antioxidant Activity

It is pretty well known that natural phenols are able to display antioxidant activity, and this activity particularly increases with the number of phenolic hydroxyl groups (Shahidi & Ambigaipalan, 2015). On urolithins, studies indicated that urolithin C and D are particularly display more antioxidant activity in comparison to major urolithins A and B, as well as parent ellagic acid molecule in DPPH radical scavenging assays (Dobroslawa, Kasimsetty, Khan, & Daneel, 2009). Moreover, in ORAC assays (oxygen radical antioxidant capacity assay), urolithins have also been found to be active (Kallio et al., 2013). In another study, urolithin A and B were found to increase superoxide dismutase expression (Cásedas, Les, López, Choya-Foces, & Hugo, 2020). This is particularly important considering that superoxide dismutase has function in the metabolism of superoxide radical, one of oxygen radicals.

3.6.2 Anti-Inflammatory Activity

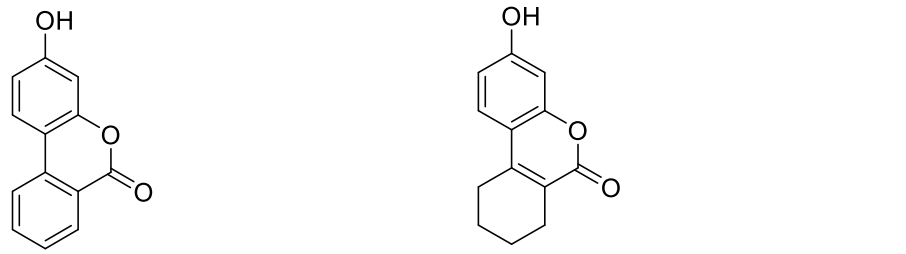
There have been many studies displaying the anti-inflammatory activity of extracts of ellagitannin containing dietary sources. Particularly, it has been shown that pomegranate itself has anti-inflammatory effect within the gastrointestinal system (Colombo, Sangiovanni, & Dell'Agli, 2013). In a study on pure urolithin A, it was displayed that this compound reduce the paw edema in rats in 1h after oral administration (Ishimoto et al., 2011). In another study, ellagic acid, urolithin A, and B were found to possess anti-inflammatory activity following interleukin 1B stimulation. It was observed that ellagic acid was not active, while urolithin A and B displayed anti-inflammatory activity through activating Nuclear factor kappa B and inhibiting PGE_2 production via down regulation of COX-2 (González-Sarrías, Larrosa, Toms-Barberán, Dolara, & Espín, 2010).

3.6.3 Anti-Cancer Activity

In recent years, there is more focus on anticancer activity of urolithins. This is also related to findings with the anticancer activity of pomegranate extract. Urolithin A, B, C, and D were found to be inhibitor of cell proliferation through acting on cells at S and G_2/M phases (González-Sarrías et al., 2017). Furthermore, urolithin A was able to display potentiation of 5- Fluorouracil effect (González-Sarrías, Tomé-Carneiro, Bellesia, Tomás-Barberán, & Espín, 2015). Urolithin A itself were determined to be an inhibitor of metastasis in human sw620 colorectal cancer cells (Zhao et al., 2018). Moreover, urolithin A and C inhibited the proliferation of LNCaP prostate cancer cells, as shown in another study (Stolarczyk et al., 2013). In a very recent work, the effect of urolithin B on the suppression of hepatocellular carcinoma was also pointed out (Li, Li, Chen, & Zhou, 2020).

3.6.4 Cholinesterase Inhibitory Activity

There are limited studies so far made on the investigation of potential of urolithin to inhibit cholinesterase enzymes. In 2014, Gulcan et al. described urolithin B and tetra hydro urolithin analogue (3-Hydroxy-7,8,9,10-tetrahydro-6H-benzo[c]chromen-6-one) having potential to inhibit cholinesterase enzymes. Beside that there has been no known study investigated the potential of urolithins to inhibit cholinesterase enzymes (Figure 15) (Gulcan et al., 2014b).



Urolithin B 3-Hydroxy-7,8,9,10-tetrahydro-6H-benzo[c]chromen-6-one

Figure 15: Structure of Urolithin B and 3-Hydroxy-7,8,9,10-tetrahydro-6H-benzo[c]chromen-6-one

Beside these, there have been studies conducted to design urolithin analogues. In these studies, very potent inhibitors have been designed and synthesized and some of them were found to be active compared to galantamine and rivastigmine. Some representative examples are shown in Figure 16 (Norouzbahari et al., 2018).

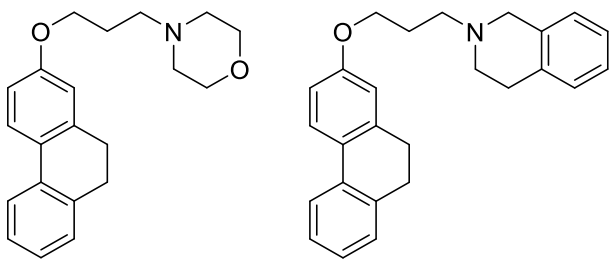


Figure 16: Representative cholinesterase inhibitors derived from urolithins

Chapter 4

EXPERIMENTAL

4.1 Material

The chemicals used in this research were analytical reagent grade and used as received without further purification. 2-Iodobenzoic acid, resorcinol, phloroglucinol, 2-bromo-5-hydroxy benzoic acid, 2-bromo-4,5-dimethoxy benzoic acid, 2-bromo-5-methoxybenzoic acid, methyl iodide, copper (II) sulfate, sodium hydroxide, hydrochloric acid, ethanol, ethyl acetate, dimethylformamide were obtained from Acros organic, Merck and Sigma Aldrich, cholinesterase enzyme, acetylthiocholine iodide, galanthamine hydrobromide all from Sigma Aldrich. COX activity assay kit from Cayman chemical, monoamine oxidase (MAO) assay kit from Sigma Aldrich (All solutions were prepared by using deionized water).

4.2 Instrument

4.2.1 Infrared Spectra

FT-IR Spectra were determined using a Shimadzu FT-IR Prestige 21 Spectrophotometer. For each sample, 30 mg of dry compound was used without KBr disc. Functional groups were investigated and analyzed employing the IR spectrums obtained.

4.2.2 Thin Layer Chromatography

For TLC, Merck aluminum-precoated plates of silica were utilized to monitor the reactions. For this purpose the following solvent systems were used (V/V ratio):

System 1: Ethyl acetate - n-Hexane -Methanol (70:20:10)

System 2: Ethyl acetate - n-Hexane (80:20)

System 3: Ethyl acetate - n-Hexane - Methanol- Chloroform (60:20:10:10)

4.2.3 Mass Spectroscopic Analysis

The mass analyses were conducted employing a Waters Alliance ZQ Micromass spectrometer (Waters Corporation, Milford, MA, USA in ESI (+) mode). The capillary was arranged to 2500 V.

4.2.4 Melting Point

To determine the melting point an electronic apparatus, IA9200*6 MK2 model instrument, was used. For these analyses, the capillary tubes filled with 3 mg of each sample and inserted inside the instrument.

4.2.5 Nuclear Magnetic Resonance Spectroscopy (NMR)

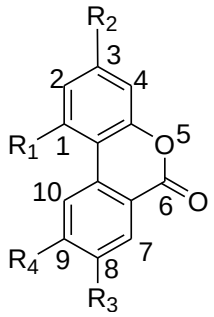
H1-NMR and C13-NMR spectra were recorded on a Bruker-400 NMR spectrometer using tetramethyl silane (TMS) as internal standard and DMSO-d as solvent; all chemical shifts were reported in parts per million (ppm, δ).

4.3 Synthetic Methodologies

The following urolithin derivatives have been synthesized within the scope of this study. The numbering system of benzo[c]chromen ring and the title compounds are shown in the Table 1:

Table 1: The numbering system of benzo[c]chromen ring and the title compounds synthesized.

	R_1	R_2	R_3	R4
1 (Urolithin B)	-H	-OH	-H	-H
2	-H	$-OCH_3$	-H	-H
3	-OH	-OH	-H	-H
4	-OCH3	-OCH3	-H	-H
5 (Urolithin A)	-H	-OH	-OH	-H
6	-H	-OCH3	-OCH3	-H
7	-OH	-OH	-OCH3	-OCH3
8	-H	-OH	-OCH3	-H
9	-OH	-OH	-OH	-H
10	-OCH3	OCH3	-OCH3	-H
11	-H	-OH	-OCH3	-OCH3
12	-OH	-OH	-OCH3	-H



4.3.1 General Synthetic Protocols

The appropriate 2-halobenzoic acid derivative was reacted with either resorcinol or phloroglucinol in basic aqueous containing NaOH under reflux conditions. $CuSO_4$ aqueous solution was added to finalize the synthesis of hydroxylated benzo[c]chromen-6-one derivatives and to make them precipitate. The methoxy derivatives were obtained through methylation reaction employing methyl iodide. General synthetic protocol is presented in Figure 17.

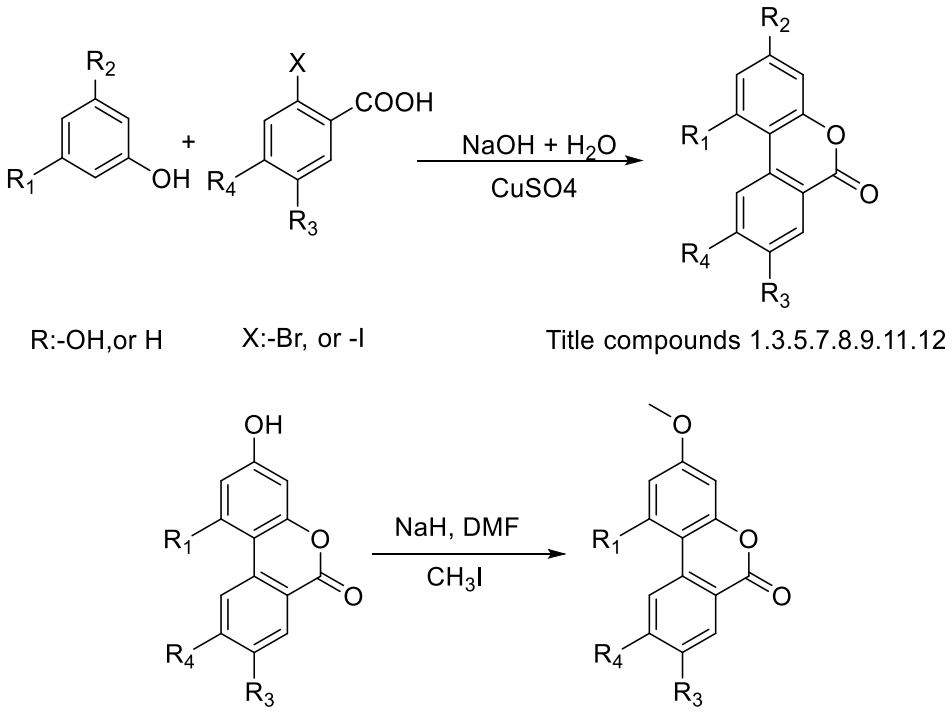


Figure 17: General Synthetic Protocols

4.3.2 Synthesis of Hydroxyl Substituted Analogues

A mixture of 6 mmol 2-halobenzoic acid (hydroxybenzoic acid, or 2-bromo-5-methoxy benzoic acid, or 2-bromo-4,5-dimethoxy benzoic acid depending on the reaction), 18 mmol resorcinol (phloroglucinol) and 22.2 mmol NaOH in 25 ml of water , was refluxed for 1 hour. At the end of the reaction period, 10 ml of 25% $CuSO_4$

aqueous solution was added dropwise. The product precipitated was filtered off and washed with ice water.

4.3.3 Synthesis of Methoxy Substituted Analogues

2.2 mmol of hydroxyl substituted urolithin derivative was dissolved in 25 ml DMF. NaH was added into this solution and mixed for 5 min at rt. Following that, appropriate amount of CH_3I was added. The amount of CH_3I and NaH were calculated as 1.1×number of hydroxyl group×2.2 mmol, depending on the number of hydroxyl groups. The reaction was stirred at rt for 2 hours. At the end of the reaction period 15 ml of 1N HCl was added. The product was obtained through 3 times extraction with 20 ml ethyl acetate, and evaporating the organic phase.

4.4 Cholinesterase Inhibition Assays

For the measurement of cholinesterases inhibition (AChE, and BuChE) Modified Ellmann's method was employed (Šinko, Čalić, Bosak, & Kovarik, 2007). Accordingly, each reaction mixture contained 168 µL of 50 mM of Tris HCl buffer (pH 8.0), 10 µL of 6.8 mM DTNB solution (i.e., 0.34 mM final), 20 mM $MgCl_2$ and 100 mM NaCl, 10 µL of AChE or BuChE solution (0.4 U/mL from Human recombinant AChE or 1.64 U/mL from Human recombinant BuChE, from Sigma Aldrich, Dorset, England), and 2 µL of each sample solution with a total volume of 200 µL. The reactions were initiated by the addition of 10 µL of 10 mM acetylthiocholine iodide or 10 µL of 1.5 mM butyrylthiocholine iodide. After incubation for 15 min at 27°C, the measurements were obtained at 412 nm using 96-well microplate reader (Varioskan Flash, Thermo Scientific,USA). Utilizing this formula (E-S)/E x 100, the percentage of inhibition of AChE and BuChE was calculated by the comparison of rates of reaction of samples relative to blank sample (DMSO and methanol). In the formulation factor E is the activity of enzyme in the

absence of test sample and factor S is the activity of enzyme in the presence of sample. By plotting the percent inhibition against the concentration of test molecules, the concentration of test molecules and reference molecules which determined 50% inhibition of the AChE or BuChE activity (IC_{50}) was calculated. Each concentration was assessed in triplicate and each measurement was employed to obtain three IC_{50}s under the experimental conditions. The mean ± standard deviations of these IC_{50}s were represented.

4.5 COX Inhibition Assays

To measure COX inhibition, the fluorescent assay method was used. For this measurement ADHP (10-acetyl-3,7-dihydroxyphenoxazine) was used as substrate for the peroxidase component of the COX enzymes. The enzyme catalyzed reaction product is resorufin which is a fluorescent compound. The amount of resorufin was measured with an excitation wavelength of 530 nm and an emission wavelength of 585 nm (i.e., COX Fluorescent Inhibitor Screening Assay Kit from Cayman Chemical, Product number 700100, Michigan 48108 USA). Each title urolithin derivative concomitant to ibuprofen and flurbiprofen (as reference molecules), were analyzed in triplicates at various concentrations to measure IC_{50} values. Also to control the experiments, the enzyme-free and inhibitor-free assay systems were used. The plot of the percent inhibition against the concentration of test molecules was drawn and then IC_{50}s were calculated. The mean ± standard deviations of IC_{50}s were represented.

4.6 MAO-B Inhibition Assays

To measure the potential of the title urolithin derivatives to inhibit MAO-B a fluorescent method was utilized. In this assay substrate was p-tyramine and the amount of enzyme catalyzed hydrogen peroxide was measured with a fluorescent dye reagent (i.e., excitation wavelength of 530 nm and an emission wavelength of 585 nm, Sigma-

Aldrich MAO Assay Kit, Catalog number MAK-136, Dorset, England). Accordingly IC_{50} values for each test compound and pargyline as reference molecule was analyzed in triplicates at various concentrations. To control the experiment the enzyme-free and inhibitor-free assay systems were utilized. The IC_{50}s of test compounds and reference materials was calculated via plotting the percent inhibition against the concentration of test materials. The mean ± standard deviations of these IC_{50}s were represented.

4.7 DPPH Radical Scavenging Activity

UV technique was employed to screen the DPPH radical scavenging activity. For this aim, the solution of title urolithin derivatives and gallic acid as reference were prepared in 50 µM and 100 µM and 20 µL methanol was used as solvent. After that 180 µL of 0.15 mM DPPH in methanol was added to each solution. Then the mixtures were incubated for 20 minutes at rt. After this period the amount of remaining DPPH was measured at 520 nm. For measuring the percent DPPH radical scavenging activity the following formula $[(A_{blank} - A_{sample})/A_{blank}] \times 100$ was used. The A blank is the result obtained without test sample and A sample is the result obtained for each sample or the reference. The experiment performed in triplicate and the mean ± standard deviation mean was calculated.

4.8 Docking Studies

The X-ray structures of the proteins under investigation were retrieved from the Protein Data Bank (PDB) [35] (PDB IDs: 1EQG, 5IKR, 6F7Q, 6O4W, 6FVZ) and prepared with Schrödinger's Protein Preparation Wizard tool. Water molecules and residues defined as heteroatoms in PDB were removed, except FAD in the crystal structure of MAO-B (PDB ID: 6FVZ). Hydrogen atoms and missing side chain residues were added to the protein structures. The hydrogen bonding network was optimized taking into account the protonation states predicted by PROPKA at pH 7.0.

Finally, a restrained energy minimization step was performed using the OPLS3 force field with default settings. Inhibitor structures were drawn by means of Maestro 2D sketcher, then converted to their corresponding 3D structures and prepared using Schrödinger's LigPrep tool. Possible tautomers and stereoisomers were generated at pH 7.0 ± 2.0 with Epik and OPLS3 force field was used for energy minimization. Conformers were subsequently generated with ConfGen: a maximum of 25 conformers was allowed and energy minimization of the output conformations was performed using the default force field (OLPS_2005). [39-The Receptor Grid Generation tool was employed and grid boxes were generated by selecting the co-crystallized inhibitors as the center of the grid. Molecular docking studies were carried out using Glide Standard Precision mode from the Schrödinger suite. The option "sample ring conformation" was turned on and a maximum of three docking poses were output for each ligand; all other settings were kept as default. The first poses were then subjected to protein-ligand complex refinement. Residues within 2 Å from the ligand were treated as flexible and VSGB and OPLS3 were used as solvation model and force field. The ability of the above-mentioned protocol was firstly evaluated via redocking studies for all proteins under investigation. In all cases, the protocol was able to reproduce the co-crystallized inhibitor structures with low root-mean-square deviation (RMSD) values. Specifically, the following RMSD values were obtained (values refer to heavy atoms): 1EQG = 1.049 Å, 5IKR = 0.749 Å, 6O4W = 0.836 Å, 6F7Q = 0.631 Å, 6FVZ = 0.109 Å.

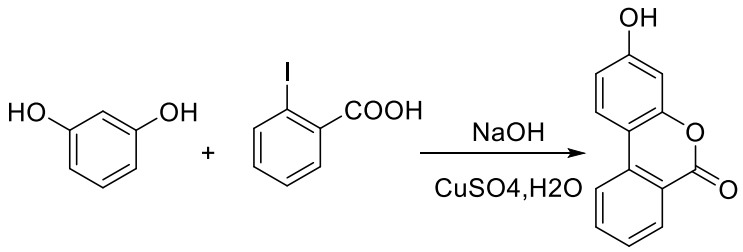
Chapter 5

RESULTS AND DISCUSSION

5.1 Chemistry Synthesis and Analyses of the Title Compounds

There are various methods displayed so far for the synthesis of substituted urolithin derivatives. The most sophisticated techniques involve the employment of Bringmann concept and Suzuki reactions (Melville & Triano, 2014). For certain derivatives of urolithins, it is also possible to use the classical urolithin synthesis method in which 2-halobenzoic acid can be reacted with resorcinol under basic condition. Particularly, the appropriate substituents with no electrophile character on these derivatives can accommodate to these reactive conditions. Regarding this point, we have employed this final strategy on the presence of $CuSO_4$ catalyst.

5.1.1 Synthesis of 3-Hydroxy-6H-benzo[c]chromen-6-one (1)

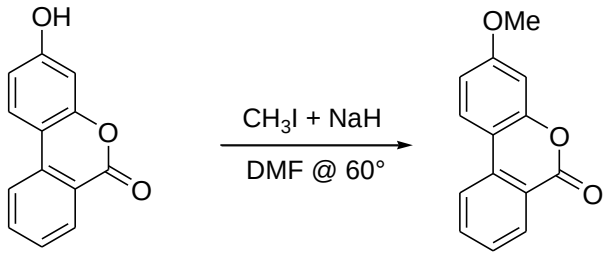


resorcinol 2-iodobenzoic acid 3-hydroxy-6H-benzo[c]chromen-6-one

Product was white-yellow powder and yield obtained: 76%. 1H NMR (DMSO-d, 400 MHz): δ = 10.30 (s, 1H), 8.15–8.12 (m, 3H), 7.83 (t, 1H, J = 6.1Hz), 7.50 (t, 1H, J = 6.1Hz), 6.77–6.74 (m, 2H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 160.5 ppm for lactone carbonyl. IR (cm^{-1}): 1702 (lactone carbonyl). (+)-ESI-MS: m/z 212.76 (M^+).

R_f: S-1, S-2, S-3; 0.12, 0.62, 0.2 (UV lamp 254nm)

5.1.2 Synthesis of 3-methoxy-6H-benzo[c]chromen-6-one (2)

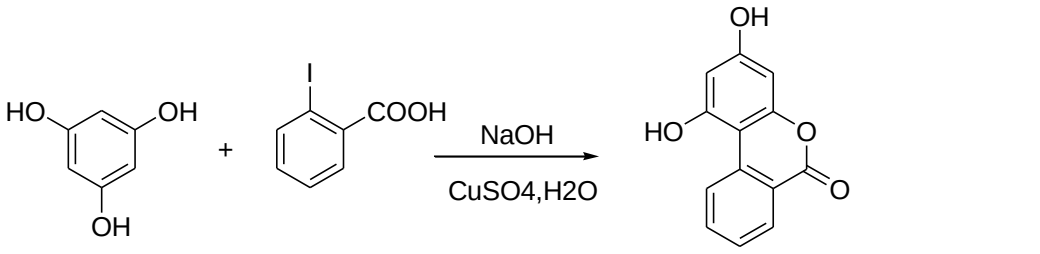


3-hydroxy-6H-benzo[c]chromen-6-one 3-methoxy-6H-benzo[c]chromen-6-one

The product was a green solid and yield obtained: 68%. 1H NMR (DMSO-d, 400 MHz): δ = 8.38-8.19 (m, 3H), 7.89 (t, 1H, J = 6.4 Hz), 7.58 (t, 1H, J = 6.4Hz), 7.02-7.10 (m, 2H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 161.0 ppm for lactone carbonyl. IR (cm^{-1}): 1732 (lactone carbonyl). (+)-ESI-MS: m/z 227.73 (M^+).

R_f: S-1, S-2, S-3; 0.29, 0.11, 0.25 (UV lamp 254nm)

5.1.3 Synthesis of 1, 3-dihydroxy-6H-benzo[c]chromen-6-one (3)

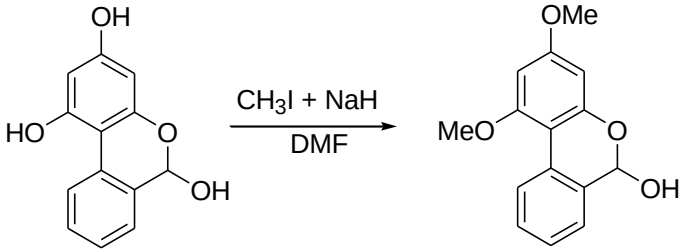


phloroglucinol 2-iodobenzoic acid 1,3-dihydroxy-6H-benzo[c]chromen-6-one

The product was a dark brown solid and yield obtained: 74%. 1H NMR (DMSO-d, 400 MHz): δ = 10.90 (s, 1H), 10.15 (s, 1H), 8.92 (d, 1H, J = 6,8Hz), 8.24 (d, 1H, J = 6.8 Hz), 7.78 (t, 1H, J = 8,0Hz), 7.41 (t, 1H, J = 8,0Hz), 6.37 (s, 1H), 6.25 (s, 1H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 180.5 ppm for lactone carbonyl. IR (cm^{-1}): 1707 (lactone carbonyl). (+)-ESI-MS: m/z 227.81 (M^+).

R_f: S-1, S-2, S-3; 0.14, 0. 61, 0.43 (UV lamp 254nm)

5.1.4 Synthesis of 1, 3-dimethoxy-6H-benzo[c]chromen-6-one (4)

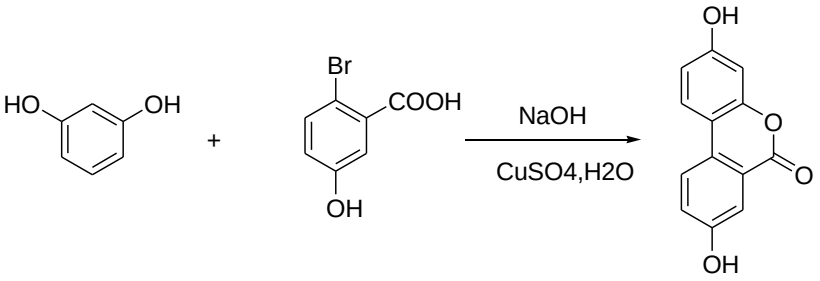


1, 3-dihydroxy-6H-benzo[c]chromen-6-one 1, 3-dimethoxy-6H-benzo[c]chromen-6-one

The product was a brown oily residue and Yield obtained: 65%. 1H NMR (DMSO-d, 400 MHz): δ = 8.88 (d, 1H, J = 8.1 Hz), 8.29 (d, 1H, J = 7.6Hz), 7.83 (t, 1H, J = 6.9Hz), 7.56 (t, 1H, J = 6.9Hz), 6.89-6.80 (m, 2H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 188.5 ppm for lactone carbonyl. IR (cm^{-1}): 1726 (lactone carbonyl). (+)-ESI-MS: m/z 255.89 (M^+).

R_f: S-1, S-2, S-3; 0.68, 0.13, 0.65 (UV lamp 254nm)

5.1.5 Synthesis of 3, 8-dihydroxy-6H-benzo[c]chromen-6-one (5) (Urolithin A)

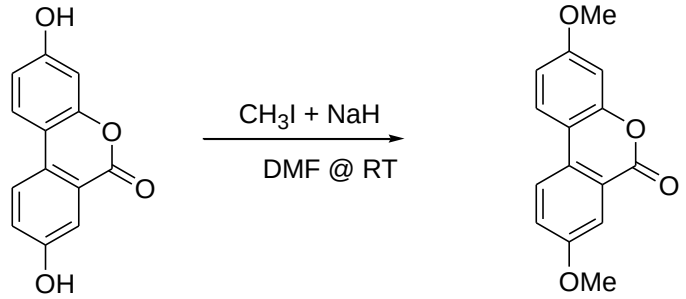


resorcinol 2-bromo-5-hydroxy benzoic acid 3,8-dihydroxy-6H-benzo[c]chromen-6-one

The precipitated product was brown and yield obtained: 71.5%. 1H NMR (DMSO-d, 400 MHz): δ = 10.2 (s, 1H), 10.1 (s, 1H), 8.05 (d, 1H, J = 8.4Hz), 7.95 (d, 1H, J = 6.8Hz), 7.48 (s, 1H), 7.28 (d, 1H, J = 6.8Hz), 6.77 (d, 1H, J = 8.4Hz), 6.69 (s, 1H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 184.3 ppm for lactone carbonyl. IR (cm^{-1}): 1701 (lactone carbonyl). (+)-ESI-MS: m/z 227.73 (M^+).

R_f: S-1, S-2, S-3; 0.1, 0.62, 0.13 (UV lamp 254nm)

5.1.6 Synthesis of 3, 8-dimethoxy-6H-benzo[c]chromen-6-One (6)

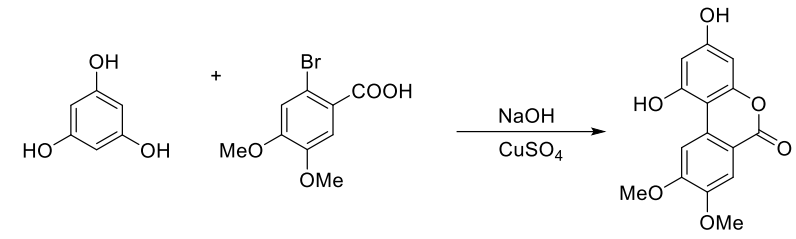


3,8-dihydroxy-6H-benzo[c]chromen-6-one 3,8-dimethoxy-6H-benzo[c]chromen-6-one

The product was obtained as yellow residue and yield obtained: 68%. 1H NMR (DMSO-d, 400 MHz): δ = 8.83 (d, 1H, J = 7.9Hz), 8.25 (d, 1H, J = 7.0Hz), 7.82 (s, 1H), 7.55 (d, 1H, J = 7.0Hz), 6.88 (s, 1H), 6.65 (d, 1H, J = 7.9Hz), 3.98 (s, 3H), 3.83 (s, 3H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 182.7 ppm for lactone carbonyl. IR (cm^{-1}): 1745 (lactone carbonyl). (+)-ESI-MS: m/z 256.88 (M^+).

R_f: S-1, S-2, S-3; 0.29, 0.75, 0.4 (UV lamp 254nm)

5.1.7 Synthesis of 1,3-dihydroxy-8,9-dimethoxy-6H-benz[c]chromen-6-one (7)

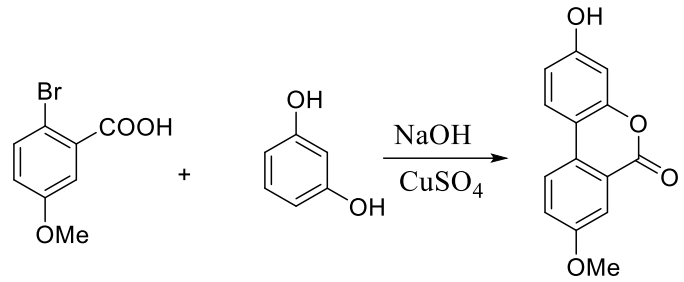


Phloroglucinol 2-bromo-4,5-dimethoxy benzoic acid 1, 3-Dihydroxy-8, 9-Dimethoxy-6H-Benzo[c] Chromen-6-One

The product was a white solid and yield obtained: 78%. 1H NMR (DMSO-d, 400 MHz): δ = 10.84 (s, 1H), 10.13 (s, 1H), 8.57 (s, 1H), 7.51 (s, 1H), 6.36 (s, 1H), 6.23 (s, 1H), 3.93 (s, 3H), 3.81(s, 3H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 180.5 ppm for lactone carbonyl. IR (cm^{-1}): 1742 (lactone carbonyl). (+)-ESI-MS: m/z 287.86 (M^+).

R_f: S-1, S-2, S-3; 0.2, 0.13, 0.4 (UV lamp 254nm)

5.1.8 Synthesis of 3-hydroxy-8-methoxy-6H-benzo[c]chromen-6-one (8)

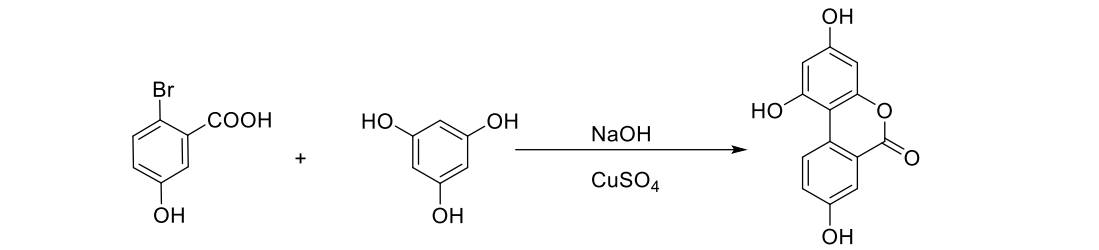


2-Br-5-methoxy benzoic acid resorcinol 3-Hydroxy-8-Methoxy-6H-Benzo[c] Chromen-6-One

The product was green powder and yield obtained: 81%. 1H NMR (DMSO-d, 400 MHz): δ = 10.23 (s, 1H), 8.22 (d, 1H, J = 7.8Hz), 8.05 (d, 1H, J = 6.9Hz), 7.60 (s, 1H), 7.45 (d, 1H, J = 6.9Hz), 6.75 (d, 1H, J = 7.8 Hz), 6.61 (s, 1H), 3.85 (s, 3H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 182.9 ppm for lactone carbonyl. IR (cm^{-1}): 1698 (lactone carbonyl). (+)-ESI-MS: m/z 241.70 (M^+).

R_f: S-1, S-2, S-3; 0.6, 0.67, 0.69 (UV lamp 254nm)

5.1.9 Synthesis of 1, 3, 8-trihydroxy-6H-benzo[c]chromen-6-one (9) (Urolithin C)

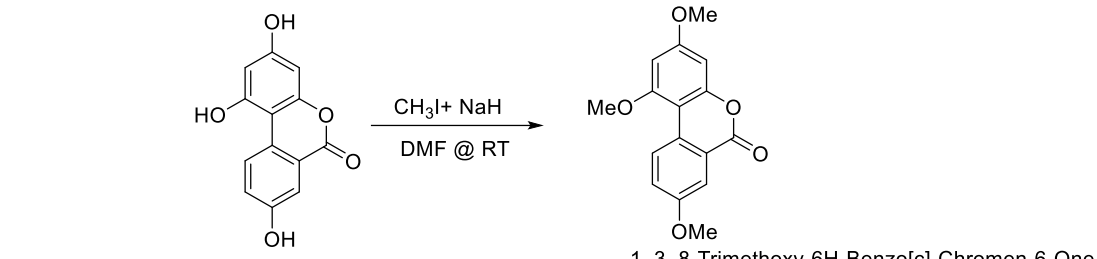


2-bromo-5-hydroxy-benzoic acid phloroglucinol 1,3,8-trihydroxy-6*H*-benzo[*c*]chromen-6-one

The product was white powder and yield obtained: 71%. 1H NMR (DMSO-d, 400 MHz): δ = 10.71 (s, 1H), 10.07 (s, 1H), 9.95 (s, 1H), 8.81 (d, 1H, J = 8.2Hz), 7.54 (s, 1H), 7.28 (d, 1H, J = 8.2Hz), 6.39 (s, 1H), 6.24 (s, 1H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 180.1 ppm for lactone carbonyl. IR (cm^{-1}): 1703 (lactone carbonyl). (+)-ESI-MS: m/z 243.79 (M^+).

R_f: S-1, S-2, S-3; 0.23, 0.11, 0. 8 (UV lamp 254nm)

5.1.10 Synthesis of 1, 3, 8-trimethoxy-6H-benzo[c]chromen-6-one (10)



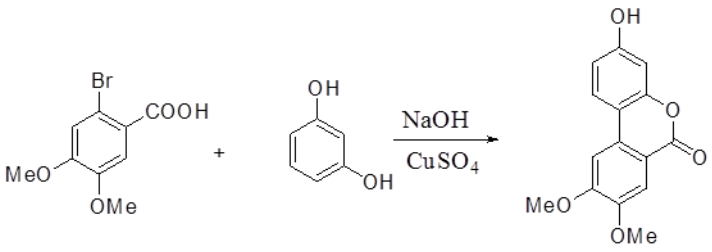
1, 3, 8-Trihydroxy-6H-Benzo[c] Chromen-6-One

1, 3, 8-Trimethoxy-6H-Benzo[c] Chromen-6-One

The product was a brown solid and yield obtained: 69%. 1H NMR (DMSO-d, 400 MHz): δ = 7.94 (s, 1H), 7.14-7.26 (m, 2H), 6.44-6.55 (m, 2H), 3.80 (s, 3H), 3.71 (s, 3H), 3.65 (s, 3H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 180.7 ppm for lactone carbonyl. IR (cm^{-1}): 1736 (lactone carbonyl). (+)-ESI-MS: m/z 286.81 (M^+).

R_f: S-1, S-2, S-3; 0.26, 0.13, 0.19 (UV lamp 254nm)

5.1.11 Synthesis of 3-hydroxy-8,9-dimethoxy-6H-benzo[c]chromen-6-one (11)

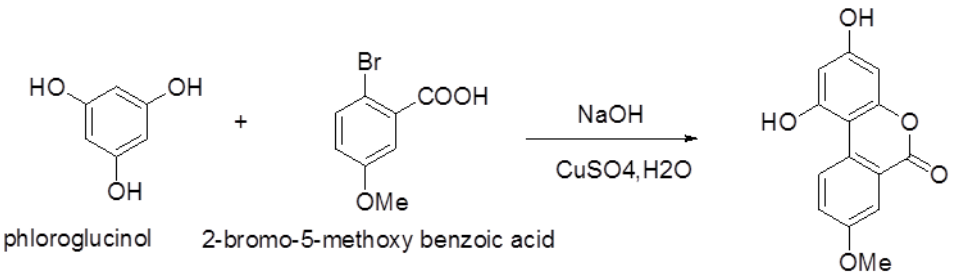


2-bromo-4,5-dimethoxy benzoic acid resorcinol 3-hydroxy-8,9-dimethoxy-6H-Benzo[c] Chromen-6-One

The product was brown powder and yield obtained: 78%. 1H NMR (DMSO-d, 400 MHz): δ = 10.25 (s, 1H), 8.21 (d, 1H, J = 7.8Hz), 7.70 (s, 1H), 7.50 (s, 1H), 6.75 (d, 1H, J = 7.8Hz), 6.65 (s, 1H), 3.95 (s, 3H), 3.83 (s, 3H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 179.5 ppm for lactone carbonyl. IR (cm^{-1}): 1693 (lactone carbonyl). (+)-ESI-MS: m/z 271.87 (M^+).

R_f: S-1, S-2, S-3; 0.1, 0.2, 0.3 (UV lamp 254nm)

5.1.12 Synthesis of 1,3-dihydroxy-8-methoxy-6H-benzo[c]chromen-6-one (12)



phloroglucinol 2-bromo-5-methoxy benzoic acid

1, 3-Dihydroxy-8-Methoxy-6H-Benzo[c] Chromen-6-One

The product was brown and yield obtained: 68%. 1H NMR (DMSO-d, 400 MHz): δ = 10.71 (s, 1H), 10.05 (s, 1H), 8.82 (d, 1H, J = 8.0Hz), 7.67 (s, 1H), 7.42 (d, 1H, J = 8.0Hz), 6.38 (s, 1H), 6.23 (s, 1H), 3.83 (s, 3H). 13C NMR (DMSO-d, 125 MHz) δ (ppm) major rotamer: 179.7 ppm for lactone carbonyl. IR (cm^{-1}): 1696 (lactone carbonyl). (+)-ESI-MS: m/z 257.83 (M^+).

R_f: S-1, S-2, S-3; 0.1, 0. 2, 0.2 (UV lamp 254nm)

5.2 The Potential of Compounds to Inhibit Cholinesterase, MAO-B, and COX Enzymes

The results obtained in enzyme inhibition studies for the title compounds are shown

Table 2.

Table 2: The potential of title urolithin derivatives to inhibit cholinesterases, MAO-B, and cyclooxygenases.

Title Compounds	IC_{50} (µM)				
	AChE	**BuChE**	**MAO-B**	**COX-1**	**COX-2**
1	> 50	> 50	46,3 ± 1,4	> 50	> 50
2	12,4 ± 1,3	19,4 ± 2,0	> 50	> 50	> 50
3	> 50	> 50	28,5 ± 0,8	> 50	> 50
4	14,3 ± 0,2	21,7 ± 1,1	> 50	> 50	> 50
5	> 50	> 50	9,3 ± 0,3	> 50	> 50
6	18,0 ± 0,4	24,1 ± 1,4	28,1 ± 0,9	44,0± 0,9	> 50
7	> 50	> 50	29,4 ± 1,0	23,2± 1,1	27,3± 1,3
8	45,4 ± 0,5	49,3 ± 0,3	36,4 ± 0,7	> 50	> 50
9	47,1 ± 0,1	49,6 ± 1,0	11,3 ± 0,3	11,1± 0,6	12,9± 1,2
10	11,1 ± 0,2	14,5 ± 0,5	41,3 ± 0,5	> 50	> 50
11	> 50	> 50	23,5 ± 0,8	43,2± 2,4	47,1± 0,3
12	> 50	> 50	16,9 ± 0,5	14,2± 0,2	13,1± 0,5
Donepezil	0,15±0,02	7,1 ± 0,4	NT	NT	NT
Rivastigmine	33,2 ± 0,2	11,8 ± 0,9	NT	NT	NT
Pargyline	NT	NT	2,8 ± 0,4	NT	NT
Ibuprofen	NT	NT	NT	11,8± 0,8	22,9± 2,1
Flurbiprofen	NT	NT	NT	2,4 ± 0,3	17,5± 1,6

Accordingly, some of the compounds were found to show potential to inhibit both AChE and BuChE. In particular, the title molecules 2, 4, 6, and 10 were the most potential inhibitors for both cholinesterases. Although none of the compounds were found to possess high activity in comparison to the effect of donepezil, the activities obtained for these compounds (i.e., 2, 4, 6, and 10) were found comparable or superior to the activity of rivastigmine. On the other hand, the activities were found to be slightly selective for AChE, since for each active compound, the IC_{50}s were found lower for AChE. In terms of structure activity, it was observed that the methoxy substitution generated more potential to inhibit cholinesterase enzymes. In general, the hydroxyl substitutes almost totally diminished the activity as seen in the example of title molecules 1 and 2, and 5 and 6 comparisons, respectively. The SAR analysis also pointed out an important outcome. Pomegranate juice is known to have positive effect on cognition. Moreover, it is also a fact that particularly the methoxy substituted urolithin analogues present in CNS, since hydroxyl substituted derivatives have narrow CNS penetration capacity. The results obtained within this group of study indicate the possible role of methoxy substituted analogues positive effect on cognition through inhibition of cholinesterase enzymes. Once considering the major urolithin A and B present in systemic circulation, these methoxy ether metabolites appear to have more function for cognitive enhancement.

The results were also evaluated concomitant to the findings in docking studies in which the most active compounds (2 and 6) found were investigated. Figure 18A, B, C, and D segments represent the interaction of compounds 2 and 6 with AChE and BuChE. Accordingly, the compound bonding to AChE and BuChE appear to be different in such a way that the compounds interact with the peripheral site of AChE while it is the active site for BuChE. The π–π stacking interactions with Try341 and some

hydrophobic contacts with Try72, Trp286 and Try377 and hydrogen bonding with Phe295 with the carbonyl group were found to be the main interactions with AChE. The establishment of π–π stacking with Trp82 in the active site of BuChE and a hydrogen bond with Thr120 as well as hydrophobic interactions with Try128, Try332, and Trp430 were found the main interactions with AChE enzyme. Beside compound 6 makes another hydrogen bond with Try128.

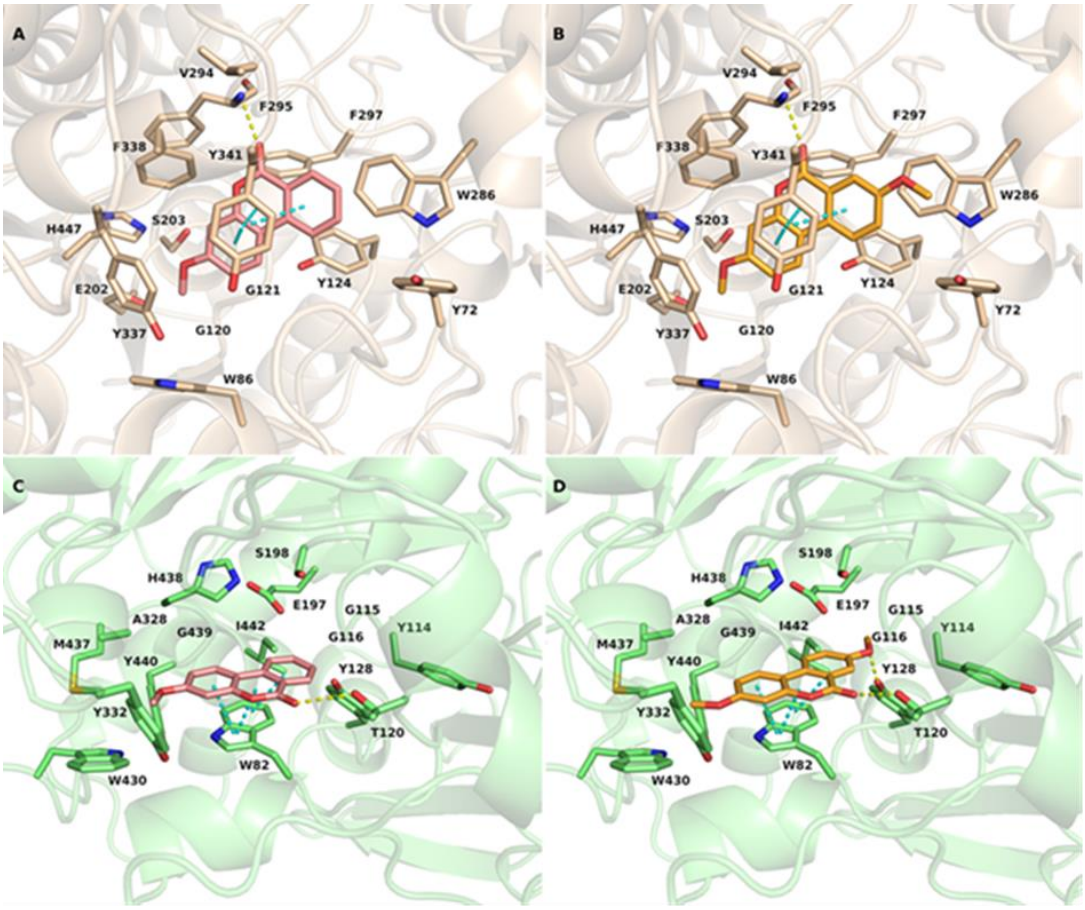


Figure 18: Predicted binding modes of compound 2 (A and C) and compound 6 (B and D) to hAChE and hBuChE (PDB IDs: 6O4W and 6F7Q). Only the side chains of the surrounding amino acid residues are shown for clarity and they are displayed as beige sticks for hAChE (A and B) and green sticks for hBuChE (C and D); instead, for residue F295, the main chain is shown. The docking poses are depicted as sticks and colored in pink (compound 2) and bright orange (compound 6) while hydrogen bonds are represented with dashed yellow lines and π–π stacking interactions with dashed cyan lines

Considering the activities obtained in MAO-B inhibitor studies, in general, compounds displayed lower activity in comparison to the reference molecule, pargyline. The compounds 5 and 9 were found to be the most active within the series. It was observed that, opposite of the finding with cholinesterases, the methoxy substitutions in general appear to lower the activity. In other words, hydroxyl substitution particularly on position 3 was found significant for higher MAO-B inhibitor potential. Indeed, the compounds in which position 3 hydroxyl group is missing (i.e., compounds 2, 4, 6, and 10) the activities were all measured decreased.

Regarding the importance of dopamine and its metabolism in CNS particularly with MAO-B, the findings have important outcomes. First of all, the major urolithin metabolite (compounds 5, urolithin A) was found to possess MAO-B inhibitor activity. Its possible availability in CNS through the oxidative demethylation of the methoxy metabolite, the antidepressant potential of the ellagitannin rich food might be explained. This might be further extrapolated to other hydroxyl containing urolithin metabolites.

The docking studies conducted on compound 5 as shown in figure 19 A and B (i.e., respectively for molecules 5 and 9), the presence of hydroxyl group on position 1, 3, and 8 were found significant to establish hydrogen bonding with Ile199, Leu171, and Pro102.

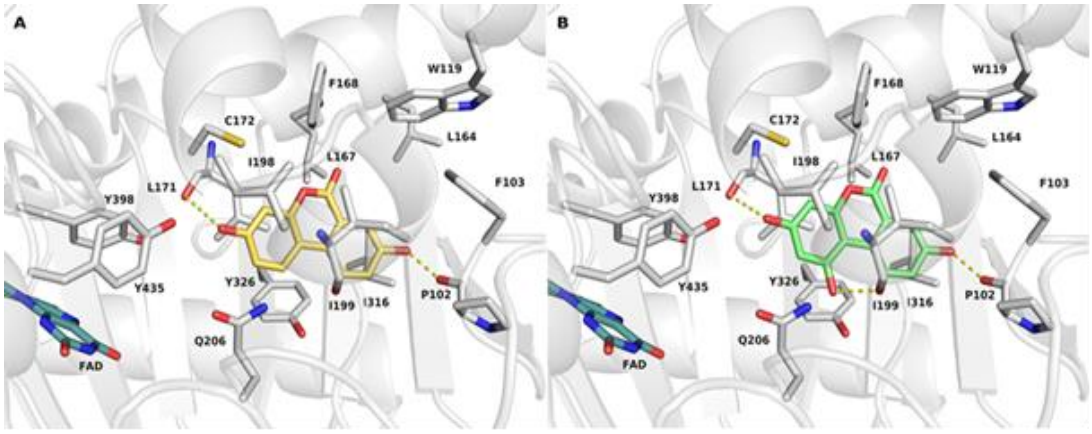


Figure 19: Predicted binding modes of compound 5 (A) and compound 9 (B) at the hMAO-B active site (PDB ID: 6FVZ). Only the side chains of the surrounding amino acid residues are shown for clarity and they are displayed as white sticks. For residue P102, L171, and I199 the main chains are shown. The docking poses are depicted as sticks and colored in yellow (compound 5) and green (compound 9), while hydrogen bonds are represented with dashed yellow lines

The results obtained for COX inhibitor assays also pointed out some of the title molecules as important scaffolds for COX inhibitor. Although in many of the compounds screened for their activity found inactive under experimental conditions, the title molecules 9 and 12 displayed comparable activity to reference drug molecules ibuprofen and flurbiprofen. Although the reference molecules are relatively COX-1 selective, the active urolithin analogues have been shown to possess a balanced inhibition on COX-1 and COX-2. Including compound 7, another active molecule in the series, the common substitution was found to be 1,3,8-tri substitution to have COX inhibitor potential. Particularly, 1,3-dihydroxyl and 8-substitution (i.e. either methoxy- or hydroxyl-) generated active compounds.

The docking studies (in figure 20 for the most active compounds 9 and 12 for COX-1 and COX-2) displayed the predicted binding mode. Accordingly 1-hydroxyl and 8-hydroxyl/methoxy substitution generated hydrogen bonding in the active sites of COX-1 and 2 and this aided in stabilization. It was very important to observe hydrogen bonding with Ser530 with the benzo[c]chromene-6-one scaffold, since this residue is the key residue for the interaction of many non-steroidal anti-inflammatory drugs. The hydroxyl on the position 1 and hydroxyl and methoxy on position 8 were found to establish hydrogen bonding with Met522, and Tyr355 residues, respectively.

Regarding the recent investigations on the beneficiary effects of some non-steroidal anti-inflammatory drugs for the prevention and treatment of AD, the COX inhibitor potential of urolithins, displayed in this work, is also an important outcome.

Overall, the results obtained within these enzyme inhibition assays, displayed some mechanism aspect of the activity of urolithins on the treatment of AD. Moreover, for

the design and generation of novel molecules, the results also pointed out the significance of urolithin backbone to be utilized as an important scaffold.

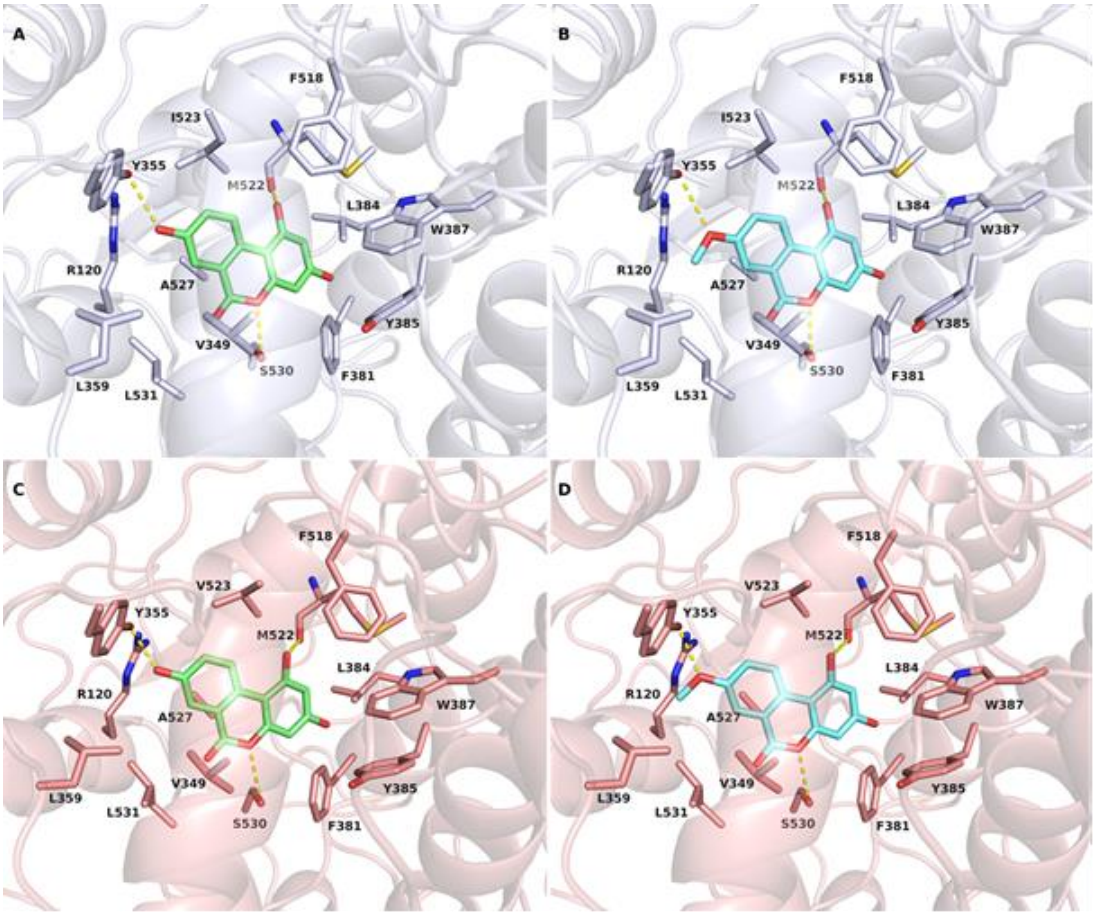


Figure 20: Predicted binding modes of compounds 9 and 12 at the active site of (A) oaCOX-1 (PDB ID: 1EQG) and (B) hCOX-2 (PDB ID: 5IKR). Only the side chains of the surrounding amino acid residues are shown for clarity and they are displayed as sticks (oaCOX-1 colored in light grey; hCOX-2 colored in pink). For residue M522, the main chain is shown. The docking poses are depicted as sticks and colored in green (compound 9, A and C) and cyan (compound 12, B and D), while hydrogen bonds are represented with dashed yellow lines

5.3 DPPH Radical Scavenging Activities

The result obtained in Table 3 for DPPH radical scavenging activities of the compounds displayed that the compound 9 had the highest activity. Compound 5 was another active molecule. It is known that the number of phenolic hydroxyl group is in possible relationship with the activity. Indeed compound 9 has three and compound 5 has two phenolic hydroxyl groups. The other active molecules have at least one available phenolic hydroxyl group. The overall results also pointed out also possible antioxidant activity of urolithin analogues and this is depended on the number of phenolic hydroxyl groups.

Table 3: Antioxidant activity of the title urolithins in DPPH radical scavenging assay.

Title Compounds	IC_{50} (µM)	
	50µM	100µM
1	18.03 ± 0.07	21.67 ± 0.08
2	6.07 ± 0.08	12.72 ± 0.02
3	22.66 ± 0.04	26.62 ± 0.08
4	4.06 ± 0.06	8.13 ± 0.02
5	33.14 ± 0.04	38.86 ± 0.07
6	8.19 ± 0.02	13.95 ± 0.21
7	28.62 ± 0.02	31.15 ± 0.07
8	16.54 ± 0.07	19.22 ± 0.18
9	42.26 ± 0.04	50.41 ± 0.01
10	7.90 ± 0.01	18.64 ± 0.07
11	7.38 ± 0.01	14.45 ± 0.03
12	18.66 ± 0.04	29.46 ± 0.25
Gallic acid	53.88 ± 0.09	72.39 ± 0.24

Chapter 6

CONCLUSION

The worldwide research studies on the treatment of AD have been continuing in parallel to the findings with the pathophysiology of the disease. One aspect of these studies is related to the design of original molecules related to a specific target. Much emphasis have been provided on the design of cholinesterase, MAO, beta-secretase, gamma-secretase, and amyloid beta aggregative inhibitors. Indeed, quite accumulated data obtained so far through these strategies. However, another important aspect of research studies for the treatment of AD employs, some natural sources known to have beneficiary effects.

Pomegranate juice, extracts and other ellagitannin rich food effect on the prevention and treatment of AD have been known for a long time. Considering the bioavailability problem of ellagitannins, much of their biological effects have been attributed to urolithins, since they are the bioavailable forms in systemic circulation following to exposure.

Within this study we have synthesized the main urolithin compounds and some of their metabolites concomitant to their other synthetic alternatives, the potential of compounds to inhibit AChE, BuChE, MAO-B, COX-1, and COX-2 have been determined in parallel to findings obtained in DPPH scavenging activities.

The results, for the first time, described out a SAR work on the effect of urolithins on specified targets. It was clearly shown that, the possible treatment and protective effect of urolithins on the AD can be explained with diverse and combinational activities on different targets.

Furthermore, the title compounds 2-10, 5-9, and 9-12, have been found as representative molecules as starting point for the design of original molecules to inhibit AChE-BuChE, MAO-B, and COX-1-2 enzymes, respectively.

As a final evaluation, cholinesterase enzymes, MAO-B, and cyclooxygenase enzymes are involved in the biological activities of urolithins concomitant to their antioxidant properties depending on the number of hydroxyl substituents.

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