INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE (COMT) ENZYME ACTIVITY BY SOME PLANT-DERIVED ALKALOIDS AND PHENOLS

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ABSTRACT

INHIBITION OF CATECHOL-*O*-METHYLTRANSFERASE (COMT) ENZYME ACTIVITY BY SOME PLANT-DERIVED ALKALOIDS AND PHENOLS

In this study, inhibition potentials of harmal seed alkaloids and olive leaf polyphenols were investigated on COMT catalyzed methylation reaction. Inhibition performances of natural alkaloids and polyphenolics whose purities were attempted to be increased by extraction and fractionation were compared with standards of these compounds and the best known COMT inhibitor, 3,5-dinitrocatechol.

COMT enzyme inhibition experiments were performed with sentitive fluorometric method. In this method, K_m values for SAM and aesculetin substrates were found as 3.5 ± 0.3 μM and 6.4 ± 0.4 μM, respectively. As a result of the inhibition study, the highest inhibitory effect is observed for harmine and harmaline rich fractions among harmal seed alkaloids. Inhibition constants indicating degree of competitiveness and noncompetitiveness (K_i and αK_i) of the harmine fraction which has the highest inhibition performance were calculated as 0.15±0.07 μg/ml and 1.28±0.06 μg/ml, respectively.

In the studies performed with standards of olive leaf polyphenols, while it was observed that rutin has inhibitory effect, oleuropein was found less effective. However, oleuropein rich fraction of olive leaf extract showed higher inhibitory effect than crude extract and rutin. It was also observed that polyphenolic extracts obtained from vitex, terebinth and mastic leaves showed high inhibition capacities, it was thought that fractionation should be performed for these plants.

As a more general conclusion, it was revealed that harmal seed alkaloids and olive leaf fractions with high purity are promising natural COMT inhibitors. By performing this study, the kinetic inhibition constants of extracts, their fractions and standards could be determined and they could be reported into literature.

ÖZET

KATEŞOL-*O*-METİLTRANSFERAZ (KOMT) ENZİM AKTİVİTESİNİN BİTKİLERDEN ELDE EDİLEN BAZI ALKALOİTLER VE FENOLLERLE ENGELLENMESİ

Bu çalışmada, üzerlik tohumu alkaloitleri ve zeytin yaprağı polifenollerinin KOMT tarafından katalizlenen metillenme reaksiyonunu engelleyebilme potansiyelleri araştırılmıştır. Ekstraksiyon ve fraksiyonlama işlemleri ile saflıkları arttırılmaya çalışılmış alkaloitlerinin ve polifenollerinin KOMT enzim inhibisyonu performansları, bu bileşiklerin standartları ve bilinen en iyi KOMT inhibitörü olan 3,5-dinitrokateşol ile kıyaslanmıştır.

KOMT enzim inhibisyon deneyleri, hassasiyeti yüksek ve ekonomik olan florometrik metot ile gerçekleştirilmiştir. Bu metotta metil verici ve metil alıcı substrat olarak kullanılan SAM ve Eskuletin için inhibitörsüz ortamdaki K_m değerleri sırasıyla 3,5 ± 0,3 μM ve 6,4 ± 0,4 μM olarak hesaplanmıştır. Toplam 18 adet standart, özüt ve fraksiyonun kullanıldığı inhibisyon çalışması sonucunda, üzerlik tohumu alkaloitleri arasından harmin ve harmalin alkaloitleri için en yüksek inhibisyon etkisi görülmüştür. İnhibisyon performansı en yüksek olan üzerlik tohumunun harmin fraksiyonunun kompetitiflik ve nonkompetitiflik derecelerine ait inhibisyon sabitleri (K_i ve αK_i) sırasıyla 0,15±0,07 μg/ml ve 1,28±0,06 μg/ml olarak hesaplanmıştır.

Zeytin yaprağı polifenollerinin standartları ile yapılan çalışmada rutinin KOMT enzimini inhibe etme özelliği görülürken oleuropeinin daha az etkili olduğu görülmüştür. Zeytin yaprağı özütünün oleuropeince zengin fraksiyonu ise oleuropein standardı, ham özüt ve rutine göre daha yüksek inhibisyon etkisi göstermiştir. Ayrıca, laden, menengiç ve sakız özütleri yüksek inhibisyon kapasitesine sahip olduklarından dolayı fraksiyonlama yapılması gereken bitki türleri olarak belirlenmiştir.

Sonuç olarak, üzerlik tohumu alkaloitleri ve saflığı arttırılmış zeytin yaprağı fraksiyonları umut vaad eden doğal KOMT inhibitörleri oldukları tespit edilmiş ve inhibisyon kinetiği sabitleri literatüre kazandırılmıştır. Ayrıca, bu alkaloit ve polifenolik bazlı doğal bileşiklerin bilinen antioksidan ve antimikrobiyal özelliklerinin de olması nedeniyle çok fonksiyonlu bileşikler olarak bir çok sektörde kullanılabilecekleri anlaşılmıştır.

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COMT	Catechol *O*-methyltransferase
SAM	S-(5′-Adenosyl)-L-methionine
L-DOPA	3-(3,4-dihydroxyphenyl)-L-alanine (levodopa)
AChE	Acetylcholine esteraz
MAO	Monoaminoxidase
AADC	Aromatic aminoacid decarboxylase
CNS	Central Nervous System
BBB	Blood-Brain Barrier
CSF	Cerebrospinal Fluid
S-COMT	Soluble catechol *O*-methyltransferase
MB-KOMT	Membrane bound catechol *O*-methyltransferase
3,5-DNC	3,5-dinitrocatechol
V_{max}	Maximum velocity of enzymatic reactions
$V_{max,app}$	Maximum velocity of enzymatic reactions in presence of inhibitors
K_m	Affinity of substrate
$K_{m,app}$	Affinity of substrate in presence of inhibitors
α	Alpha (constant determining the inhibition mechanism)
K_i	Inhibition constant (affecting to competitive inhibition)
αK_i	Inhibition constant (affecting to noncompetitive inhibition)
IC_{50}	50 % inhibitory concentration
HPLC	High Performance Liquid Chromatography
BSA	Bovine Serum Albumin
DHAP	3,4-dihydroxyacetophenone (substrate; spectrophotometric method)
ES	Aeskuletin (substrate; fluorometric method)
SAH	S-(Adenosyl)-homocystein
3H4MAP	*O*-methylated form of DHAP
4H3MAP	*O*-methylated isoform of DHAP
FP1	Harmal seed fraction rich in harmol and harmalol
FP2	Harmal seed fraction rich in harmine

FP3	Harmal seed fraction rich in harmaline
FO1	Olive leaf fraction rich in oleuropein (water fraction)
FO5	Olive leaf fraction rich in rutin and other glycosides (ethanol fraction)

CHAPTER 1

INTRODUCTION

Catechol *O*-methyltransferase (COMT) enzyme catalyzes methylation of the *O*-hydroxyl groups in the structure of catechol by using S-(5'-Adenosyl)-L-methionine (SAM) as the methyl source (Mannistö et al., 1999). In mammalians, COMT presents in brain and peripheral tissues. The highest activity of COMT exists in liver, kidney and gastrointestinal tract (stomach-bowel) in rats and humans (Guldberg et al., 1975). COMT enzyme presents in its soluble form in many tissues but mainly bound to the membrane in the human brain. Primary physiological function of COMT enzyme is deactivation of catechols in and out of the cell having biological activity.

Catecholestrogens, catecholamine neurotransmitters (dopamine, norepinephrine and epinephrine) and 3-(3,4-dihydroxyphenyl)-L-alanine) L-DOPA (levodopa), are the important substrates of the COMT. Among these, dopamine and levodopa, are known as in relationship with each other and the most important substrates. L-DOPA synthesized through hydroxylation of tyrosine in the sympathetic neuron terminals and adrenal glands, transfroms to 2-(3,4-dihydroxyphenyl)-ethylamine (dopamine) as a result of the reaction catalyzed by a pyridoxal phosphate enzyme dopa decarboxylase.

Destruction of the dopaminergic neurons in the brain leads to the lack of dopamine and cause the starting of symptoms of the Parkinson disease. During pharmacological therapy, by taking of levodopa with dopa decarboxylase inhibitors, peripheral metabolism of levodopa is reduced thereby dopamine level in the brain can be increased (Bonifati et al., 1999; Dingemanse, 1997). Moreover, use of COMT inhibitors together with the levodopa and dopa carboxylase inhibitors plays a significant role in the therapy of the disease (Singh et al., 2007; Lerner et al., 2003).

Among the COMT inhibitors that have been developed since 1960s, inhibitors that have been used clinically such as entacapone and tolcapone are known as the synthetic nitrocatechol compounds. Human liver besides being one of the most important tissue where compounds containing these types of catechols are metabolized, is one of organs where the highest activity of COMT presents (Vieria-Coelho et al., 1996; Guldberg et al., 1975). Since the cytotoxic effects of the nitrocatecholic inhibitors

used in the therapy of Parkinson disease are known, the discovering and development of non-toxic natural inhibitors have ultimate importance.

Moreover, it is known that in Parkinson patients, increase in the lipid peroxidation in the region of the brain known as *substantia nigra* (black matter) leads to increase in the production of free radicals (Sudha et al., 2003). In the researches, it has been stated that parkinsonism progresses due to increase in neuron degeneration resulted from the formation of free radicals. Thus, importance given to the use of natural compounds having antioxidant capacity as COMT inhibitors has increased (Chen et al., 2006; Esposito et al., 2002; Chiueh et al., 2000).

Phytochemicals that are the indispensable chemicals of the traditional medicine and can be metabolized easily in the body are known have less toxic effects. Besides, due to that phytochemicals have antioxidant, antimicrobial and antitumour activities, they have been used increasingly in the modern therapy. Recently, the studies on the utilization of natural compounds in therapy of neurodegenarative diseases have gained importance. (Chen et al., 2005; Perry et al., 1999). In literature, for the therapy of Parkinson disease, utilization of many plant species that are rich in alkaloids and polyphenolics as especially COMT inhibitors has been investigated and the results have been reported.

Alkaloids are compounds that can be biologically synthesized and have several pharmacological activities. Since the ancient times, they are not only used as medicine but also as a source of madder. Several kinds of these compounds that structurally contain nitrogen exist in nature. Tropane, ergot, and indole alkaloids are classes that are widely known and are usually used for narcotic purposes. The harmal plant (*Peganum harmala*), which is rich in one of the subclass of indole alkaloids that are β-carboline derived alkaloids, is a plant belonging to the wild cummin (*zygophyllaceae*) family. It is known that the harmal has been used for pharmaceutical purposes in ancient Greek and India as well as it is used today in modern medicine for worm omitting and narkotic puposes and as stimulant on the central nervous system. In the seeds of the harmal, alkaloids including harmaline, harmine, harmalol and harmol, and etheric oils present. In the studies on the harmal, it has been revealed that the harmal alkaloids prevent the degeneration of acetylcholine and dopamine by inhibiting acetylcholine esterase (AChE) and monoamin oxidase (MAO) enzymes that have important roles on the metabolic pathways of the Parkinson disease (Theodore et al., 1999). Furhermore, a red dye can be extracted from its seeds (Akalın, 2003).

Another important class of the phytochemicals is known as polyphenols. Similar to alkaloids, polyphenols have also been utilized in pharmaceutics, chemicals and food applications since ancient times and most of them have been extracted mainly from plants.

Compounds such as oleuropein and rutin which majorly exist in the olive (*Olea europaea*) leaves and apigenin glycosides belong to the widest class of polyphenols and they are known as flavanoids. These subtances for which antimicrobial and antioxidant activities have been stated by the researches, can neutralize or delay the effects of the free radicals causing many diseases including hearth disease, cancer, diabetes, aging and cataract (Garcia et al., 2000). On account of these properties, it has been thought that they may prevent the free radicals formed upon neuron degeneration causing to the Parkinson disease and especially oleuropein ve rutin can inhibit the COMT enzyme.

This study aims investigation of effects of olive leaf polyphenols such as oleuropein ve rutin having antioxidant, antimicrobial, anticarcinogenic and antiviral activities and of harmal seed alkaloids including harmaline, harmine, harmalol and harmol as COMT inhibitors. In accordance to this purpose, the extracts obtained from these plants are fractionated and also performances of each fraction are examined as COMT inhibitors. The performances of the natural inhibitors are compared with those of the synthetic COMT inhibitors that are commercially used currently and have some adverse health effects.

CHAPTER 2

LITERATURE REVIEW

2.1. Neurodegenerative Diseases

Neurodegenerative diseases, one of the matters that could not been illuminated by the medical science, involves many negative situations resulted from destruction of especially central nervous system (CNS) neurons and their chronic dysfunctions. Mitochondrial defects, protein aggregation, high metal concentration and oxidative stress are known as the most significant reasons for death and destruction of the neurons (Cavalli et al., 2008).

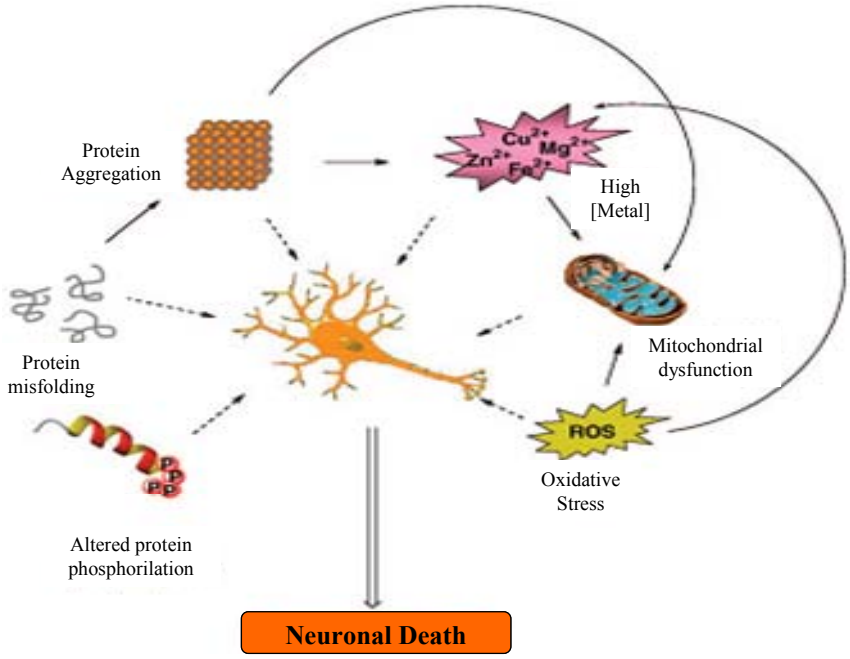

Figure 2.1. Factors resulting in neuron death and general impression mechanisms (Source: Cavalli et al., 2008).

Many neurological disorders such as multiple sclerosis (MS), Alexander disease, amiyotrophic lateral sclerosis (ALS), bovine spongioform encephalopathy (BSE), Creutzfeldt-Jakob disease, schizophrenia, epilepsy and lethargy that are still under etiologic investigation occur in humans. Alzheimer and Parkinson diseases are known

as the most important diseases among the neurological disorders that are commonly seen at older ages as revealed by the researches (Institute of Neurodegenerative Diseases, 2009). Alzheimer disease is a kind of neurological disorder is characterized primarily by comprehension loss and dotage. In early periods of the Alzheimer disease, which begins with dotage generally above the age of 65 and progresses, symptoms such as loss in comprehension and memory are observed while symptoms such as speech disturbance, depression and mental instability are also frequently observed in the late periods (Houghton et al., 2005).

2.1.1. Parkinson Disease

Parkinson disease was first described in 1817 by an English surgeon Sir James Parkinson as a syndrome of trembling of muscles unwillingly (Zhu, 2004). From pathological point of view, although the etiology of Parkinson disease has not been explained clearly it is characterized as chronic and ascending disorders of the CNS neurons and known that it is caused by degeneration of dopaminergic neurons existing in the nucleus part of the midbrain called as *substantia nigra pars compacta* (SNpc).

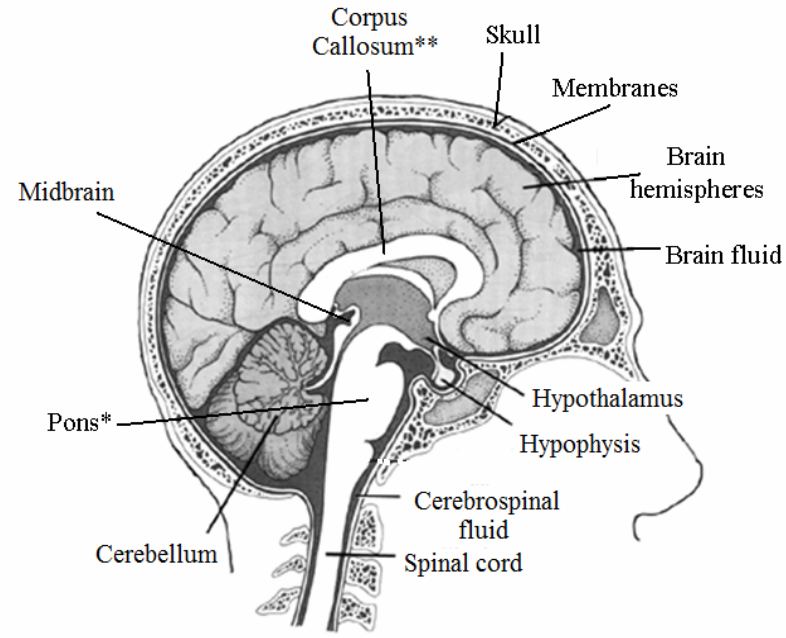

Figure 2.2. Important parts of brain. (*Pons: Mass consisted of CNS nerve cells and fibers in front of the cerebellum **Corpus Callosum: Center controlling generally paresis ve physical disorders)

In addition, since presence of neurons deposited in the cytoplasm called Lewy bodies and increased oxidative stress depending on increase in the reactive oxygen species in the midbrain lead to death of the dopamine producing neurons, they are shown amongst the causes of the disease (Houghton et al., 2005). The other important reasons of Parkinson disease related to these factors are known as;

- Decrease in dopamine level in the brain,
- Imbalance between neurotransmitters (Gamma-aminobutyric acid (GABA), Acetylcholine, Norepinephrine, Serotonin),
- Formation of free radicals.

Although Parkinson disease seen in many people commonly in elders, even rarely it has been observed also in people below the age of 40 (Houghton et al., 2005). Increase in tremors in arms and legs, muscular rigidity (dyskinesia), slowing down of movement (bradykinesia) and loss of balance as well as cognitive dysfunction and memory loss that are also seen in Alzheimer disease are known as clinical symptoms of Parkinson disease (Zhu, 2004; Dingemanse, 1997). In later stages of the disease, depression, impairment in speech, mental confusion, sleeping disorder, dotage, and constipation are observed as secondary symptoms (Polymeropoulos et al., 1996).

2.1.2. Treatment Methods for Parkinson Disease

Until the late of 1950s, memory loss and tremor have been known as common symptoms of many neurodegenerative disorders and the patients have generally been using common treatment methods and drugs against these symptoms.

Currently, it has been partly possible to distinguish between neurodegenerative disorders that have not been treated yet exactly and specific drugs to each of them have been developed by also taking the advantage of the knowledge and experiences (Houghton et al., 2005). Various drugs developed for the treatment of Parkinson disease have been categorized into subclasses and these have been used clinically either solely or together depending on the stage of the disease. Furthermore, surgical operations have been applied for the treatment at later stages of the disease.

2.1.2.1. Pharmacotherapy (Drug Treatment)

The commercially available drugs that control the various symptoms of Parkinson disease include:

- Dopamine agonists (substances exhibiting similar effects of dopamine),
- Levodopa/dopa decarboxylase inhibitors (benserazide, carbidopa),
- Anticholinergic agents[1],
- Amantadine[2],
- Monoamine oxidase-B (MAO-B)[3] inhibitors (selegiline),
- Catechol *O*-methyltransferase (COMT) inhibitors (Nadeau, 1997).

Although these drugs can not behave exactly as dopamine and can not supply the lack of dopamine which is the main reason of Parkinson disease, they can prevent the decrease and balance the levels of dopamine and acetylcholine in the brain. The drugs clinically used in the treatment of Parkinson disease can not treat the disease completely but can provide improvement by decreasing the symptoms observed in the patients (Nadeau, 1997).

It is the most important criteria to determine the stage of the disease in selection of the drug that will be applied in the pharmacological treatment. Before deciding on any drug, the Parkinsonizm level of the patient must be determined. The algorithm for the treatment methods applied depending on the stage of the disease is shown in Figure 2.3.

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[1] Anticholinergic agents: substance or agent withstanding the effects of acetylcholine and paralyzing the glands and muscle elements inerved by the cholinergic fibres (atropine, scopolamine)

[2] Amantadine: compound (drug) which is known to have dopaminergic, adrenergic and slightly anticholinergic affects that diminishes the conditions such as nervousness, insomnia and anxiety as well as the Parkinsonism symptoms.

[3] Monoamine oxidase-B: Monoamine oxidase enzyme catalyzes the oxidation rection of the monoamines. It metabolizes amine neurotransmitters such as dopamine, norepinefrine and serotonin. This enzyme is present on the outer membrane of mitochondria and is synthesized by monoamine oxidase A gene. It has two types called MAO-A and MAO-B. Both are present in neurons and astroglias. MAO-A is present in liver, digestion system and plesanta as well as in central nervous system. MAO-B is present mostly in blood platelets.

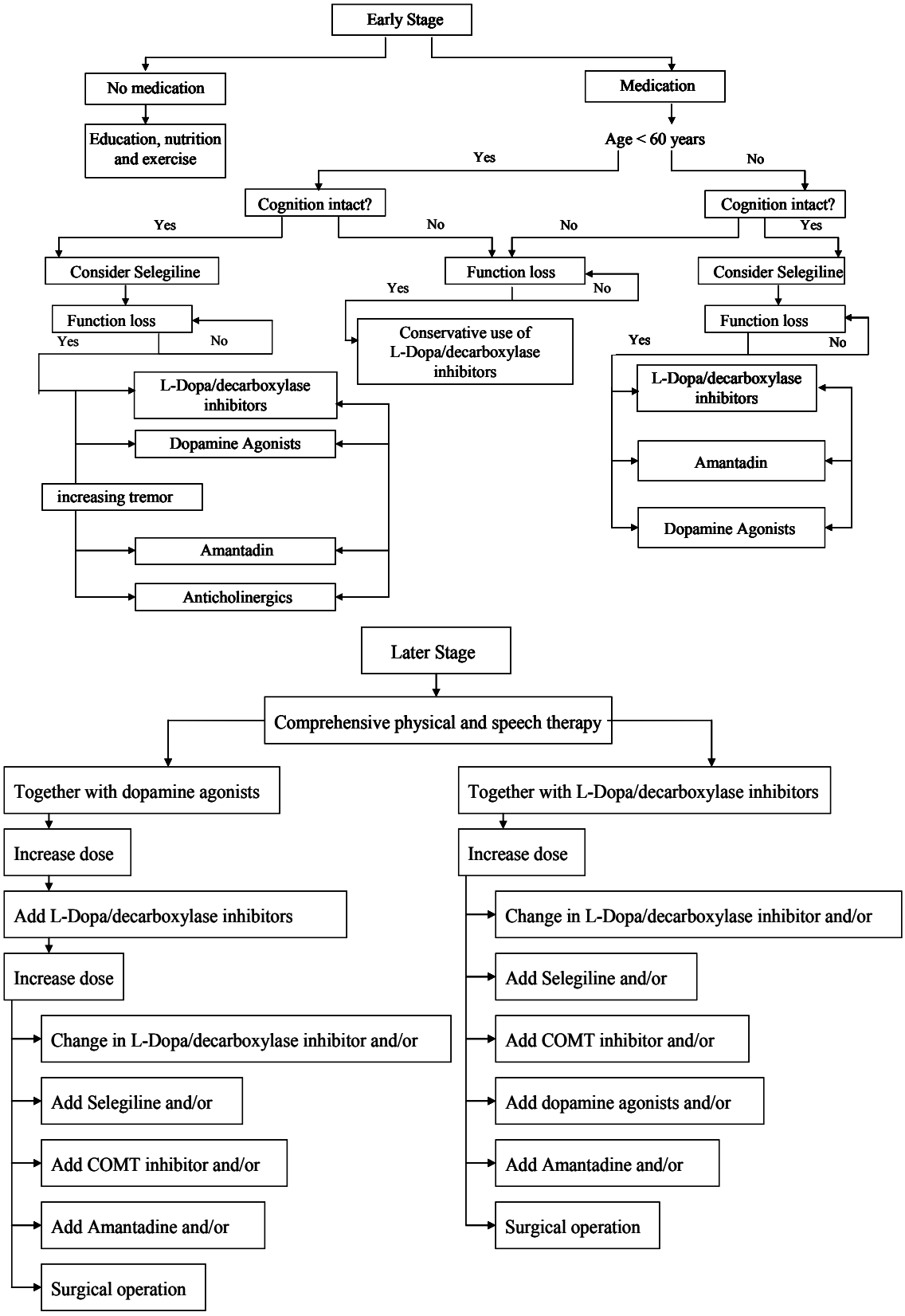

Figure 2.3. Algorithm for the treatment method of Parkinson disease depending on its stage

2.1.2.2. Surgical Operations

Before the development of L-dopa which is the main drug for treatment of Parkinson disease, the patients have been subjected to surgical operations at early stages of the disease. As seen from the algorithm given in Figure 2.3, today surgery is the last choice at the stage where the drug treatment applied has no use in relief of the symptoms. The main purpose of the surgery is breaking off the nerve connections in thalamus[1] and globus pallidus[2] parts in the brain (Lozano et al., 1998).

- Stereotaxic technique: It was firstly employed in 1950 before the development of L-dopa. This technique is used in neurosurgery and neuroradiology for the purpose of 3-dimensional radiographic and topographic investigations of the deep parts of the brain by means of an electrode inserted through a hole opened on the skull (Singh et al., 2007).
- Pallidotomy: This technique firstly employed in 1940's is still used for the surgical treatment of Parkinson disease (Lozano et al., 1998). In the application of pallidotomy, again a hole is opened through the skull and the globus pallidus part is burned by means of a heated wire inserted through the hole. Although this technique carries many risks, the symptoms of the disease can be alleviated by breaking off the connection of the dopamine circuit with the thalamus. After the pallidotomy application, the patients continue to take their drugs at the previous dosage (National Institute of Neurological Disorders and Stroke, 2009; National Parkinson Foundation, 2009; Singh et al., 2007).
- Thalamotomy: Tthis technique carrying risks as much as pallidotomy was first applied in 1950's. During application, lesions formed in the nucleus of the thalamus are removed using an extremely cold metal rod and thereby the tremblings of the patients are reduced (Lozano et al., 1998).
- Stimulation of the Brain: In this technique, which was first practisized in 1997, the brain is stimulated by use of electrodes inserted in specific parts of the thalamus (Singh et al., 2007).

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[1] Thalamus: Center which is present in the inner center of the brain controlling all senses except for the scent and responsible for transmission of knowledge to other control centers.

[2] Globus Pallidus: One of the important ganglions presents out of and above the thalamus including nerves responsible for transmission of movement, emotions and senses.

- Transplantation: In transplantation method, cells taken from medulla of the patient's own adrenal gland are transplanted in place of the diseased cells (Parkinson's Disease Society, 2009; Singh et al., 2007).

Due to severity and intensity of the methods applied for the treatment of Parkinson disease including pharmaceutical therapy and surgery, they are still hot topics for the researchers. From this point, pathology, etiology, synthetic and natural treatment methods of the disease have been extensively investigated.

2.1.3. Important Criteria in Drug Development for the Treatment of Parkinson Disease

Similar to the most of neurodegenerative diseases, difficulty in delivery of substances used as drug in the treatment of Parkinson disease to the specific parts of the brain is one of the most important problems encountered. Therefore, during drug discovery and development, structural characteristics and functions of the certain parts, naturally present in the structure of the brain where transition of chemical substances takes place, have been investigated.

2.1.3.1. Blood-Brain Barrier

Blood-Brain Barrier (BBB) is a highly specific barrier system that is consisted of epithel cells. Besides, it includes pericyte, astrocyte and neuronal cells in its structure (De Boer et al., 2007). Since the epithel cells in the form of segments that are attached to each other allow partial transition of the molecules, they provide most of selectivity to the barrier. The most vital function of the BBB that is responsible for the transition of molecules, DNA and enzymes is to protect the brain stability and to separate the brain cells from the blood (De Boer et al., 2007; Sumio, 2004). Moreover, BBB has functions of transition of agents that can be used in diagnosis and treatment of the neurological diseases and consequently transmission of those agents to the brain.

Although dopamine, for which decrease in its level in the brain is the main reason of Parkinson disease, is a neurotransmitter that can not pass through the BBB, L-dopa, which is known as the precursor of dopamine, can pass through the BBB (Dingemanse, 1997). Yet, since transition of most of the therapeutic molecules like dopamine and genes through the BBB in desired amounts cannot be allowed, it is a

criteria that should be taken into account during the drug development. The potential transport types through the BBB are explained by the known diffusion mechanisms in Figure 2.4 (De Boer et al., 2007).

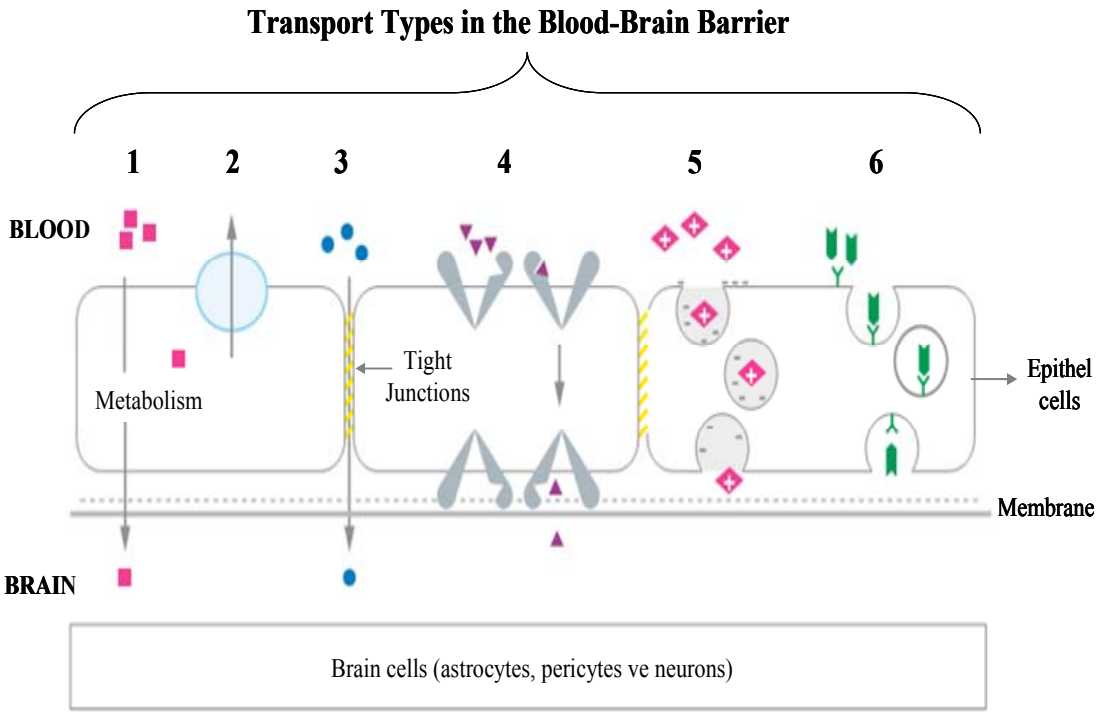


Figure 2.4. Possible transport types through BBB. (**1.** Lipophilic transport, **2.** Ionic transfer, **3.** Hydrophilic transport, **4.** Transport with carriers, **5.** Transport by adsorption, **6.** Transport with receptors)

2.1.3.2. Brain-Spinal Cord (Cerebrospinal) Fluid Barrier

The fluid in the subaracnoid gap and in the spinal cord channel which is one of the brain membranes is called as cerebrospinal fluid (CSF). CSF is generally formed within the glomus of capillaries (coroid plexus) extended to specific regions on the wall of small voids (ventricules). CSF, which is a dynamic fluid, joins to the blood circulation through being absorbed by the veins in the brain. This liquid formed in the coroid plexus, plays an important role in the drug metabolism and transfer as well as in the cell regeneration and neuron transmission (De Boer et al., 2007; Zheng et al., 2004). Moreover, CSF helps the phase I-II and III enzymes present in the coroid plexus to preserve their activation (De Boer et al., 2007).

The activation of COMT, which is one of the important phase I-III enzymes as explained in detail in Section 2.1.4, and regulation of the metabolic reactions related to this enzyme are also controlled by CSF (Zheng et al., 2004).

2.1.4. COMT Enzyme and Its Role in Parkinson Disease

The drugs used in the treatment of neurodegenerative disorders are first adsorbed and then delivered to the specific targets in the body. The drugs reached to the target are metabolized and excreted (Lautala, 2000). The metabolic transformation of the drugs occurs through many enzymatic reactions. The enzymes taking part in the metabolic reactions are called as phase I-II and III enzymes. The phase I enzymes such as Cytochrome P-450 and MAO contribute to the oxidation, reduction and hydrolysis reactions and thereby the forms of the drugs that can bind to the related functional groups are obtained (De Boer et al., 2007; Lautala, 2000). The phase II enzymes, mainly including glucuronozyl, glutathione, sulfo, acetyl and methyltransferases, catalyze the reactions required for the metabolizing of the conjugated forms of the drugs (Lautala, 2000). As a result of these reactions, pharmacologically active compounds are formed. Stability of the formed active compounds and the microenvironment where they present is preserved by the phase III enzymes known as sodium-dicarboxylate, ascorbic acid, organic anion and cation carriers (De Boer et al., 2007).

Catechol *O*-methyltransferase (COMT; EC 2.1.1.6), which is one of the phase II enzymes, present in kidney, liver and brain in mammalians. Furthermore, the activity of COMT was also observed in the gastrointestinal channel, muscles, erytrocytes, and in some organs and tissues (Reenilä, 1999; Guldberg et al., 1975). The COMT enzyme, which is coded by a single gene and different promoters, has two different forms having different amino acid chain numbers (Lotta et al., 1995). The activities of the soluble (S-COMT) and membrane-bound (MB-COMT) forms change depending on their cellular distribution, sex and age (Bonifacio et al., 2007; Lautala, 2000; Gogos et al., 1998; De Santi et al., 1998).

S-COMT that present in humans and recombinant S-COMT isolated from rat exhibited structural similarities in 81% as revealed by the molecular biologists (Lautala, 2000). Hence, in the studies on determination of COMT activity *in vitro*, recombinant S-COMT isolated from rat has been used. Firstly in 1994, Vidgren and co-workers studied the three dimensional structure of the complex that is formed by the enzyme and

its substrates and the inhibitor in order to elucidate the activity of recombinant S-COMT isolated from rat and molecular mechanism of the methylation reaction (Bonifacio et al. 2007; Lautala, 2000). According to the results of this study on the crystal structure of S-COMT, complex β-sheets and eight α-helixes surrounding them are present in the center of the enzyme. This topology is also observed in other methyltransferases that use S-adenosyl-L-methionine (SAM) like DNA and RNA methyltransferases. Also, it was revealed that the catalytic region of the COMT enzyme is present at the center and on the surface of the giant protein structure (Bonifacio et al., 2007). In the catalytic region of the enzyme schematically illustrated in Figure 2.5, there are two distinct regions, the one where SAM binds and the other where catecholic substrate binds (Bonifacio et al., 2007). SAM, which is the methyl source, is buried in the structure at the catalytic region, while the other region, where the catecholic substrate binds, is in the space on the surface (Lautala, 2000).

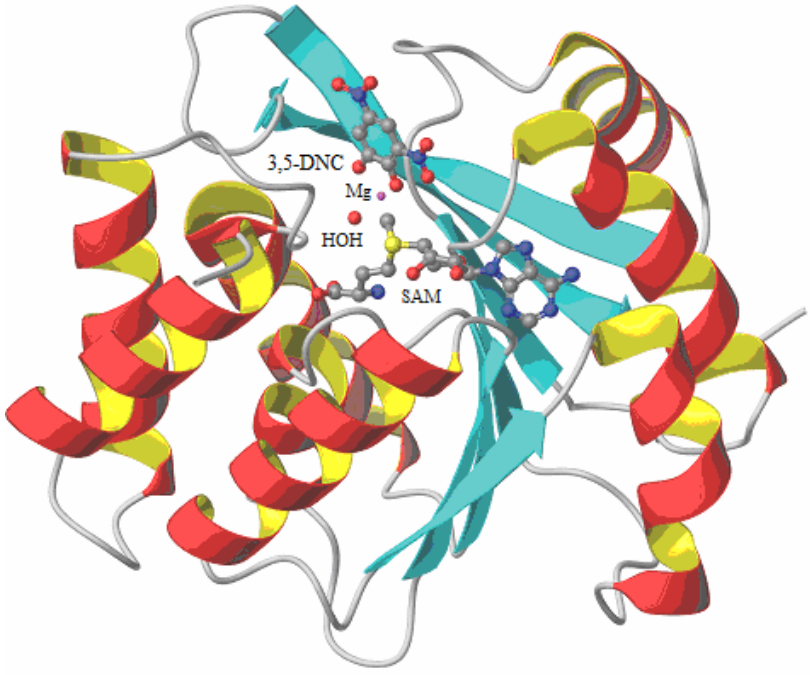


Figure 2.5. 3D representation of S-COMT isolated from rat. (3,5-DNC: 3,5-dinitro-catechol (COMT inhibitor))

The magnesium ion (Mg^{2+}), which is used as cofactor by COMT, is coordinated by a water molecule, two aspartic acid residues (Asp141, Asp169), an asparagine residue (Asn170) and hydroxyl groups of the catechol molecule via hydrophobic forces (Bonifacio et al., 2007; Lautala, 2000). The giant protein structure of the enzyme is preserved by this coordination. The structurally important Mg^{2+} lowers the pK_a values of the hydroxyl groups of the catecholic substrate and causes them to ionize faster (Lautala, 2000). One of the ionized hydroxyls is stabilized by forming a hydrogen bond to the negatively charged carboxyl group of the glutamic acid residue (Glu199) as shown in Figure 2.6. The other hydroxyl gives a proton to the lysine residue (Lys144) and breaks bond of the electron deficient methyl group of SAM (Bonifacio et al., 2007; Lautala, 2000). This reaction, which is explained by the nucleophilic substitution mechanism (S_N2 type) shown in Figure 2.7, is completed by binding of the methyl group broken from the SAM to the proton-deficient hydroxyl group of the catecholic ring (Lautala, 2000).

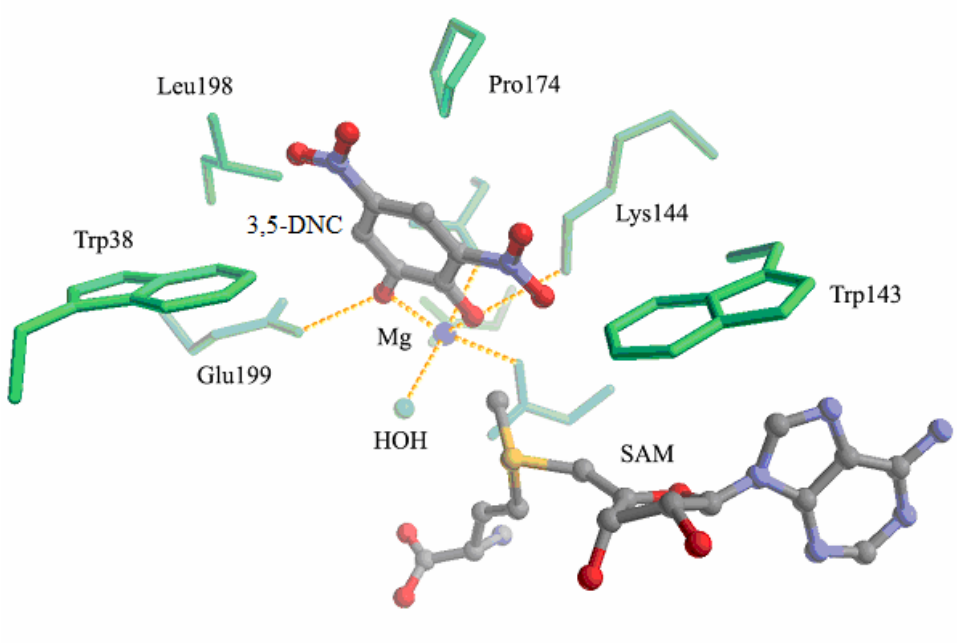


Figure 2.6. Catalytic region of S-COMT.

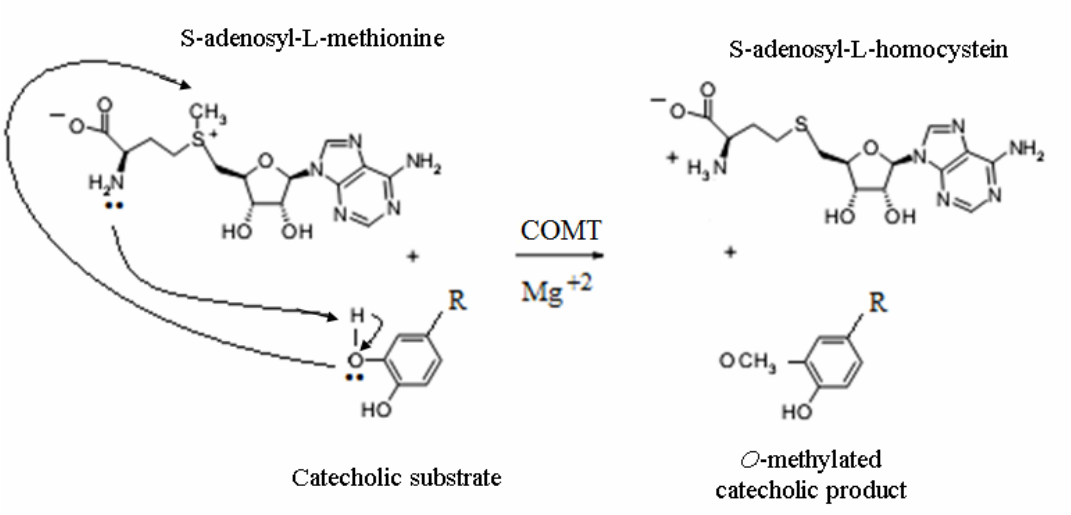


Figure 2.7. General mechanism of the COMT catalyzed methylation reaction.

In the molecular level studies related to the COMT enzyme, the best known inhibitor of the enzyme 3,5-dinitrocatechol, has been used as the catecholic compound which are given in Figures 2.6 and 2.7. However, biologically active or xenobiotic endogenous and exogenous catecholic compounds are the substrates of physiological COMT and metabolized through *O*-methylation reaction (Bai et al., 2007; Kurkela et al., 2004; Erdal et al., 2002; Ruottinen et al., 1998; Lotta et al., 1995). The catecholamines (dopamine, epinephrine, norepinephrine), catecholestrogens (2 and 4-hydroxyestradiol), L-dopa (levodopa) and ascorbic acid that can be methylated by COMT enzyme are the well-known endogenous catecholic substrates (Chen et al., 2005; Mannistö et al., 1999; Lotta et al., 1995). The exogenous catecholic substrates are known as compounds in our dietaries and some medicines such as triphenols, flavonoids, benserazide, carbidopa and dihydroxyphenyl serine (Bai et al., 2007; Chen et al., 2005; Lautala, 2000; Mannistö et al., 1999).

These catecholic compounds, also used as substrates of COMT, are administered solely or in accompaniment to the other drugs as previously mentioned in the Section 2.1.2.1. The metabolic roles of the COMT inhibitors, of catecholic compounds, especially of levodopa which can pass through the blood-brain barrier (BBB), and of aromatic L-aminoacid decarboxylase (AADC) and MAO-B inhibitors in the treatment of Parkinson disease are summarized in Figure 2.8.

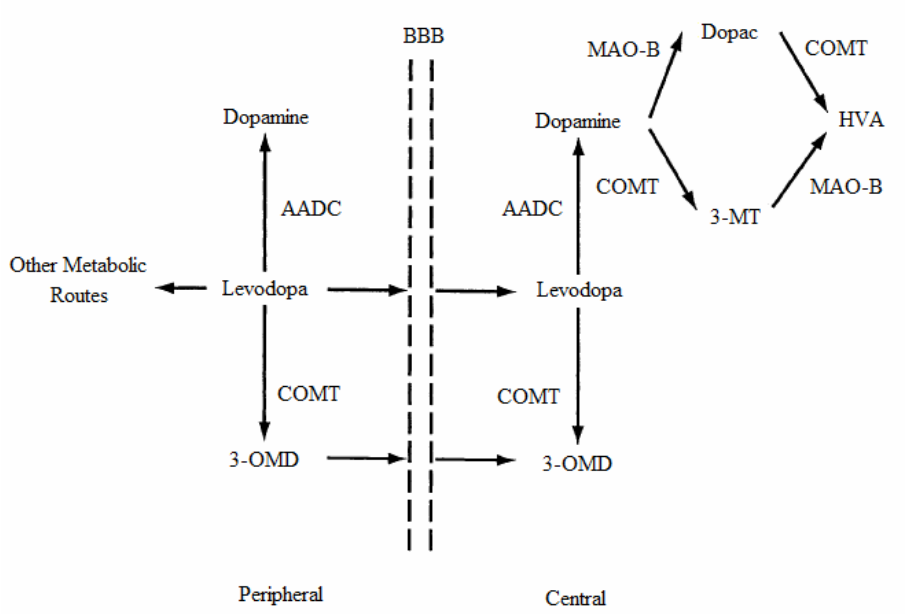


Figure 2.8. Metabolic routes effective in Parkinson disease. 3-OMD: 3-*O*-methyldopa, Dopac: 3,4-dihydroxyphenylacetic acid, 3-MT: 3-methoxytyramine, HVA: homovanillic acid (Source: Dingemanse, 1997).

It was previously stated in detail in the Section 2.1.1 that the deficiency of dopamine level in the brain (central) is one of the most important reasons of Parkinson disease. In the treatment of the disease, it was claimed that co-administration of compounds like carbidopa, which is used as the AADC inhibitor, with the COMT inhibitors leads to decrease in the peripheral dopamine level (Dingemanse, 1997). However, by this treatment method, the level of levodopa, which is the precursor of dopamine and can pass through the blood-brain barrier (BBB), can be increased and thereby the central dopamine level can be adjusted to the desired levels. In Figure 2.8, the reactions catalyzed by MAO-B and COMT enzymes causing transformation of the central dopamine are shown. From this point, during the treatment depending on the stages of disease, the MAO-B inhibitors such as selegiline is used in addition to the COMT inhibitors so that the central dopamine level can be adjusted at the desired level (Mannistö et al., 1992).

2.2. COMT Inhibitors

Towards the end of 1950's, the purification techniques have been improved and studies on discovery and development of inhibitor for the structurally-characterized COMT enzyme have been emerged. The standard substances used as COMT inhibitors or their chemically-synthesized forms can be classified into the groups based on their historical development.

2.2.1. Synthetic Inhibitors

2.2.1.1. First Generation COMT Inhibitors

Pyrogallol which was invented by Axelrod and LaRoche in 1959 was defined as the first potential COMT inhibitor and has been used in the following years as the model inhibitor in the studies on the inhibitor development (Guldberg et al., 1975). The substances developed as the first generation inhibitors including gallic acid, caffeic acid, 3`,4`-dihydroxy-2-methyl-propiophenon (U-0521), 2-hydroxyestrogen are the derivatives of pyrogallol and catechol (Bonifacio et al., 2007; Bailey et al., 2005). Among the non-catecholic substances, ascorbic acid, tropolone, 8-hydroxyquinoline derivatives, 3-hydroxylated pyrones and pyridones are also used as the first generation COMT inhibitors (Bonifacio et al., 2007; Mannistö et al., 1999). Furthermore, some flavonoids such as quercetin and rutin are also known as inhibitors of this class of inhibitors (Bonifacio et al., 2007).

Although the first generation COMT inhibitors have high *in vitro* inhibition performances, results of the animal tests did not show the successful inhibition. These inhibitors with low *in vivo* inhibition properties and selectivity, with short efficacy periods and having toxicity could not been found a role in clinical applications (Mannistö et al., 1999; Dingemanse, 1997; Guldberg et al., 1975).

2.2.1.2. Second Generation COMT Inhibitors

New COMT inhibitors most in nitrocatecholic structure were synthesized in the late 1980's. As structurally shown in Figure 2.9, entacapone [OR-611; (*E*)-2-cyano-*N,N*-diethyl-3-(3,4-dihydroxy-5-nitrocinnamide)], nitecapone [OR-462; 3-(3,4-dihydroxy-5-nitro-benzylidine)-2,4-pentadion], tolcapone [Ro 40-7592; 4`-methyl-3,4-dihydroxy-5-nitro-benzophenon], nebicapone [BIA 3-202; 1-(3,4-dihydroxy-5-nitrophenyl)-2-phenyl-ethanone], some nitrobenzaldehydes and nitronoradrenalines are nitrocatecholic substances developed as second generation COMT inhibitors (Bonifacio et al., 2007; Loureiro et al., 2006; Mannistö et al., 1999). Other than the nitrocatecholic inhibitors, the inhibitor coded as CGP 28014, a pyridine derivative, is also known as second generation COMT inhibitor (Mannistö et al., 1999; Dingemanse, 1997).

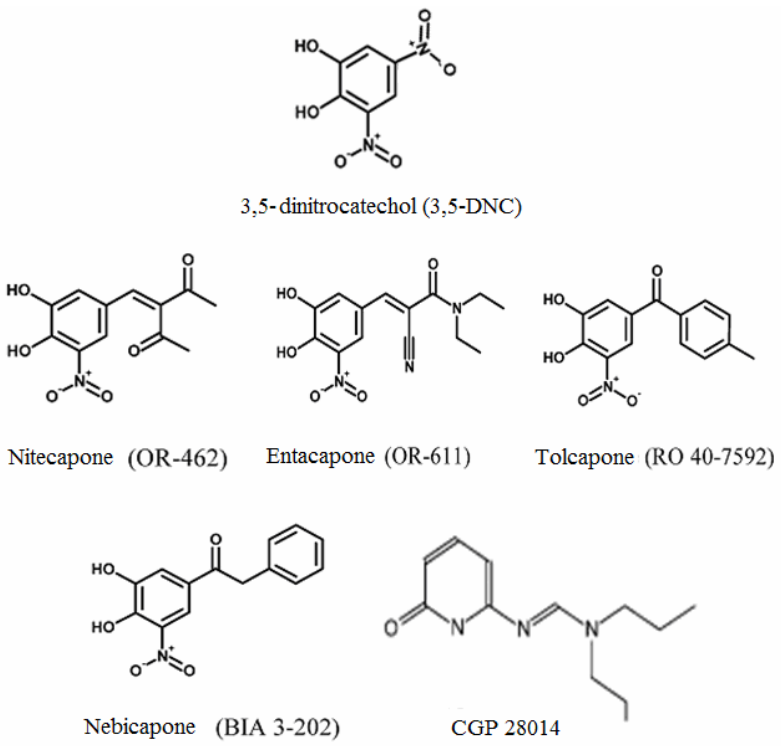


Figure 2.9. Some second generation COMT inhibitors
(Source: Bonifacio et al., 2007; Palma et al., 2006; Mannistö et al., 1999).

The nitrocatecholic inhibitors are synthesized from the well-known synthetic COMT inhibitor 3,5-dinitrocatechol (3,5-DNC) compound. As shown in Figure 2.9, the synthesis is performed by binding of another functional group in place of one of the nitro groups which is present in *orto* and *para* positions of the catechol ring (generally

para position) in the structure of 3,5-DNC with respect to the same hydroxyl group (Bonifacio et al., 2007; Learmonth et al., 2002; Bäckstrom et al., 1989). The CGP 28014 coded pyridine derivative inhibitor is obtained by addition of specific functional group after being transformed to pyridone (Waidmeier et al., 1990).

Among the inhibitors with higher potential and selectivity as compared to the first generation inhibitors, nitecapone, entacapone and tolcapone are known as the second generation inhibitors which have been used clinically (Ruottinen et al., 1998; Dingemanse, 1997). It was reported that these inhibitors in nitrocatecholic structure are known as reversible and tight binding inhibitors of COMT (Bonifacio et al., 2007; Palma et al., 2006; Mannistö et al., 1999). The concentrations of nitecapone, entacapone and tolcapone required to decrease the peripheral COMT activity by 50 percent (IC_{50}) were reported as 300 nM, 150 nM and 36 nM, respectively. The IC_{50} values of them for the brain COMT activity were found to be 10 nM, 20 nM and 0.3 nM, respectively (Bonifacio et al., 2007; Mannistö et al., 1999). These values indicated that the nitrocatecholic inhibitors were 1000 times more effective than the first generation inhibitors (Bonifacio et al., 2007).

It was found that mode of action of nitecapone is primarily effective on duodenum so, its use in the treatment of Parkinson disease has been limited (Nissinen et al., 1988). Currently, the other potential COMT inhibitors (entacapone and tolcapone) are used in combination with MAO-B and AADC inhibitors, and levodopa (Singh et al., 2007; Ruottinen et al., 1998; Dingemanse, 1997).

According to the results of the studies on treatment methods applied to the Parkinsonians, entacapone showed high peripheral COMT inhibition, while tolcapone, which can pass through the blood-brain barrier, decreased the both central and peripheral COMT activity. Moreover, the efficacy period of tolcapone was found to be longer than that of entacapone almost twice (Bonifacio et al., 2007; Mannistö et al., 1999; Ruottinen et al., 1998).

Although new COMT inhibitors as effective and clinically applicable as these two compounds have not been developed yet, it is known that their use in high concentrations caused some toxic or carcinogenic effects on the liver (Singh et al., 2007; Bonifacio et al., 2007; Mannistö et al., 1999; Ruottinen et al., 1998). The administration of tolcapone, which has higher hepatoxicity as compared to entacapone, has been banned in some countries (Europe and Canada) (Singh et al., 2007; Mannistö et al., 1999; Nissinen et al., 1992).

The researches on the development of catecholic based inhibitors with high selectivity have been going on in spite of the inhibitors in nitrocatecholic structure which are highly effective in inhibition but have significant adverse side effects.

2.2.1.3. Last Generation – Non-typical COMT inhibitors

According to the classification of the COMT inhibitors based on their historical development, CGP 28014 coded compound defined as a second generation inhibitor has been later included in the group of non-typical COMT inhibitors. This compound that is unsuccessful in *in vitro* COMT inhibition, it resulted in significant inhibition effect especially on the central COMT inhibition (Mannistö et al., 1999, 1992; Dingemanse, 1997). However, studies on mode of action and toxicity of this compound have not been completed yet.

Many COMT inhibitors included in this class are compounds that are structurally modified from the nitrocatecholics and are gained some specific properties (Bonifacio et al., 2007; Paulini et al., 2006).

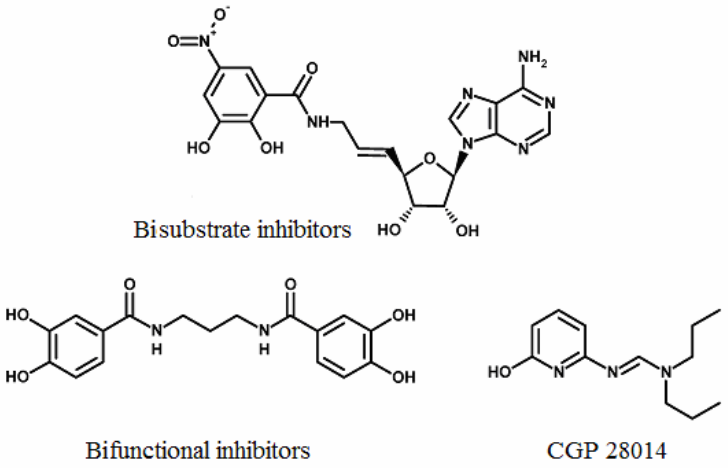


Figure 2.10. Some last generation COMT inhibitors.

Bisubstrate inhibitors are synthesized as structures that can bind to the SAM and catechol binding regions in the active site of COMT enzyme and thereby it might prevent the interaction of the enzyme with its substrates that may lead to the disease (Paulini et al., 2006; Learmonth et al., 2005; Lerner et al., 2003). Among the bisubstrate inhibitors having several structurally-characterized derivatives, the *in vitro* IC_{50} value

for the [N-{(*E*)-3-[(2R,3S,4R,5R)-5-(6-Amino-9*H*-purin-9-yl)-3,4-dihydroxytetrahydro-furan-2-yl]prop-2-enyl}-2,3-dihydroxy-5-nitrobenzene-1-carboxa-mide] compound was found as 9 nM (Bonifacio et al., 2007; Lerner et al., 2003).

The bifunctional inhibitors are formed through synthesis of two catechol rings, a nitro group is added to each. It was claimed that *in vitro* inhibition potentials of these types of compounds are higher than those of their analogs with single catechol ring (Bailey et al., 2005).

On the other hand, *in vivo* data on the bisubstrate inhibitors as well as the bifunctional ones has not been confirmed, the mechanisms of them have not been elucidated and the toxicity studies have not been performed yet (Bonifacio et al., 2007).

2.2.2. Natural Inhibitors

During last years, researches on the potential utilization of some natural compounds that are generally obtained from plants (phytochemicals) in the treatment of neurodegenerative diseases have been increased. Since plant-based compounds can be metabolized easily in the body and have less side effects, their utilization in inhibition of enzymes catalyzing some metabolic reactions causing neurodegenerative diseases is extremely important (Perry et al., 1999).

In the literature, within the large class of phytochemicals, studies on inhibition of AChE (acetylcholine esterase), COMT (catechol-*O*-methyltransferase) and MAO (monoaminoxidase) enzymes playing important roles in Alzheimer and Parkinson diseases by especially flavonoids, flavonoid glycosides, catechins and alkaloids are available (Chen et al., 2006; Kadowaki et al., 2005; Kong et al., 2004; Perry et al., 1999). On the other hand, according to these studies, in order to accept a substance that can inhibit at least one of these enzymes as a new drug, the interactions of this compound with the other enzymes, metabolites and receptors must be elucidated. Therefore, the plants employed to inhibit AChE, COMT and MAO enzymes are summarized in Table 2.1.

Table 2.1. Plants of which therapeutic potentials have been investigated in the treatment of Alzheimer and Parkinson diseases.

Family	Botanical name	Known name	Active Ingredients	Metabolic Functions	Reference (s)
Theaceae	*Camellia sinensis*	Tea (green)	Catechin and catechin gallats	COMT inhibition	Chen et al., 2005 Nagai et al., 2004 Zhu et al., 2004
Arecaceae	*Areca catechu*	Areca nut palm	alkaloids (pilocarpin), tannin and gallic acid	AChE inhibition	Houghton et al., 2005
Piperaceae	*Piper betel*	Betel pepper	catechins and alkaloids	AChE inhibition	Houghton et al., 2005
Labiateae	*Salvia officinalis*	sage	Flavonoids and essential oils	AChE inhibition	Houghton et al., 2005
Labiateae	*Melissa officinalis*	melisse	monoterpenes (essential oils)	AChE inhibition, antidepressant	Houghton et al., 2005 Wake et al., 2000
Labiateae	*Salvis Lavandulaefolia*	Not known	monoterpenes (essential oils)	AChE inhibition	Perry et al., 2002
Ginkgoaceae	*Ginkgo biloba*	Ginkgo, Maidenhair Tree	Flavonoid glycosides, terpenes	AChE and MAO inhibition	Sloley et al., 2000 Perry et al., 1999
Malpighiaceae	*Banisteriopsis caapi*	Ayahuasca, caapi	alkaloids (beta carbolines)	COMT and MAO inhibition	Schwarz et al., 2003
Cannabaceae	*Cannabis sativa*	cannabis	Cannabinoids	Cholinergic and dopaminergic activity increase	Joy et al., 1999
Cupressaceae	*Biota orientalis*	*Platycladus*	Not known	Anticholinergic agent, MAO inhibition	Howes et al., 2003 Lin et al., 2003
Liliaceae	*Lilium brownii*	*Brownii lily*	steroid saponines and alkaloids	MAO inhibition	Lin et al., 2003
Rubiaceae	*Gardenia jasminoides*	Gardenia	Glycosides and phenolic acids	MAO inhibition	Lin et al., 2003
Rubiaceae	*Uncaria rhynchophylla*	caper berry	alkaloids (indole)	AChE and MAO inhibition	Hou et al., 2005 Mamedov, 2005
Huperziaceae	*Huperzia serrata*	cinque foli	alkaloids (huperizin A)	AChE inhibition	Cheng et al., 1998, 1996 Zhang et al., 1991

(continued on next page)

Table 2.1. (cont.)

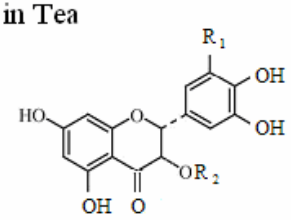
Family	Binomial name	Known name	Active ingredients	Metabolic Functions	Reference (s)
Labiatae	*Origanum majorana*	Marjoram	Flavonoids and essential oils	AChE inhibition	Chung et al., 2001
Apiaceae	*Bacopa monniera*	Coastal waterhyssop, Brahmi	Alkaloids and saponins (bacosite)	Learning and comprehension strength	Howes et al., 2003 Perry et al., 1999
Solanaceae	*Withania somnifera*	chinese lantern	Steroids (witanolites)	Anticholinergic agent	Perry et al., 1999 Schliebs et al., 1997
Guttiferae	*Hypericum perfaratum*	St.John's wort	flavonoids, tannins, naphtodiantrons (hyperphorin, hypericin)	Memory strength, antidepressant	Mamedov, 2005 Perry et al., 1999 Chatterjee et al., 1998
Zygophyllaceae	*Peganum harmala*	Harmal	Alkaloids (beta carbolins)	KOMT and MAO inhibition	Akalın, 2003 Schliebs et al., 1997
Rosaceae	*Rosa Damascena*	Rose	Essential oils	Learning and comprehension strength	Köse et al., 2007
Fabaceace	*Mucuna pruriens*	A kind of aquarium plant	Not known	Dopamine agonist	Manyam et al., 2004
Papaveraceae	*Papaver somniferum*	Poppy	Alkaloids (papaverin, codein)	AChE and COMT inhibition	Perry et al., 1999
Alliaceae	*Galanthus nivalis*	Snowdrop	Alkaloids (galantamine)	AChE inhibition, memory strength	Olin et al., 2001a, 2001b

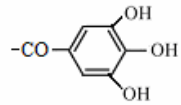
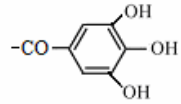
Regarding the divergence of the plants given in Table 2.1, they serve many resources that can be applied for the treatment of diseases of those etiologies have not been entirely defined yet and seen in many people around the world such as Alzheimer and Parkinson diseases. It was reported that plants that are specifically rich in alkaloids, terpenes, flavonoids and flavonoid glycosides as active ingredients have potential therapeutic applications.

However, since isolation of each of the active ingredients of plants separately, given in Table 2.1, requires time and money, the raw extracts of these plants and rarely their standards have been used in the studies. Among the plants that are active in inhibition of COMT enzyme, generally the *Camellia sinensis* (green tea) extract rich in catechin and catechin derivatives and the standards of the fractions has been studied in detail.

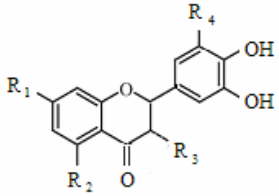
Tea catechins and flavonoids found in abundant amounts in our dietaries are compounds having catechol ring and the structures of those used in the COMT inhibition are represented in Figure 2.11 (Zhu et al., 2004).

Phenolics in Tea



SAMPLE	R_1	R_2
(-) Epicatechin	-H	-H
(+)-Epicatechin	-H	-H
(-)-Epigallocatechin (EGC)	-OH	-H
(-)-Epicatechin gallat (ECG)	-H	
(-)-Epigallocatechin gallat (EGCG)	-OH	

Bioflavonoids



SAMPLE	R_1	R_2	R_3	R_4
Quercetin	-OH	-OH	-OH	-H
Ficetin	-H	-OH	-OH	-H
Luteolin	-OH	-OH	-H	-H
Rhamnetin	$-OCH_3$	-OH	-OH	-H
Myricetin	-OH	-OH	-OH	-OH

Figure 2.11. Some polyphenolics used as COMT inhibitors.

In the studies performed using the compounds shown in Figure 2.11, it was reported that the structure-activity relationship between polyphenolics and catecholestrogens found in human liver for their COMT catalyzed O-methylation

inhibition (Zhu et al., 2008; Bai et al., 2007). It was revealed that catechins containing galloil type D-rings (ECG and EGCG) have higher potential than quercetin which is known as a strong inhibitor (Chen et al., 2005; Nagai et al., 2004; Zhu et al., 2001). It was suggested that 4`,4``-di-*O*-methyl-EGKG which is a metabolite formed by methylation of (-)-Epilgallocatechin gallate (EGCG), which exhibits the highest inhibition effect in the enzyme kinetic analysis, might also be a new type of COMT inhibitor (Chen et al., 2005; Kadowaki et al., 2005; Weinreb et al., 2004).

It has become a desired property that the drugs developed especially for the treatment of cancer and neurological diseases have multifunction. It was also known that Parkinson disease is a neurological disease that is caused by several mechanisms. Among these mechanisms, increase in oxidative stress (increase in free radical formation) is one of the mechanisms triggering the disease as previously mentioned (Cavalli et al., 2008). Consequently, the compounds administered against Parkinson disease should have antioxidant capacity as well as they have ability to inhibit the COMT enzyme.

It was reported that due to decrease in glutathione concentration in the *substantia nigra* of the Parkinson patients, lipid peroxidation has increased (Akyol, 2004). This substance is used as co-enzyme in the transformation reaction of peroxyl radicals to water molecule which is catalyzed by glutathione peroxidase enzyme (Akyol, 2004; Sirnonian et al., 1996). It was also known that increasing peroxyl radicals due to glutathione deficiency causes to loose the activity of alkaline phosphatase enzyme which is responsible for prevention of many proteins in the body (Bertolini et al., 2007). As a result of these mechanisms which are closely interrelated, destruction or misfolding of protein structures and increase in oxidative stress have been observed. Therefore, neuron deaths triggering Parkinson disease occur (Cavalli et al., 2008).

In order to prevent these effects of the free radicals, quercetin which can inhibit COMT enzyme and possesses antioxidant capacity has been employed in the studies. Quercetin, shown as one of the synthetic inhibitors of COMT enzyme, was found to have higher protective effect on alkaline phosphatase activity and its structure than currently-used drug tolcapone. Moreover, its potential to deactivate the free radicals is known higher than those of most nitrocatecholic COMT inhibitors (Bertolini et al., 2007).

Although it was confirmed that antioxidants can prevent Parkinson disease and retard its progress, they increase risk of especially breast cancer due to decrease in the

COMT activity (Chen et al., 2006; Van Duursen et al., 2004; Esposito et al., 2002; Suk Yin, 2002; Chiueh et al., 2000). It was reported that transformation of catecholestrogens and catecholamines, due to they can not undergo the methylation reaction, to the quinone forms raises the cancer risk (Van Duursen et al., 2004; Suk Yin, 2002).

2.2.2.1. Harmal (*Peganum harmala*) Seed

Harmal (*Peganum harmala*), one of the plants listed in Table 2.1, is a plant of the family *zygophyllaceae*. In ancient times, medicine obtained from the seeds of this plant was used as an anthelmintic (to omit parasitic worms) and as narcotics. It was also used to prepare antidotes against poisoning and snake bites. Greeks and Romans used harmal against diarrhea. Today, in modern medicine, harmal is used as an anthelmintic and as a central nervous system stimulant (Akalın, 2003). Moreover, in ancient societies, since it has aphrodisiacal properties, it was used to increase sexual power (Akalın, 2003).

Harmal seeds contain several types of alkaloids including betacarboline, tetrabetacarboline, quinasoline derivatives in more than 25 percent (Juma, 2005; Molchanov et al., 1996). The remaining part includes etheric oil, flavonoids and glycosides (Akalın, 2003; Sharaf et al., 1997). The seeds mostly contain betacarboline derivative alkaloids in 2-7 percent. Especially harmaline and harmine and other betacarboline derivative alkaloids such as harmalol, harmol, harman and peganin are the major alkaloids present in harmal seeds (Figure 2.12) (Grella et al., 1998; Ott, 1996; Khan, 1990). Additionally, a red dye can be extracted from the harmal seeds (Akalın, 2003; Kartal et al., 2003).

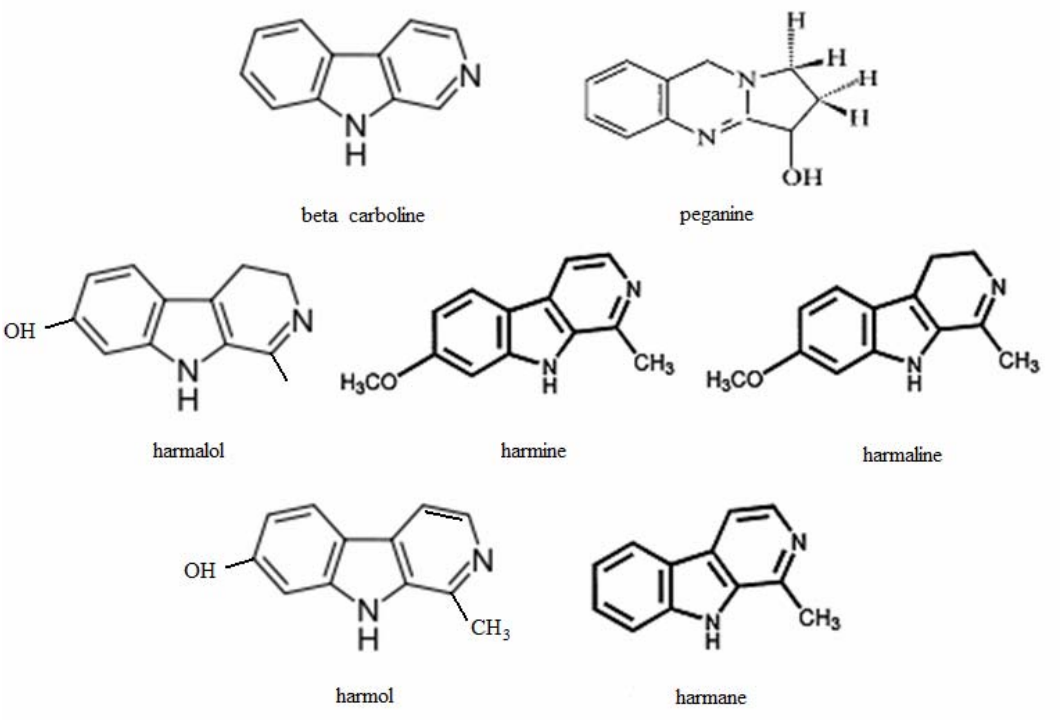


Figure 2.12. Some major alkaloids found in harmal seed.
(Source: Srivastava et al., 2001; Grella et al., 1998; Khan, 1990).

The numerous studies using especially harmaline and harmine as well as other harmal alkaloids shown in Figure 2.12 have been performed. These alkaloids can substitute serotonin, inhibit MAO enzyme, stimulate central nervous system, have amplification effect on immune system as well as hallucinogenic and antioxidant effects (Halpern, 2004; Kartal et al., 2003).

Moreover, peganine, which can pass through the blood-brain barrier, inhibits acetylcholine esterase and monoaminoxidase enzymes and thereby controls the acetylcholine and dopamine levels in the brain (Theodore, 1999). Although for most of the plants containing alkaloids, *in vivo* and cytotoxicity studies have not been completed yet, it was claimed that the alkaloids extracted from the plants in the belladonna, harmala and aporphine families can be utilized in the treatment at early stages of Parkinson disease (Theodore, 1999).

2.2.2.2. Olive (*Olea europaea*) Leaf

Olive tree, the most important member of the *oleaceae* family, is known as symbol of Mediterranean and peace since ancient times (Bonilla et al., 2006; Al-Azzawie et al., 2006). Olive leaves, which are referred even in the Bible, have been used as a medicine against fever and malaria since ages (Lee-Huang et al., 2003). Furthermore, since it can decrease the blood pressure and prevent spasms, it has been used for treatment purposes in animals and humans (Garcia et al., 2000). Today, the clinical studies and our daily experiences have showed that olive leaf extract is effective against diabetes, hepatitis, flu, open wounds, bacterial and viral infections and even HIV (Page, 2002).

It was stated that the high polyphenolics content of olive leaves is responsible for the health beneficial and therapeutic properties of olive leaves against several diseases. It was reported that oleuropein and its derivatives (verbascoside); flavones (luteolin-7-glycoside, apigenin-7-glycoside, diosmetin-7-glycoside, luteolin and diosmetin); flavonoles (rutin); flavan-3-ols (catechins) and substituted phenolics (tyrosol, hydroxytyrosol, vanillin, vanillic acid and caffeic acid) are the most widely present polyphenols in olive leaves (Garcia et al., 2000).

Among these polyphenolics, it was revealed that rutin, luteolin, hydroxytyrosol and oleuropein have the highest antioxidant capacities (Baycin et al., 2007; Garcia et al., 2000). Moreover, it has been possible to isolate the major polyphenolics rutin and oleuropein present in olive leaves of which the chemical structures are shown in Figure 2.13 by the silk fibroin adsorption technique (Baycin et al., 2007; Savournin et al., 2001; Garcia et al., 2000).

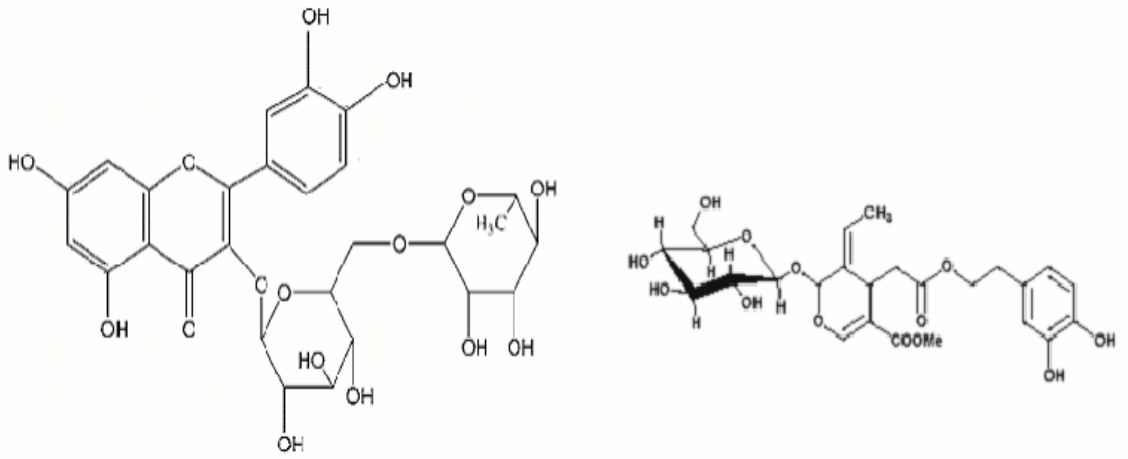


Figure 2.13. Chemical structures of major polyphenolics in olive leaf.

The studies on major olive leaf polyphenolics have shown that they have several health beneficial properties. Moreover, it was known that oleuropein has blood pressure and immune system regulating and therapeutic effects mainly against heart related diseases and diabetes as well as has antibacterial and antitumour activities. Besides, it was revealed that oleuropein and hydroxytyrosol which is an oleuropein derived metabolite have significant antioxidant capacity (Ranalli et al., 2006; Al-Azzawie et al., 2006).

There exists also infection preventing, antitumour, antibacterial, and antioxidant effects of rutin that is the secondary major polyphenolics present in olive leaves (Ghica et al., 2005). In addition, rutin is a compound which has been used for treatment against several diseases due to its preventing effect against DNA destruction and also used mostly in our dietaries (Kurisawa et al., 2003).

The researches on the use of phytochemicals against neurological disorders as well as several diseases have still been performed (Perry et al., 1999). The importance of these compounds in the drug discovery and development point of view has been increasing due to their possible uses in several metabolic routes as substrates, protective and reaction initiating agents and also stabilizers (Sheu et al., 2004).

2.3. Enzyme Activation and Inhibition Determination Methods

Most of the enzymatic reactions can be explained using equations known as simple Michaelis kinetics. According to Michaelis kinetics, reaction velocity (v) is mathematically associated with enzyme (E), substrate (S), enzyme-substrate complex (ES), product (P) and reaction constants (k_1, k_{-1} and k_2) as given in Equation 2.1.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$\frac{-d[S]}{dt} = \frac{d[P]}{dt} = v = \frac{V_{\max}[S]}{K_m + [S]} \tag{2.1}$$

where $V_{\max} = k_2[E]_0$ and $K_m = {(k_{-1} + k_2)}/{k_1}$.

The enzyme activity revealing the kinetic progression (Figure 2.14) of reaction can be followed by many physicochemical methods. Spectrophotometric, fluorometric and radioactive methods are referred as the most widely used techniques in the literature (Copeland, 2000). However, as given in the equations above, since the reaction rate can be calculated according to substrate and the product, it is quite important to define the materials of which the kinetic data will be recorded in choosing kinetic definition method.

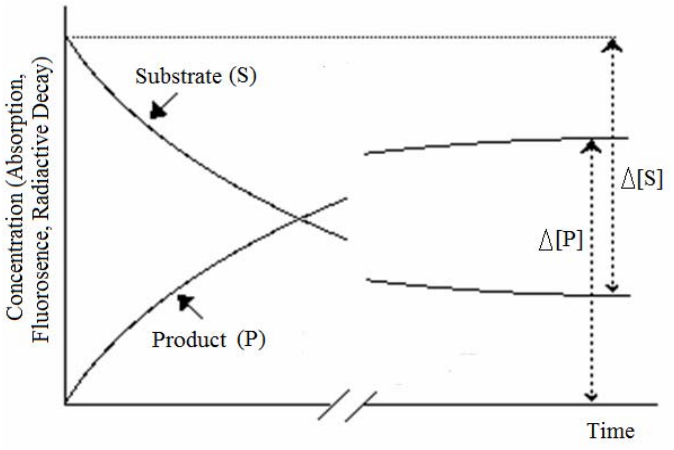


Figure 2.14. General enzymatic reaction progression curve

2.3.1. Spectrophotometric Method

In spectrophotometric method, the changes in the electronic orientation of a molecule after exposure to a light source are observed. The light energy absorbed by the molecule and which will cause required amount of change in the electronic orientation of the molecule, is present in regions of electromagnetic spectrum known as ultraviolet (UV) and visible regions (totally 200-800 nm range) (Copeland, 2000).

In reactions followed with spectrophotometric method, the molecule, kinetic data of which will be collected, should firstly be exposed to a total spectral energy range. Thus, the wavelength (λ_{max}) of the maximum light energy absorbed by the molecule is defined and this wavelength is used for most accurate kinetic results.

Absorption values and concentrations of materials in the solution obtained as a result of kinetic measurements at maximum wavelength are analytically associated using Beer Law (Copeland, 2000). According to Beer Law;

$$A = \varepsilon c l$$

where A (absorbance) is a unitless quantity, and it is shown that it changes in correlation with concentration value defined as C (molarity, M), l (the pathlenght of light, distance passed by the light in the solution, cm) and ε (molar absorptivity coefficient, $(M.cm)^{-1}$) (Copeland, 2000).

2.3.2. Fluorometric Method

As in spectrophotometric method, in fluorometric method the molecule is exposed to a light source. However, the light absorbed by the molecule in this method does not cause a change in electron orientation. Electrons, using the applied light energy, achieve high energy level for a short while and then turn to their initial energy level. The energy used during this change is given to reaction medium and recorded as increases or decreases in fluorescence (Copeland, 2000).

In fluorometric method, 2 maximum wavelengths are defined for the molecule whose kinetic data will be collected. Maximum wavelengths where the light is absorbed (emission) and electrons reach high energy level (excitation) are found with spectral scanning and measurements at these wavelengths are recorded.

The relationship between concentration and fluorescence can be calculated with a method similar to Beer's law used in spectrophotometric method. In fluorometric calculations, molar quantum constant (φ) is used instead of molar absorptivity. However, it is recommended that in practice a calibration curve is obtained for the material whose kinetic data will be collected instead of using this constant (Copeland, 2000).

Since fluorescence devices are more sensitive and less interference is observed, this method is more advantageous than absorption and used more for enzymatic reactions (Copeland, 2000).

2.3.3. Radiochemical Method

The basic strategy in this method is the use of radioisotopes combined with substrate (such as carbon-14, phosphorus-32, tritium, etc.). Such materials which are subject to radioactive decay pass to the product as a result of enzymatic reaction. After substrate and product fractions are obtained using appropriate separation techniques, reaction rate is determined according to the amount of radioactive material left in the substrate (or passed in the product) (Copeland, 2000).

Radioactive decay is expressed with Curie unit (Ci). 1 Ci equals to complete decay rate of 1 gram of radium-226. After the amount of decay in one minute is defined for every isotopes used in the reaction, the reaction rate can be calculated (Copeland, 2000).

Physically, the light emerging as a result of scintillation by the radioactive material is absorbed by other materials in the reaction medium. The absorbed light is recorded by a scintillation counter. In this method which requires a separation, the scintillation counter is usually connected to a chromatography device. This method is known as a method as sensitive as fluorescence and is widely used.

Other than these methods used for kinetic measurements of enzymatic reactions, some different techniques such as polarography, electrophoretic separation, chromatographic separation and immunological reactivity are also used (Copeland, 2000).

2.3.4. COMT Enzyme Activity/Inhibition Assays

It is known that the COMT enzyme, which is usually obtained by isolating from livers, kidneys and brains of animals and humans, can be used many catecholic substrates together with SAM as substrate. In studies since 1958, COMT enzyme activity is tried to be determined by fluorometric, spectrophotometric and radioactive methods. Among these, radioactive method is preferred since it does not require a reference standard. In this method, usually carbon-14 (^{14}C) and tritium (^{3}H) radioisotopes bound SAM is used as methyl donor substrate. However, because of inability to separate several isomeric *O*-methylated products formed in *in vitro* experiments by using liquid-liquid extraction and primitive chromatography techniques, radioactive methods can not always be applied (Lautala, 2000).

With the help of improved separation methods in recent years, it is possible to use multiple detectors compatible with liquid and gas chromatography devices. Thus, spectrophotometric, fluorometric and radioactive methods with increased sensitivity have been used in many studies in order to determine COMT activity. However, since fluorometric and radioactive methods are preferred more since they have the capacity to measure very low concentration values (Lautala, 2000; Reenilä, 1999).

In Table 2.2, the studies employed in order to determine enzyme activity or inhibition, are summarized by giving information about reaction medium, substrate selections and some kinetic data. As it can be seen from this table, COMT activity can be determined by using a variety of methods from simple optical methods to modern techniques.

Table 2.2. COMT activity/inhibition assays

Reference(s)	Assay conditions	Method of Analysis	Catecholic Substrate	[Catecholic Substrate]	[SAM]	Enzyme source (amount)	Additional information
Tsunoda et al., 2002	pH=7.8, 37 °C, total rxn. volume=300 µl, incubation time=60 min.	HPLC-Fluorescence (excitation 430 nm; emission 505 nm)	Norepinephrine (NE)	2 mM	200 µM	human S-COMT	[NE] concentration range 10-2000 µM
Tsunoda et al., 2002	pH=7.4, 37 °C, total rxn. volume=300 µl, incubation time=60 min.	HPLC-Fluorescence (excitation 430 nm; emission 505 nm)	Norepinephrine (NE)	0.1 mM	200 µM	human MB-COMT	[NE] concentration range 1-100 µM
Salyan et al., 2006	pH=7.5, 37 °C, total rxn. volume=200 µl, incubation time =90 min.	LC-MS[1]	1) Dopamine 2) DOBA[2]	2-128 µM (for both substrates)	10 µM	S-COMT (2 µg/ml)	K_m (DOBA) = 11 µM K_m (Dopamine) = 42 µM
Aoyama et al., 2005	pH=7.8, 37 °C, total rxn. volume=250 µl, incubation time =60 min.	HPLC-Fluorescence (excitation 430 nm; emission 505 nm)	Norepinephrine (NE)	1.5 mM	200 µM	S-COMT (62 µg/ml)	K_m (NE) = 503 µM (inhibition with pyrogallol and 3,5-dinitrocatechol)
Shen et al., 2005	pH=7.8, 37 °C, total rxn. volume=1 ml, incubation time =60 min.	HPLC-Fluorescence (excitation 260 nm; emission 400 nm)	DOBA	25 µM	100 µM	S-COMT (0.5 mg/ml)	
Lautala et al., 2001, 1999	pH=7.4, 37 °C, total rxn. volume=250 µl, incubation time =30-60 min.	Radioactivity-UV detection (260 nm) HPLC	DOBA	5-300 µM	150 µM	S-COMT (12.5-100 µg)	Inhibition and Validation study

(continued on next page)

Table 2.2. (cont.)

Reference(s)	Assay conditions	Method of Analysis	Catecholic Substrate	[Catecholic Substrate]	[SAM]	Enzyme source (amount)	Additional Information
Chen et al., 2005	pH=7.4, 37 °C, total rxn. volume=100 µl, incubation time=30 min.	HPLC Electrochemical Detection (ECD)	1) 2-OH-E_2[3] 2) 4-OH-E_2[4]	50 µM	60 µM	Human liver COMT (0.2 mg)	Inhibition study (substrate concentrations vary between 5-200 µM)
Bailey et al., 2004	pH=7.45, 37 °C, total rxn. volume=60 µl, incubation time=60 min.	HPLC - UV detection (370 nm)	Catecholic Azo-dyes	0.5-150 µM	20 mM	Porcine liver COMT (125 U/ml)	Inhibition study (inhibitor concentrations vary between 0-21 µM)
Kurkela et al., 2004	pH=7.4, 37 °C, total rxn. volume=100 µl, incubation time=60 min.	Microplate Reader - Fluorescence (excitation 355 nm; emission 460 nm)	Esculetin	Not given	10 µM	Human liver COMT (15 µg/ml)	Inhibition study (6 different catecholic compound)
Veser, 1987	pH=7.4, 37 °C, total rxn. volume=500 µl, incubation time=30 min.	Fluorescence (excitation 390 nm; emission 465 nm)	Esculetin	4 µM	600 µM	*Candida tropicalis* S-COMT (20-50 µl)	[Esculetin] = 1.2-5 µM [SAM] constant at 460, 46 and 7.7 µM
Hirano et al., 2007	pH=7.8, 37 °C, total rxn. volume=200 µl, incubation time=30-120 min.	HPLC-Fluorescence (excitation 430 nm; emission 505 nm)	Norepinephrine (NE)	1.5 mM	200 µM	rat brain S-COMT	Activity test for COMT isolated from several regions of rat brain

(continued on next page)

Tablo 2.2. (cont.)

Reference(s)	Assay conditions	Method of Analysis	Catecholic Substrate	[Catecholic Substrate]	[SAM]	Enzyme source (amount)	Additional Information
Hirano et al., 2007	pH=7.0, 37 °C, total rxn. volume=200 µl, incubation time=30-120 min.	HPLC-Fluorescence (excitation 430 nm; emission 505 nm)	Norepinephrine (NE)	200 µM	200 µM	rat brain MB-COMT	Activity test for COMT isolated from several regions of rat brain
Dhar et al., 2000	pH=7.5, 35 °C, total rxn. volume=1 ml, incubation time=30 min.	Spectroscopy (absorbance 510 nm)	Esculetin	1 mM	2 mM	*Streptomyces griseus* S-COMT (20-200 µg)	K_m (esculetin) = 500 µM K_m (SAM) = 600 µM Homocystein (product) inhibition (K_i=224 µM)
Zhu et al., 2001	pH=7.4 and 10.0, 37 °C, total rxn. volume=1 ml, incubation time=20 min.	Radioactivity-UV detection (254 nm) HPLC-MS	Tea catechins	10-100 µM	50 µM	Human liver COMT (0.5 mg/ml)	Inhibition with tea catechins (10-100 µM)
Palma et al., 2006	pH=7.8, 37 °C, total rxn. volume=1 ml, incubation time=15 min.	HPLC-EIMS[5]	Nitrobenzo-phenone derivatives (bisubstrates)	10 µM	500 µM	rat S-COMT (1 mg/ml)	Inhibition with bisubstrate inhibitors (10 µM)

[1] LC-MS: Liquid chromatography-mass spectroscopy
[2] DOBA: 3,4-dihydroxy-benzoic acid
[3] 2-OH-E_2: 2-hydroxy-estradiol
[4] 4-OH-E_2: 4-hydroxy-estradiol
[5] HPLC-EIMS: High pressure liquid chromatography - electrospray ionization mass spectroscopy

2.4. Enzyme Inhibition Mechanisms

Activity of an enzyme can be blocked by various methods. It has been known that hundreds of natural compounds and thousands of synthetic chemicals can inhibit catalytic activity of enzymes. Basically these compounds cause reduction in rate of the enzyme catalyzed reactions (Leskovac, 2003; Copeland, 2000). Inhibition is a situation, which is sometimes undesirable although it is necessary for some enzymatic reactions. Especially, inhibition of enzymatic reactions in living organisms is widely discussed in food and pharmaceutical fields.

Inhibition is simply caused by materials which are directly or indirectly added to the reaction medium. These materials are called inhibitors and they are classified according to their effects on the reaction. The equations derived by Michaelis and Menten in order to calculate the rate of a simple enzymatic reaction given in Section 2.3, are modified according to the type of the inhibitor. Despite the fact that, new rate equations are derived in order to explain complex mechanisms nowadays, most enzymatic reactions can be explained with Michaelis-Menten approach (Pekin, 1970). Therefore in this study, equations derived for inhibitors explained by linear inhibition mechanisms are used.

2.4.1. Competitive Inhibitors

Competitive inhibitors are usually compounds having similar structures to substrate of the enzyme and therefore they are bound to the same active site of enzyme with the substrate (Leskovac, 2003). Consequently, they reduce the production rate by forming an enzyme-inhibitor complex (EI) (Figure 2.15).

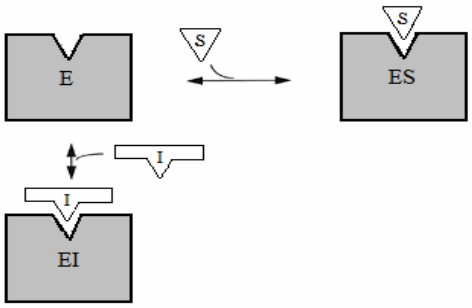
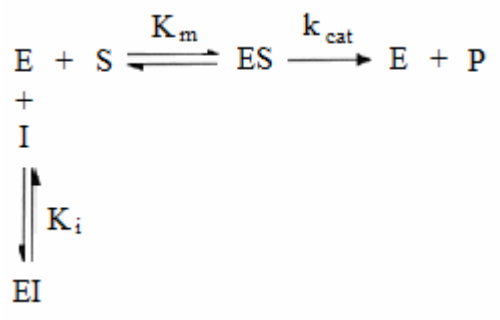


Figure 2.15. Simple competitive inhibition
(Source: Copeland, 2000).

Possible competitive inhibition reaction shown in Figure 2.15 occurs as given below.



Rate of reaction for the reaction in which the medium does not include any inhibitors is mathematically derived as given in Equation 2.1.

$$\frac{-d[S]}{dt} = \frac{d[P]}{dt} = v = \frac{V_{\max}[S]}{K_m + [S]} \tag{2.1}$$

In this equation $V_{\max} = k_{cat}[E]_0$ and it is defined as the maximum velocity of reaction. $K_m = (k_{-1} + k_2)/k_1$, which is the affinity of the substrate, is the equilibrium constant of the reaction where enzyme-substrate complex (ES) forms. In cases where there is a competitive inhibitor (I) in such reaction medium, the rate equation given in Equation 2.1 can be derived as in Equation 2.2 by Michaelis-Menten approach (mass balance is made for total E amount taking into consideration the pseudo-steady state hypothesis) (Shuler, 2001; Copeland, 2000).

$$v = \frac{V_{\max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \tag{2.2}$$

K_i, the equilibrium constant of the reaction through which the enzyme-inhibitor (EI) complex is formed, is defined as inhibition constant and it has the same unit with concentration. Using rate equations known as Michaelis-Menten equations, determination of inhibition mechanism and kinetic constants require complex calculations. For this purpose, some researchers such as Lineweaver and Burk, Hanes and Dixon have linearized these equations (Leskovac, 2003). Among these, linear

equations derived by Lineweaver and Burk are the most widely used equations in order to illuminate mechanism and kinetic constants graphically.

Linear form of competitive inhibition rate derived by the method developed by Lineweaver and Burk (double reciprocals of Equation 2.2) is given in Equation 2.3.

$$\frac{1}{v} = \frac{K_{m,app}}{V_{\max}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{\max}} \tag{2.3}$$

where $K_{m,app} = K_m\left(1 + \frac{[I]}{K_i}\right)$ and when 1/[S] graph is drawn against 1/v, it can be calculated from the intersect points on the x-axis. V_{max} value is calculated from the point intersecting the y axis as can be understood from the equation. A graphical demonstration of linear function of rate obtained for competitive inhibition and known as Lineweaver-Burk graph is given in Figure 2.16.

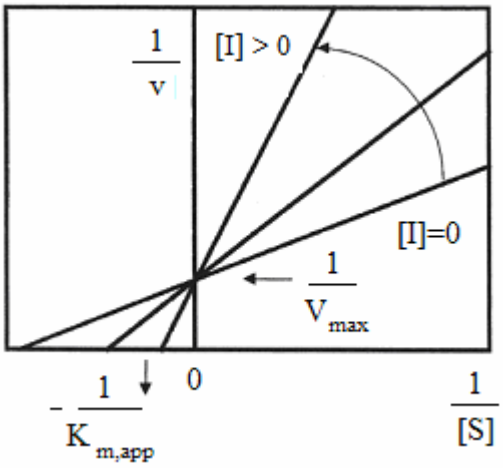


Figure 2.16. Lineweaver-Burk graph for competitive inhibition
(Source: Leskovac, 2003).

As it can be understood from Figure 2.16, maximum velocity of an enzymatic reaction is not affected by the increase in inhibitor concentration in presence of competitive inhibitor. However, increase in inhibitor concentration increases the value $K_{m,app}$.

2.4.2. Noncompetitive Inhibitors

Noncompetitive inhibitors allow the binding of the enzyme to the substrate as given in Figure 2.17. Although these inhibitors bind to the different site of enzyme from the substrate, they reduce the production rate by forming solely EI or ESI complexes or both (Leskovac, 2003).

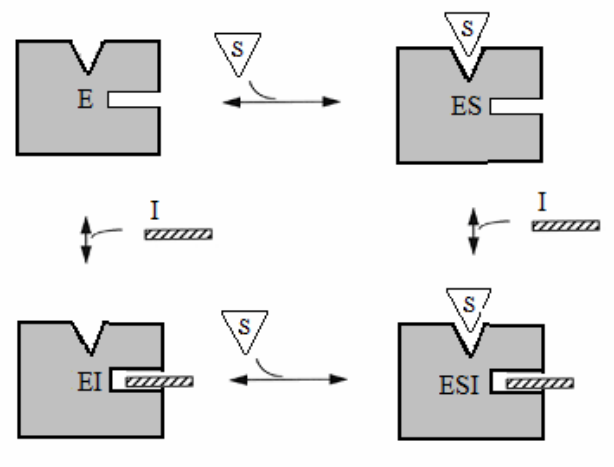
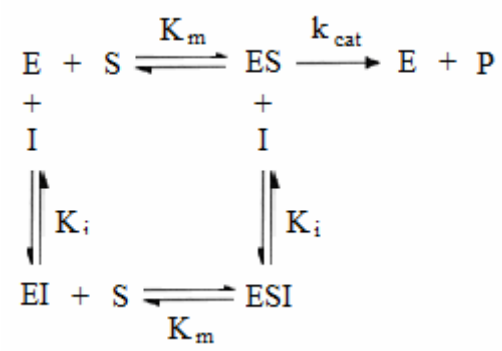


Figure 2.17. Simple noncompetitive inhibition
(Source: Copeland, 2000).

Possible kinetic model developed for noncompetitive inhibition is demonstrated below. According to this model, the equilibrium constants of the reactions, through which ES and ESI complexes and EI and ESI complexes are formed, are equal to each other. Reaction rate is derived as given in Equation 2.4, according to Michaelis-Menten approach.



$$v = \frac{V_{\max}[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]\left(1+\frac{[I]}{K_i}\right)} \tag{2.4}$$

Linear transformation of noncompetitive inhibition rate is also derived with the method developed by Lineweaver-Burk as given in Equation 2.5.

$$\frac{1}{v} = \frac{K_m}{V_{\max,app}}\left(\frac{1}{[S]}\right)+\frac{1}{V_{\max,app}} \tag{2.5}$$

In Equation 2.5, $V_{\max,app} = \left(\frac{V_{\max}}{\left(1+\frac{[I]}{K_i}\right)}\right)$ and for calculation of kinetic constants, Lineweaver-Burk graph given in Figure 2.18 can be obtained. Required calculations can be applied by using the points intersecting the axes of this graph.

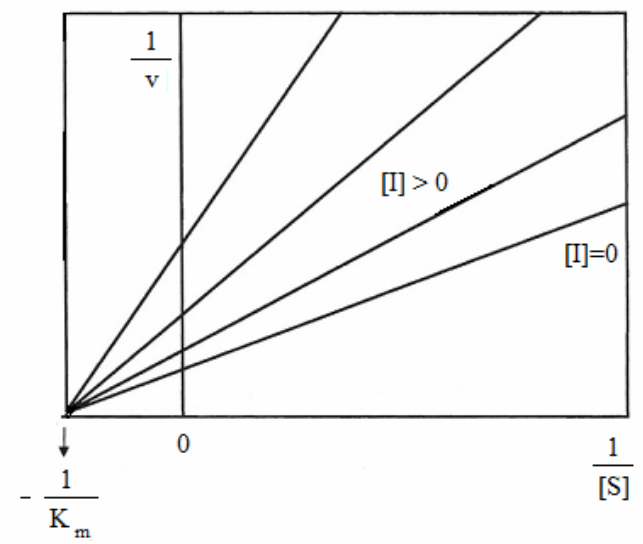


Figure 2.18. Lineweaver-Burk graph for noncompetitive inhibition
(Source: Leskovac, 2003).

Increase in the concentration of a noncompetitive inhibitor, on contrary to competitive inhibitors, does not have any effect on K_m value, while decreasing maximum velocity (V_{max}) of the reaction.

2.4.3. Uncompetitive Inhibitors

Uncompetitive inhibitors are bound to the appropriate active site which is lately formed on ES complex structure after the binding of substrate as demonstrated in Figure 2.19. Newly formed ESI is an inactive complex and thereby it reduces the catalytic activity of the enzyme by preventing product formation.

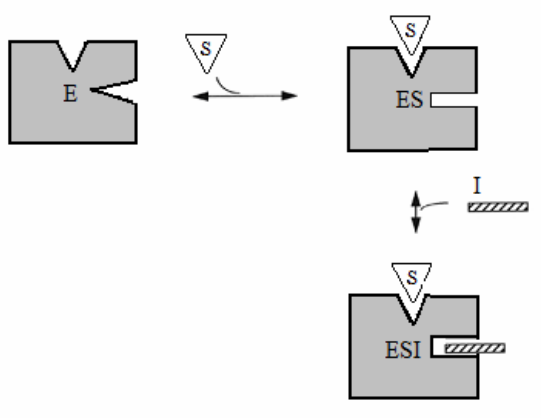
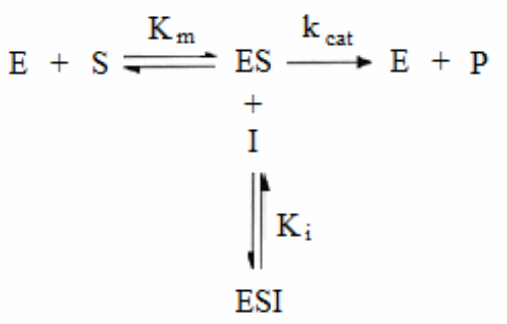


Figure 2.19. Simple uncompetitive inhibition
(Source: Copeland, 2000).

Kinetic model developed for possible uncompetitive inhibition is given below. Equilibrium constant of the reaction where ESI complex is formed is also defined as inhibition constant (K_i). In rate equation derived according to Michaelis-Menten approach (Equation 2.6) inhibition constant is indicated with an alpha (α) constant. (α) constant, which also exists in rate equations of competitive and noncompetitive inhibition but provides simplification of equations according to the value it takes, is the most important mathematical parameter determining the inhibition mechanism (Copeland, 2000). (α) constant is used in rate equations in order to explain the affinity of the enzyme to the substrate with binding degree of the inhibitor.



$$v = \frac{V_{\max}[S]}{K_m + [S]\left(1 + \frac{[I]}{\alpha K_i}\right)} \tag{2.6}$$

According to this, for values of (α) much higher than 1, (α>>1) inhibition mechanism is explained as competitive, and for its values much lower than 1 (0<α<<1) it is explained as uncompetitive. For noncompetitive inhibition (α) constant equals to 1. In mixed type inhibitions where degree of competitiveness or noncompetitiveness can be determined, it is known that the (α) constant takes a certain lower or higher values close to 1 (α<1 or α>1) (Copeland, 2000).

Linearized rate equation derived in order to explain the uncompetitive inhibition mechanism graphically is given in Equation 2.7.

$$\frac{1}{v} = \frac{K_{m,app}}{V_{\max,app}}\left(\frac{1}{[S]}\right) + \frac{1}{V_{\max,app}} \tag{2.7}$$

where $K_{m,app} = \left(\frac{K_m}{\left(1 + \frac{[I]}{\alpha K_i}\right)}\right)$ and $V_{\max,app} = \left(\frac{V_{\max}}{\left(1 + \frac{[I]}{\alpha K_i}\right)}\right)$. In Figure 2.20, Lineweaver-Burk graph obtained in order to calculate kinetic constants given in linear rate equations of uncompetitive inhibition is given.

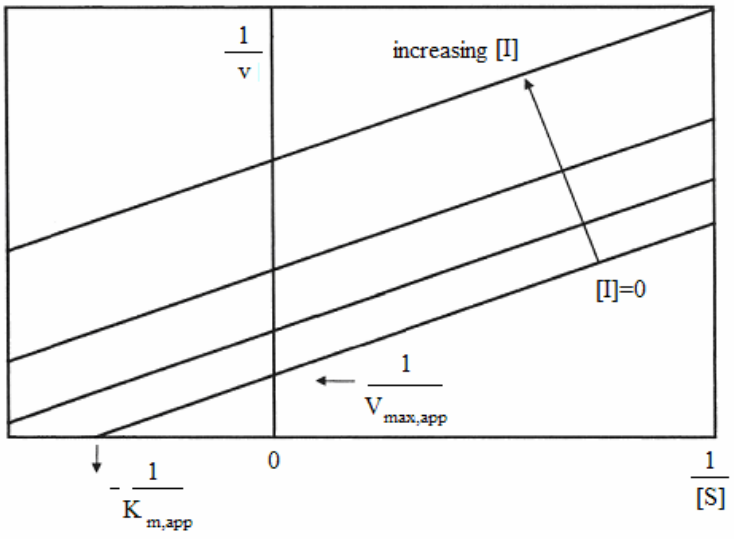


Figure 2.20. Lineweaver-Burk graph for uncompetitive inhibition (Source: Leskovac, 2003).

2.4.4. Mixed Type Inhibitors (Noncompetitive and Competitive)

In mixed type inhibitions which are similar to noncompetitive inhibition as kinetic model, affinity of inhibitor material to E and ES complex are different. (α) constant expressed in explaining uncompetitive inhibition can give information about the dominant mechanism of mixed type inhibition. In this inhibition type, whose reaction rate equation (Equation 2.8), (α) constant takes a certain lower or higher values close to 1 (α<1 or α>1).

$$v = \frac{V_{\max}[S]}{K_m\left(1+\frac{[I]}{K_i}\right)+[S]\left(1+\frac{[I]}{\alpha K_i}\right)} \tag{2.8}$$

Linearized rate equation, derived in order to explain this mechanism graphically where competitive and noncompetitive inhibitions occur simultaneously, is given in Equation 2.9.

$$\frac{1}{v} = \frac{K_{m,app}}{V_{\max}}\left(\frac{1}{[S]}\right)+\frac{1}{V_{\max,app}} \tag{2.9}$$

In this equation it is defined that; $K_{m,app} = K_m\left(1+\frac{[I]}{K_i}\right)$ and $V_{\max,app} = \left(\frac{V_{\max}}{\left(1+\frac{[I]}{\alpha K_i}\right)}\right)$.

Lineweaver-Burk graph obtained by linear rate equation of mixed type inhibition is given in Figure 2.21.

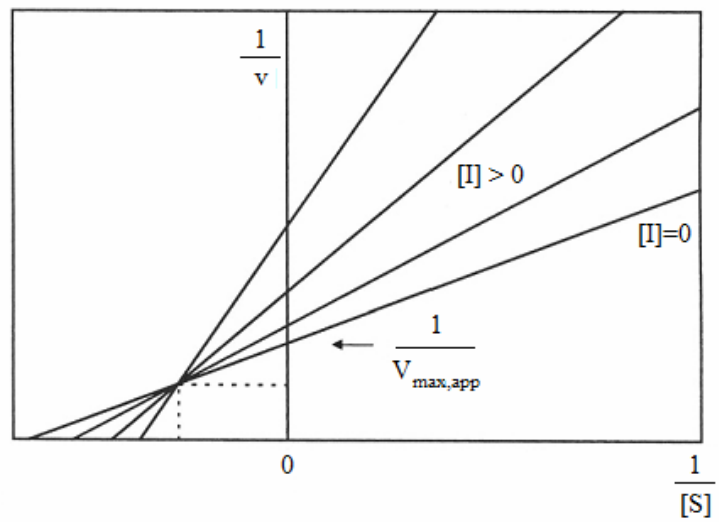
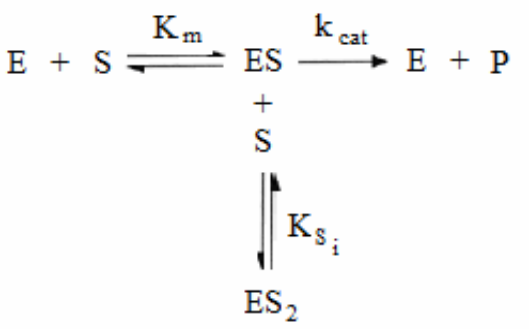


Figure 2.21. Lineweaver-Burk graph for mixed type inhibition
(Source: Leskovac, 2003).

As it can be understood from Figure 2.21, an inhibitor behavouring in mixed type manner affects both K_m and V_{max} values, however while K_m increases, V_{max} decreases.

2.4.5. Inhibitor Substrates

Despite there is no inhibitor in the reaction medium, a decrease in catalytic activity is observed. It is indicated that high substrate concentration is the main reason of this situation. Because the substrate can bind to both enzyme and ES complex simultaneously, inactive ES_2 complex forms as indicated in the kinetic model below. Inhibition of enzyme by its substrate can again be expressed mathematically by Michaelis-Menten approach and the derived rate equation is given in Equation 2.10.



$$v = \frac{V_{max}[S]}{K_m + [S]\left(1 + \frac{[S]}{K_i}\right)} = \frac{V_{max}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}} \tag{2.10}$$

As can be understood from Equation 2.10, enzyme kinetics where substrate inhibition is observed can be explained mathematically by hyperbolic functions. In this respect, Lineweaver-Burk graph developed for determining kinetic constants in case of substrate inhibition is obtained through hyperbolic linearization techniques (Figure 2.22).

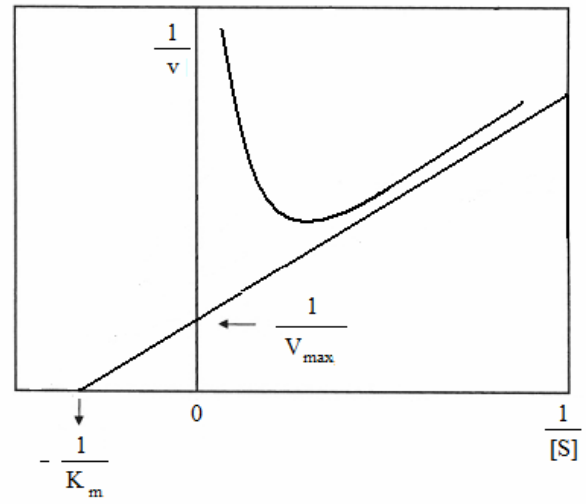


Figure 2.22. Lineweaver-Burk graph for substrate inhibition
(Source: Shuler, 2001).

In order to find the kinetic constants in points intersecting the axes, from the hyperbolic function obtained according to the substrate concentration, a linear line equation which is tangent of the area belonging to the low substrate concentrations is derived. From the points of this tangent intersecting the axes, V_{max} and K_m values can be calculated.

CHAPTER 3

OBJECTIVES

The main aim of this study is to investigate the inhibition potentials of alkaloid and polyphenolic based extracts and fractions obtained especially from harmal (*P. harmala*) seeds and olive (*O. europaea*) leaves and also from leaves of some plants which are known to have high polyphenolic content like olive against COMT enzyme activity. In accordance to this aim, the goals of the study are listed below:

- ✓ To extract of harmal seed alkaloids and olive leaf polyphenols by the highest yield methods,
- ✓ To examine the parameters affecting extraction efficiency such as temperature, pH and solvent type,
- ✓ To fractionate extracts obtained from harmal seed and olive leaf and thus to obtain alkaloid and polyphenolic compounds at high purity,
- ✓ To characterize alkaloid and polyphenolic based extracts and fractions quantitatively and qualitatively,
- ✓ To determine active protein amount of COMT enzyme,
- ✓ To determine the COMT enzyme activity spectrophotometrically and fluorometrically and to perform inhibition studies with the most sensitive method,
- ✓ To examine the inhibition potentials of eighteen alkaloid and polyphenolic based standards, extracts and fractions against COMT enzyme activity,
- ✓ To compare kinetic parameters obtained as a result of inhibition study with values reported in literature and to report these data into the literature.

CHAPTER 4

EXPERIMENTAL STUDY

4.1. Materials

The olive leaf (*Olea europaea*) and the other 4 plant leaves that were used in the inhibition studies; vitex (*Vitex agnus-castus*), terebinth (*Pistacia terebinthus*), mastic (*Pistacia lentiscus*) and cistus (*Cistus parviflorus*) were collected in May, 2006 from Izmir Institute of Technology Campus (Urla) and Karaburun region. The harmal (*Peganum harmala*) seeds were purchased from local herbalist. Analytical grade ethanol (C_2H_5OH) used in extraction of the plants other than the harmal was obtained from Sigma-Aldrich Chemie GmbH (Germany). HPLC-grade methanol (CH_3OH), ammonium hydroxide (NH_4OH) and chloroform ($CHCl_3$) solvents used in the extraction of alkaloids present in the harmal seed were also purchased from Sigma-Aldrich Chemie GmbH (Germany). Petrolium ether and hydrochloric acid (HCl) used in the extraction of the alkaloids were taken from Riedel-de Haen (Germany) and Merck KGaA (Germany), respectively. Several solvents were used in the analysis of the exctracts obtained from the plants and their fractions by High Performance Liquid Chromatography (HPLC) as mobile phases. These were acetonitrile (CH_3CN) that was purchased from Sigma-Aldrich Chemie GmbH (Germany), isopropyl alcohol (C_3H_7OH) and triethylamine ($N(CH_2CH_3)_3$) that were obtained from Riedel-de Haen (Germany), formic acid (HCOOH) and acetic acid (CH_3COOH) that were purchased from Merck KGaA (Germany).

In the experiment of determination of the protein amount by Bradford method, Bovine Serum Albumin (BSA) which was obtained from Merck KGaA (Germany) was used as the reference protein. Catechol-*O*-methyltransferase (COMT) enzyme isolated from porcine liver and the substrate used as methyl source, S-adenosyl-L-methionine (SAM), was supplied from Sigma-Aldrich Chemie GmbH (Germany). Other reactants used in the fluorometric enzyme activity assays, aesculetin (6,7-dihydroxycoumarin), magnesium chloride ($MgCl_2$) and L-cysteine, were also purchased from Sigma-Aldrich Chemie GmbH (Germany). Potassium phosphate (K_2HPO_4/KH_2PO_4) used as the buffer

solution was obtained from Fluka/Merck (Germany). Dimethylsulfoxide (DMSO) used as the solvent of the inhibitor was supplied from Carlo-Erba Reagents (Spain). The reactants used in the enzyme activity experiments performed by spectrophotometric method, dihydroxyacetophenone (substrate-DHAP), dithiothreitol (DTT), and N-Tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid (TES) used as buffer solution, and boric acid were purchased from Sigma-Aldrich Chemie GmbH (Germany). Sodium hydroxide (NaOH) used in order to adjust the pH of the buffer solution was supplied from Riedel-de Haen (Germany).

The standards used in the enzyme inhibition experiments, 3,5-dinitrocatechol (3,5-DNC), harmine ve harmol hydrochloride dihydrate, were purchased from Sigma-Aldrich Chemie GmbH (Germany), and harmaline ve harmalol hydrochloride dihydrate from Fluka (Germany). The oleuropein and rutin standards were supplied from Extrasynthese (Genay, France) and Merck KGaA (Germany), respectively.

4.2. Methods

The methods developed and used in this study consist of six steps. In the first three steps, the plant extracts and their fractions that were used as inhibitors were obtained and characterized by using High Performance Liquid Chromatography (HPLC). In the other steps, protein amount and activity of the enzyme were determined and enzyme inhibition kinetic studies were performed using the plant extracts and their fractions. Two different analysis methods were applied in the activity determination and inhibition studies and their sensitivities were compared. The schematic representation of the strategy and methods followed during the study is shown in Figure 4.1.

4.2.1. Extraction of the Inhibitors

4.2.1.1. Extraction of Harmal (*P. harmala*) Seeds

For the extraction of alkaloids present in the seed of harmal (*P. harmala*), different extraction methods reported in the literature were reviewed, applied in this study and the generally used methods including maceration, soaking and Soxhlet extraction were compared based on the yield. The details of the methods were given

below by which some parameters affecting to the extraction efficiency such as solvent type, temperature, extraction period and pH were investigated.

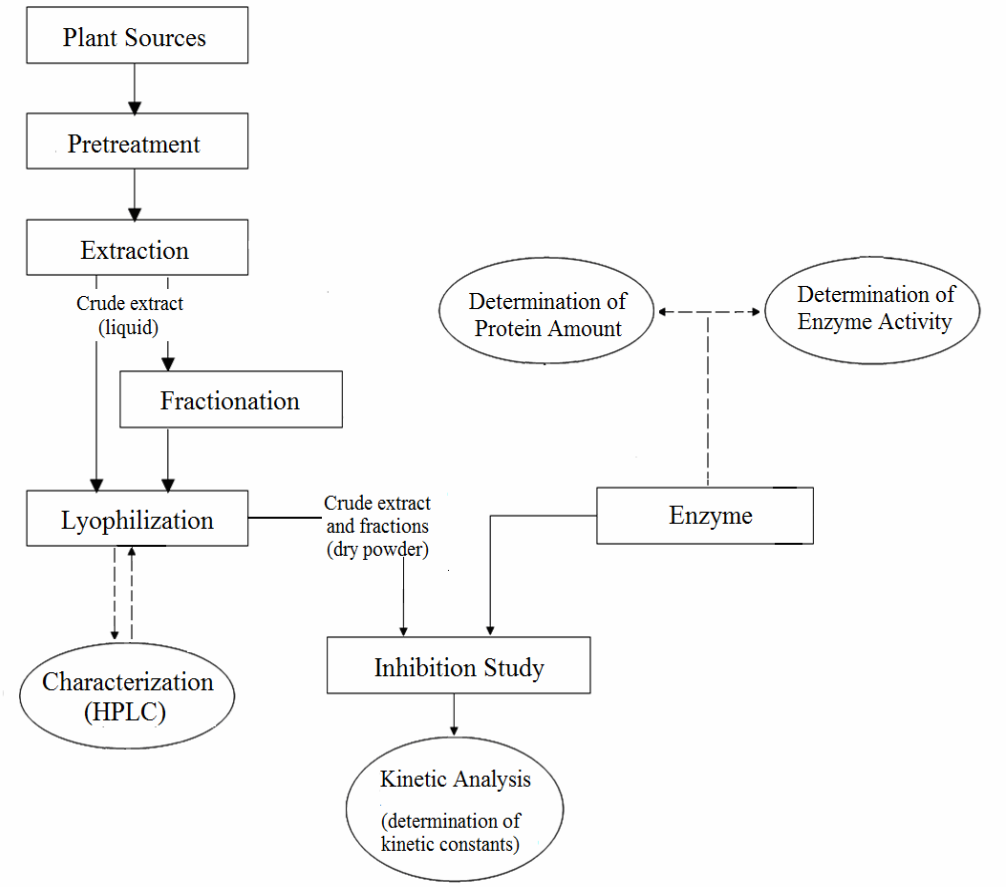


Figure 4.1. Schematic representation of the experimental studies

Method A (Maceration using Methanol): In this method where methanol was used as solvent, the effects of extraction period and pH on the extraction yield were studied.

Step 1: An amount of dried and ground *P. harmala* seeds,

System 1 (S1): was macerated using 50 ml methanol for 1 hour for each four times in a stirred jacketed heater which was kept at constant temperature of 50 ºC

System 2 (S2): was macerated using methanol for 24 hours in a water bath at 50 ^{o}C and 150 rpm.

Step 2: The methanol solutions obtained from these two systems were filtered. The methanol was removed from the filtered solutions by means of SpeedVac vacuum evaporator. The remaining part was filtered after being dissolved in 50

ml of 2% HCl. The acidic solution was subjected two times to liquid-liquid extraction using 20 ml of petrolium ether and the acidic part was collected using a separation funnel.

Step 3: In the third step, in order to examine the effect of pH on the extraction yield, pH values of the solutions obtained from the two systems were increased by addition of ammonium hydroxide. The pH values of the final solutions are given below.

System 1-1 (S1-1): pH = 10.0

System 2-1 (S2-1): pH = 11.0

System 2-2 (S2-2): pH = 8.5

Step 4: Finally the three solutions obtained with different acidity were subjected to liquid-liquid extraction using 50 ml of chloroform for four times. The chloroform parts were collected by using again a separation funnel and then chloroform was evaporated to obtain crude alkaloid extracts.

Method B (Soaking): In the extraction experiments performed according to the soaking method, 3 different systems were studied using 3 different solvents.

System 1 (S1): The same procedure as in the S1-1 system of the Method A was also applied in all the steps in Method B except for the first step. In the first step methanol was soaked to dry and ground harmal seed in a water bath at 55 ^{o}C and 150 rpm for 30 hours.

System 2 (S2): Firstly, in order to separate oil and relevant plant materials present in the harmal seeds, petrolium ether was used as first extraction solvent. After soaking petrolium ether to the seeds at 25 ^{o}C for 48 hours, filtration was applied and methanol was soaked to the seeds at 50 ^{o}C for 8 hours. After removal of the methanol the remaining solid particles were dissolved in the acidic solution as described in the Method A, S1-1 and pH of the solution was adjusted to 10.0 using basic solution. After that it was subjected to liquid-liquid extraction using chloroform.

System 3 (S3): In this system, ethyl acetate was used as the extraction solvent. After being mixed with some sodium bicarbonate, it was soaked twice to harmal seeds at 25 ^{o}C for 24 hours. After the each 24-hour soaking period, the basic ethyl acetate parts were collected by

filtration and they were subjected to liquid-liquid extraction using 25 ml of %5 HCl. The pH of the solution after the acidic portion was removed by means of a separation funnel was adjusted to 3.5 by addition of some 5M of NaOH solution.

Method C (Soxhlet Extraction): Methanol was used as solvent in this method in which Soxhlet apparatus was used. The effect of the extraction period on the extraction yield was examined. For this purpose, some ground harmal seeds were extracted at 70 ^{o}C for

System 1 (S1): 7.5 hours

System 2 (S2): 20 hours

System 3 (S3): 26 hours.

After that, the steps given in Method A were followed. In this method, the pH of the solution was adjusted to 10.0 before the extraction with chloroform.

The results obtained from different methods applied for the extraction of alkaloids in the harmal (*P. harmala*) seeds were compared with each other. The extracts obtained by the method which provided the optimum yield were stored in dark glass bottles in a cool environment.

4.2.1.2. Extraction of Olive (*O. europaea*) Leaves

The extracts from the leaves of olive and of other plants (vitex, cistus, terebinth and mastic) were obtained by the same manner as reported in the literature (Altıok et al., 2008; Baycin et al., 2007). First, the washed and dried leaves were ground and then subjected to extraction with % 70 aqueous ethanol solution. The extraction process was carried out in a thermoshaker at 25 ^{o}C and 250 rpm for 2 hours keeping solid/liquid ratio at 1/20. The extract obtained in the 70% aqueous ethanol solution after filtration was subjected to rotary vacuum evaporator in order to remove the ethanol. The extract in the remaining aqueous solution was dried in a freeze drier (Telstar Cryodos) of which the operational conditions were kept at –52 ^{o}C and at a lower pressure than 0.2 mbar. As a result of freeze drying, the crude extract was obtained and kept in dark glass bottles in a cool environment for further use in the enzyme inhibition experiments.

4.2.2. Fractionation of the Extracts

Different fractionation techniques were applied for each of the extracts rich in alkaloids and polyphenolics that were obtained by the optimized extraction methods.

4.2.2.1. Fractionation of the Harmal (*P. harmala*) Seed Extract

The raw extract of the harmal seed was fractionated by use of a fraction collector unit (Gilson) compatible with the High Performance Liquid Chromatograph (HPLC) (Agilent 1100 series). The operating conditions and properties of HPLC used for alkaloid fractionation are given in Table 4.1.

Table 4.1. The operating conditions and properties of HPLC (Alkaloid Fractionation)

Units and Parameters	Properties and Conditions
Column	Zorbax SB-C18 semipreparative column
Column length	25 cm
Column diameter	9.4 mm
Particle size	5 μm
Mobile phase(s)	Mobile phase A: Acetonitrile/isopropyl alcohol (1:1) (by volume)
	Mobile phase B: Acetonitrile/isopropyl alcohol/Deionized water/Formic acid (100:100:300:0.3) (ml) (pH of the solution was adjusted to 8.6 by addition of triethylamine)
Flow rate	1 ml/min
Temperature	30 °C
Pressure	60-70 bar
Detector type	Diode Array Detector (DAD)
Wavelength	330 nm

The optimum elution program for the alkaloid fractionation was determined by changing the polarities of the mobile phases. The changes in the concentration of the mobile phases during analysis are given in Table 4.2.

Table 4.2. HPLC elution program (Alkaloid Fractionation)

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	5	95
18	5	95
21	30	70
24	38	62
27	36	64
30	35	65
35	0	100
40	0	100

According to the results of the HPLC analysis performed for the crude alkaloid extract, the harmal seed alkaloids were collected in 3 fractions. These fractions collected being dissolved in the mobile phase, were obtained as their powder forms after removal of solvents by using vacuum evaporator. The powdered alkaloid fractions in were stored under appropriate condition until further use in HPLC characterization and enzyme inhibition experiments.

4.2.2.2. Fractionation of the Olive (*O. europaea*) Leaf Extract

The polyphenolics in olive leaf were fractionated using silk fibroin packed column as previously reported in literature (Baycin et al., 2007). Firstly, the column packed with 15 g of silk fibroin was eluted by deionized water, ethanol and again deionized water, respectively in order to remove the impurities on the packed material and to activate it. Then 20 g of the crude extract dissolved in 250 ml water was fed to the column until saturation. In order to firstly fractionate the water-soluble polyphenolics having high polarity such as oleuropein, the column was eluted with water for 9 times. The 9 water fractions were then combined into 4 main fractions based on their compositions determined by the HPLC analysis.

After that, in order to fractionate the less polar polyphenolics such as rutin and other glycosides, the column was eluted with % 70 aqueous ethanol solution for 4 times. The fractions obtained were also combined into 2 main fractions according to the HPLC analysis results. The fractions rich in oleuropein and rutin were lyophilized and stored

in appropriate conditions for further use in the enzyme inhibition and HPLC characterization experiments.

4.2.3. Characterization of the Inhibitors

The plant extracts and their fractions obtained for investigation of their enzyme inhibition potentials were first characterized by HPLC.

4.2.3.1. Characterization of the Harmal (*P. harmala*) Seed Extract

Qualitative and quantitative characterization study of the crude harmal seed extract and their fractions were performed by HPLC. The obtained samples were filtered through 0.45 μm membrane filters prior to the analysis. The properties of the HPLC system and the operating conditions for analysis proper for the characterization of the alkaloids are given in Table 4.3.

Table 4.3. Operating conditions and properties of the HPLC (Alkaloid Characterization)

Units and Parameters	Property and Conditions
Column	LiChrospher 100 RP-18 analytical column
Column length	25 cm
Column diameter	4 mm
Particle size	5 μm
Mobile phase(s)	Mobil phase A: Acetonitrile/isopropyl alcohol (1:1) (by volume)
	Mobile pahse B: Acetonitrile/isopropyl alcohol/Deionized water/Formic acid (100:100:300:0.3) (ml) (pH of the solution was adjusted to 8.6 by addition of triethylamine)
Flow rate	1 ml/min
Temperature	30 °C
Pressure	170-180 bar
Detector type	Diode Array Detector (DAD)
Wavelength	330 nm

The elution program by which the optimum separation in the fractionation was yielded was determined by changing the polarities of the mobile phases. The changes in the concentrations of the mobile phases are given in Table 4.4.

Table 4.4. HPLC elution program (Alkaloid Characterization)

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	10	90
3	10	90
6	15	85
15	15	85
18	18	82
20	0	100

The measurement wavelength of 330 nm applied in both fractionation and characterization of the alkaloids was chosen based on the spectrophotometric scanning of the crude extract, its fractions and alkaloid standards performed by using microplate reader (Multiskan, Thermo). Furthermore, calibration curves for each alkaloid standard were obtained, and detection and quantification limits for the each standard were determined as a result of the HPLC analysis (Appendix A. Calibration Curves of Alkaloids and Sample Calculation).

4.2.3.2. Characterization of the Olive (*O. europaea*) Leaf Extract

Characterization of the polyphenolics in the olive leaf extract such as oleuropein and rutin was also carried out by HPLC in a manner reported in the literature (Altıok et al., 2008; Baycin et al., 2007). The properties of the HPLC system and the operating conditions of analysis proper for the characterization of the polyphenolics are given in Table 4.5.

Table 4.5.Operating conditions and properties of HPLC (Polyphenolic Characterization)

Units and Parameters	Property and Conditions
Column	LiChrospher 100 RP-18 analytical column
Column length	25 cm
Column diameter	4 mm
Particle size	5 μm
Mobile phase(s)	Mobile phase A: % 2.5 aqueous acetic acid solution
	Mobile phase B: Acetonitrile
Flow rate	1 ml/min
Temperature	30 °C
Pressure	120-130 bar
Detector type	Diode Array Detector (DAD)
Wavelength	280 nm

In order to characterize the polyphenolics present in the olive leaf extracts, the optimum elution program for HPLC analysis which was reported in the literature was given in Table 4.6.

Table 4.6. HPLC elution program (Polyphenolic Characterization)

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	95	5
20	75	25
40	50	50
50	20	80
60	5	95

The concentrations of oleuropein and rutin, which are the major polyphenolics present in olive leaf extract which is composed of several polyphenolics, were determined based on the calibration curves prepared using their standards (Appendix B. Calibration Curves of Oleuropein and Rutin and Sample Calculation).

4.2.4. Determination of Protein Amount

It is important to determine the amount of active protein for accurate kinetic calculations of an enzymatic reaction. Among the several methods reported in the literature for protein analysis, Bradford method was preferred in this study due to its high sensitivity (Rosenberg, 1996). The main principle of the Bradford method is the measurement of change in the absorbance value of the protein solution resulted from addition of an acidic dye which tends to bind to basic and aromatic amino acids into the solutions with different protein concentrations. In this method, calibration curves for both reference and sample proteins are prepared using the absorbance values obtained from different protein concentrations (Pierce Biotechnology Inc., 2009).

Based on this principle, in the analysis of the active protein of the standard COMT enzyme isolated from porcine liver by the Bradford method, Bovine Serum Albumin (BSA) was used as the reference protein and Commassie Blue was used as the acidic dye. The stock solutions of COMT and BSA proteins in 1 mg/ml concentration were prepared. The calibration curves for the each protein were prepared based on the spectrophotometric measurements at the optimum wavelength for the 5 different concentrations prepared in the range of 0-16 μg/ml. In accordance with the results of the spectral scanning performed in wavelength range of 400-700 nm by using a microplate reader (Multiskan, Thermo), the optimum wavelength was determined as 595 nm.

The active protein amount of COMT enzyme was estimated from the ratio of the slopes of the calibration curves prepared for both BSA and COMT.

4.2.5. Determination of Enzyme Activity of COMT and Inhibition Kinetics

The enzyme activity of COMT was determined spectrophotometrically and fluorometrically according to the protocols given in the literature. The inhibition experiments were performed by the fluorometric method which is more sensitive, less time consuming and more cost effective.

4.2.5.1. COMT Activity Determination by Spectrophotometric Method

In order to determine the activity of COMT spectrophotometrically, the protocol prepared by Sigma-Aldrich Chemie GmbH was applied. According to this protocol, the absorbance values of the *O*-methylated products of DHAP formed as a result of the possible COMT catalyzed methylation reaction given below were measured at 344 nm by using a spectrophotometer (Multiskan, Thermo).

$$\text{DHAP} + \text{SAM} \xrightarrow{COMT} \text{SAH} + \text{3H4MAP} + \text{4H3MAP}$$

According to this protocol which was prepared on the basis of stop rate determination method, the reaction was carried out at 37 °C and at the pH value of 7.6. Total reaction volume was kept constant at 1 ml. While concentrations of methyl donor substrate of reaction, SAM were varied between 4-0.2 mM (serially dilutions were made 5, 10, 15 and 20 times), the concentration of the methyl acceptor substrate, DHAP was kept constant at 0.5 mM. The 150 U of COMT standard enzyme (contains 1.9 mg solid) was prepared in TES buffer solution having concentration of 2.55 mg solid/ml. In Table 4.7, the order and amount of reactants added to the reaction medium were given.

Table 4.7. Reaction medium for spectrophotometric method

Reactants	Test solution (µl)	Blank solution (µl)
TES[1]	200	300
DHAP	100	100
SAM	100	-
COMT	100	100
Sodium Borate[2]	500	500

The enzyme activities were calculated using the mathematical equation given in the applied protocol and the results obtained for this method were given in Results and Discussion section of the study.

[1] 0.2 M TES (buffer solution used in enzyme dilutions) contains 6 mM $MgCl_2$ and 20 mM DTT. The pH was adjusted to 7.6 using 1 M of NaOH.

[2] 0.4 M Sodium borate (stop solution) was prepared by adjusting the pH of boric acid solution to 10.0 using 1 M of NaOH.

4.2.5.2. COMT Activity and Inhibition Kinetics Determination by Fluorometric Method

In determining COMT activity fluorometrically, some studies employed by almost the same manner were taken as basis (Kurkela et al., 2004; Veser, 1987). In this method, Aesculetin (ES) was used as the methyl acceptor substrate of possible COMT catalyzed methylation reaction given below. The transformation rate of ES to scopoletin was measured fluorometrically at 355 nm excitation and 460 nm emission wavelengths by using microplate reader (Varioskan, Thermo) for totally 2 hours during which the fluorescences were recorded at every 30 seconds time intervals.

$$ES + SAM \xrightarrow{COMT} SAH + Scopoletin$$

The two fluorescence values measured at the first 30 min. time interval of the steady state phase of reaction were used in kinetic calculations. One unit increment in fluorescence was related to the produced scopoletin concentration by using the calibration curve prepared with the scopoletin standard (Appendix C).

In activity determination and inhibition studies, the total volume of reactions, which were carried out at 37°C and pH of 7.4, were 200 µl and 250 µl, respectively. Potassium phosphate solution was prepared at a concentration of 100 mM containing 5 mM $MgCl_2$ and 20 mM L-cystein was used as buffer solution in fluorometric method. In the fluorometric analysis, first the linear working concentrations were determined for both enzyme and substrates. As a result of this experiment, the optimum concentrations of enzyme and ES were found as 11 µg protein/ml and 4 µM, respectively and the concentrations of enzyme and ES in inhibition study were kept constant at these optimum values. SAM concentrations were varied between 10-600 µM throughout the study.

The required concentrations for plant extracts, fractions and their standards that were used in inhibition study were determined for each of them. Thus, the 3,5-DNC was prepared in the concentration range of 0.1-0.005 µg/ml, while the concentration of other inhibitors were varied between 0.5-10 µg/ml. In Table 4.8, the amounts of reactants added to reaction medium were given for both activity determination and inhibition studies.

Table 4.8. Reaction medium for fluorometric method

Reactants	Test solution (µl)	Blank solution (µl)	Test solution (µl)	Blank solution (µl)
SAM	50	-	50	-
ES	75	75	125	125
COMT	50	50	50	50
Buffer	25	75	-	50
Inhibitor(s)	-	-	25	25

4.2.6. Analysis of Enzyme Activity and Inhibition Kinetics Data

The reaction velocity data obtained from the results of studies employed for determination of activity and inhibition kinetics were analyzed on the basis of the knowledge given in Section 2.3 and 2.4. In data analysis, a nonlinear regression program known as GraphPad Prism 5.0, in which the same enzyme kinetic model equations with those of given in Section 2.4 referring also the same kinetic models reported in Copeland, 2000's book, were used. The obtained kinetic velocity data were analyzed by fitting them to the appropriate kinetic models which were previously given as Equation 2.1, 2.2, 2.4, 2.6 and 2.8. Thus, the kinetic parameters for activity and inhibition could be calculated. In order to test the convenience of the chosen model, Lineweaver-Burk graphs were also drawn for each kinetic data set. Moreover, the goodness of fit of the model was statistically analyzed by applying analysis of variance (ANOVA) to the data set which was fallowed by the Tukey's multiple comparison tests.

CHAPTER 5

RESULTS AND DISCUSSION

Results of extraction, fractionation, characterization and enzyme activity/inhibition determination experiments performed by methods explained in detail in Chapter 4 are given in this section respectively.

5.1. Obtaining and Characterization of Extracts

5.1.1. Extraction of Harmal (*P. harmala*) Seed and its Characterization

In order to obtain the alkaloids in harmal (*P. harmala)* seeds various methods whose details are given in methods section had been tried. According to this, results obtained from studies with maceration, soaking and Soxhlet extractions were compared with respect to their efficiencies.

It is known that harmal seed dominantly consists of harmine, harmaline, harmol and harmalol alkaloids. In this respect, extracts obtained using different extraction methods were initially characterized by using High Performance Liquid Chromatography (HPLC). For the characterization, firstly calibration curves are obtained for each alkaloid standards (Appendix A. Calibration Curves of Alkaloids and Sample Calculation). According to this, amounts of major alkaloids present in crude extracts obtained with each extraction method were calculated. Extraction conditions of methods, in performing which some parameters such as solvent type, temperature, extraction time and pH were changed, were summarized in Table 5.1.

Table 5.1. Operation conditions for alkaloid extraction methods

Extraction Method	Solvent Type	Temperature (°C)	pH	Extraction Time (hours)
Method A*, S1-1	Methanol	50	10.0	4
Method A, S2-1	Methanol	50	11.0	4
Method A, S2-2	Methanol	50	8.5	4
Method B*, S1	Methanol	55	10.0	30
Method B, S2	Petrolium Ether /Methanol	50	10.0	56
Method B, S3	Ethyl Acetate	25	3.4	48
Method C*, S1	Methanol	70	10.0	7.5
Method C, S2	Methanol	70	10.0	20
Method C, S3	Methanol	70	10.0	26

* Method A: maceration, Method B: Solvent soaking, Method C: Soxhlet

The alkaloid contents and efficiencies of extracts obtained with methods given in Table 5.1 were calculated using calibration curves obtained for alkaloid standards were given in Table 5.2.

Table 5.2. Alkaloid contents and efficiencies of crude extracts obtained from harmal seed

Extraction Method	Harmol (mg)	Harmaline (mg)	Harmine (mg)	Total amount of extract (mg)	Initial seed amount (mg)	efficiency (%) = (mg alkaloid*100/ mg seed)
Meth. A, S1-1	14.85	61.48	38.72	115.05	2000	5.75
Meth. A, S2-1	-	53.45	35.43	88.88	1550	5.73
Meth. A, S2-2	-	43.82	26.95	70.77	1550	4.57
Meth. B, S1	8.88	77.01	52.27	138.16	2000	6.91
Meth. B, S2	-	44.47	28.07	72.54	9770	0.74
Meth. B, S3	-	23.03	11.63	34.66	3100	1.12
Meth. C, S1	-	97.06	68.70	165.76	2500	6.63
Meth. C, S2	-	126.57	86.50	213.07	3500	6.09
Meth. C, S3	18.30	81.85	55.52	155.68	3500	4.45

It is known according to the literature that harmal seed includes 2-7% of alkaloid (Ott, 1996). When this percentage is taken into consideration, most of the extraction methods given in Table 5.2 had been found adequate with respect to efficiency. Among the extraction parameters, effect of pH on efficiency was analyzed in Method A. Therefore, as it can be understood from Tables 5.1 and 5.2, extraction efficiency was increased when the solution pH value was 10.0 or higher. Consequently, the optimum pH value was determined as 10.0.

According to the results obtained for Method B developed in order to observe the effect of solvent type, it was indicated that methanol was the most appropriate solvent for alkaloid extraction. Moreover, the highest efficiency was obtained with the S1 system of Method B in which methanol was used as solvent among the others.

Soxhlet apparatus (Method C) was used in order to analyze the effect of extraction time on efficiency. It was found that as time passed more than one day period, efficiencies of extracts decreased. As it can be seen from Tables 5.1 and 5.2, efficiency values obtained with Method C-S1 and Method B-S1 were found very close to each other.

Since, by applying Method C-S1, the required amount of efficiency could be achieved in a shorter time among these two methods, so it was defined as the optimum alkaloid extraction method. Sample chromatograms of crude alkaloid extract obtained using optimum alkaloid extraction method and of the mixture of standards prepared in order to characterize each alkaloids present in crude extract were given in Figure 5.1 and 5.2, respectively.

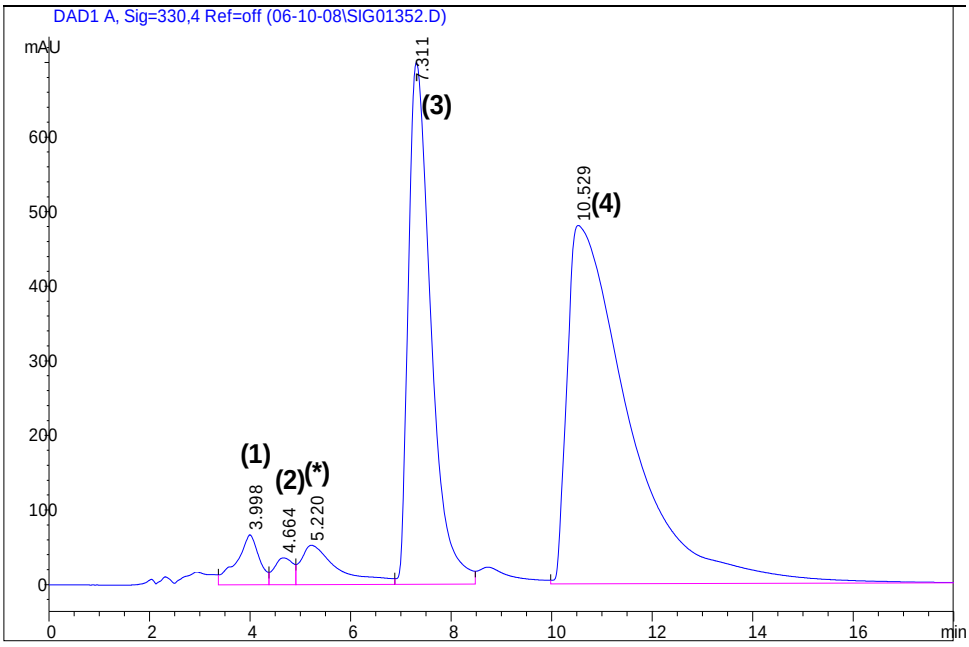


Figure 5.1. Sample chromatogram of the crude alkaloid extract (1) harmol, (2) harmalol, (3) harmine, (4) harmaline (analytical column)

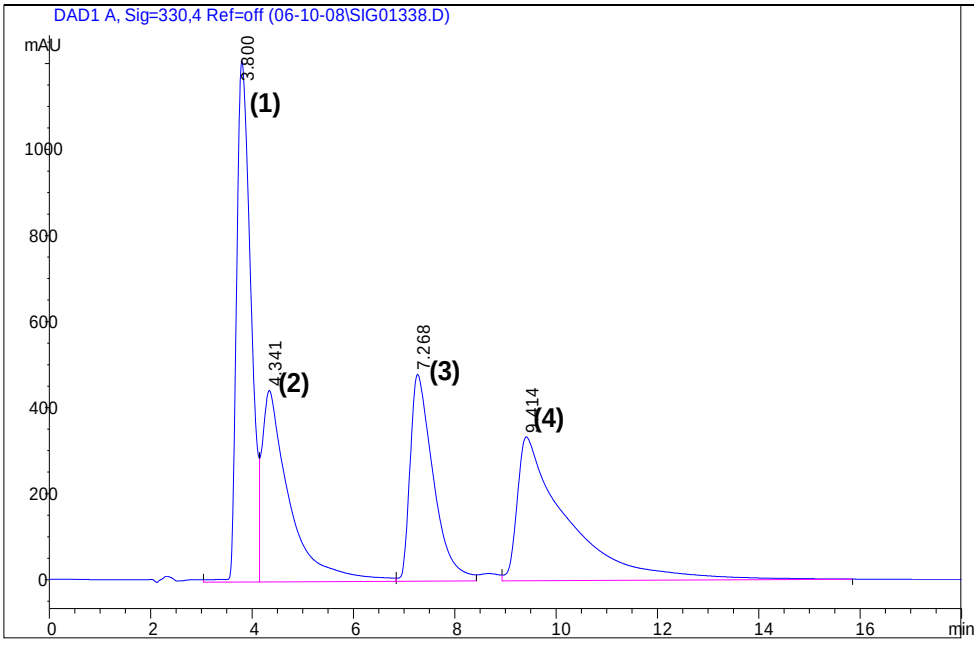


Figure 5.2. Sample chromatogram of the mixture of alkaloid standards (1) harmol, (2) harmalol, (3) harmine, (4) harmaline (analytical column)

According to HPLC chromatogram obtained for the mixture of alkaloid standards in Figure 5.2, retention times of alkaloids in non-polar column were determined respectively as 3.8 min. for Harmol, 4.3 min. for Harmalol, 7.3 and 9.4 min. for Harmine and Harmaline. Therefore, alkaloids in harmal seed extract were

characterized according to their retention times (Figure 5.1). Accordingly, elusion with highly polar mobile phases caused high polarity alkaloids to leave the column in a shorter time.

It was also observed from the HPLC analysis of crude alkaloid extracts and alkaloid standards mixture of which the obtained chromatograms given in Figure 5.1 and 5.2, a shift occurred in retention times of alkaloids in crude extract. It was thought that the shift in the retention times may be caused by other compounds obtained from plant material during extraction process, their amounts and intermolecular interaction.

As it can be observed from the sample chromatogram (Figure 5.1) given for the crude extract, harmine and harmaline were dominantly present alkaloids in harmal seed and their amounts were given in Table 5.2. Besides, harmol and harmalol content of the extract obtained by the Soxhlet method was found below the detection limits determined by their corresponding alkaloid standards, therefore amounts of them could not be calculated. Detection and quantification limits of standard alkaloids determined by their calibration concentrations were given in bold characters in Table 5.3.

Table 5.3. Concentration ranges of alkaloids used for calibration

Alkaloid Type	**Harmol (1)**	**Harmalol (2)**	**Harmine (3)**	**Harmaline (4)**
Concentration range (µg/ml)	**1050**	**1270**	**983**	**1180**
	525	635	492	590
	210	254	196	236
	105	127	98	118
	42	**51**	**39**	**47**

Moreover, an unidentified peak other than those of the four main alkaloids was observed and could be seen on the chromatogram of crude extract given in Figure 5.1. It was thought that this peak marked as (*) may be a derivative of harmol or harmalol alkaloids. However, it had not been characterized since it was not as dominant as the main alkaloids in the crude extract.

5.1.2. Extraction of Olive (*O. europaea*) Leaf and its Characterization

As given in the methods section, extraction process for obtaining olive leaf polyphenolics was performed according to the optimum conditions in the literature (Altiok et al., 2008; Baycin et al., 2007). Accordingly, the powdered olive leaves obtained after some pretreatment processes were extracted in 70 % aqueous ethanol solution for 2 hours at 250 rpm and 30 °C, solid/liquid ratio was 1/20 for the extraction of polyphenolics.

The obtained crude extract of polyphenolics were characterized by using HPLC as well as it was performed for the alkaloids and it was found that olive leaf extract were rich in oleuropein and rutin. Quantifications of these two main polyphenolics in the extract were determined by calibration of their corresponding standards. Calibration curves obtained for oleuropein and rutin standards were given in Appendix B with sample calculations. Sample chromatogram of the crude extract obtained from olive leaf was demonstrated in Figure 5.3.

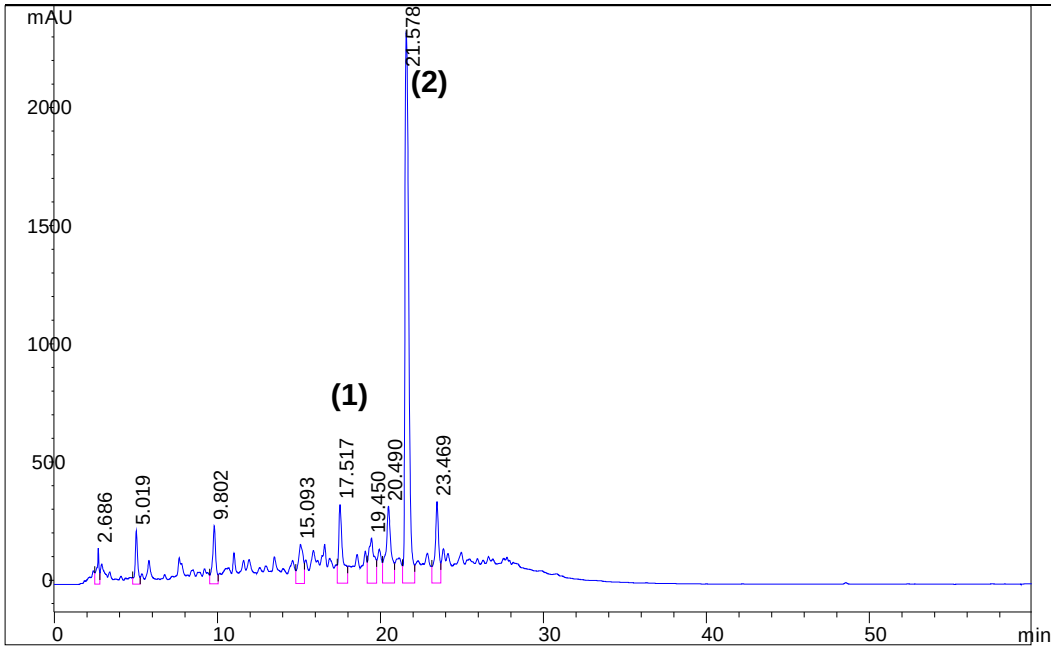


Figure 5.3. Sample chromatogram of crude olive leaf extract (1) rutin, (2) oleuropein (Mobile phase flow rate: 1 ml/min, column temperature 30 °C, analytical column)

As it can be seen from the chromatogram, it was found that oleuropein was the most dominant polyphenol present in the olive leaf extract. Based on the polarities of polyphenolics, it was observed that rutin was more polar than oleuropein and retention times of both on the column were determined as 17.5 and 21.6 minutes. Other peaks which were not quantitatively dominant were characterized as glycosides and glycoside derivative polyphenolics such as luteolin-7-glycoside, verbascoside, apigenin-7-glycoside and luteolin in the study of Altiok et al., 2008.

Moreover, according to calibration curves obtained for oleuropein and rutin standards, their amounts in the crude olive leaf extract were found as 9.0% and 0.9%, respectively.

5.2. Fractionation and Characterization of Extracts

The fractionation techniques applied for extracts rich in alkaloids and polyphenolics which were extracted at optimum conditions were given in detail in methods section.

5.2.1. Fractionation and Characterization of Harmal Seed Extract

Harmal seed alkaloids obtained with Soxhlet extraction method (70 °C, 7.5 hours) were collected as three different fractions by using HPLC compatible fractioning apparatus as given in the methods section. Fractions were characterized also by using HPLC-analytical column. In Figure 5.4, the chromatogram of the crude harmal seed extract performed in HPLC-semipreparative column conditions was given.

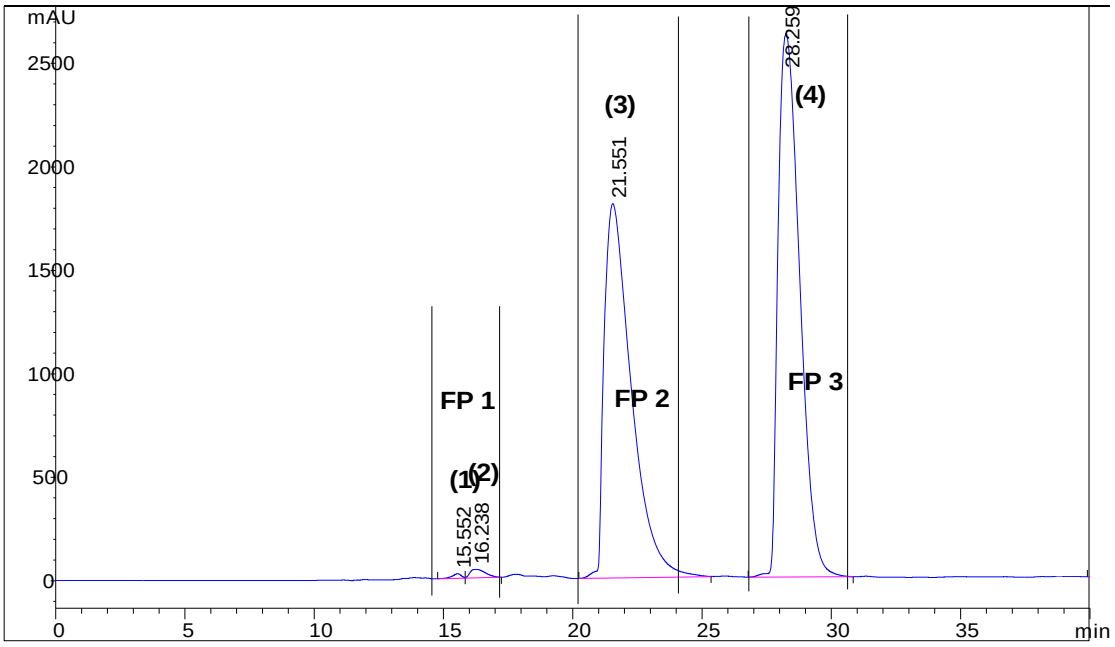


Figure 5.4. Sample chromatogram of crude harmal seed extract before fractioning (1) harmol, (2) harmalol, (3) harmine, (4) harmaline (Mobile phase flow rate: 1 ml/min, column temperature 30 °C, semipreparative column)

According to the HPLC analysis of crude alkaloid extract with semipreparative column, retention times of alkaloids were found 15.6 min. for harmol, 16.2 min. for harmalol and 21.6 min. and 28.3 min. for harmine and harmaline, respectively. Crude extract was separated into 3 parts according to the retention times and their majorities in the extract during fractioning. As shown on the chromatogram given in Figure 5.4, while 1st and 2nd alkaloids detected between 14th and 18th minutes were determined as the first *P. harmala* fraction (FP 1), harmine fraction (FP 2) was collected between 20th and 24th minutes. Harmaline fraction (FP 3) was obtained by collecting between 27th and 31st minutes.

The quantifications of the compounds in each fraction were determined according to calibration curves obtained for alkaloid standards after being analyzed them by using HPLC-analytical column. In Figure 5.5 and 5.6, chromatograms of harmine and harmaline fractions obtained as a result of HPLC analytical column analyses were given, respectively.

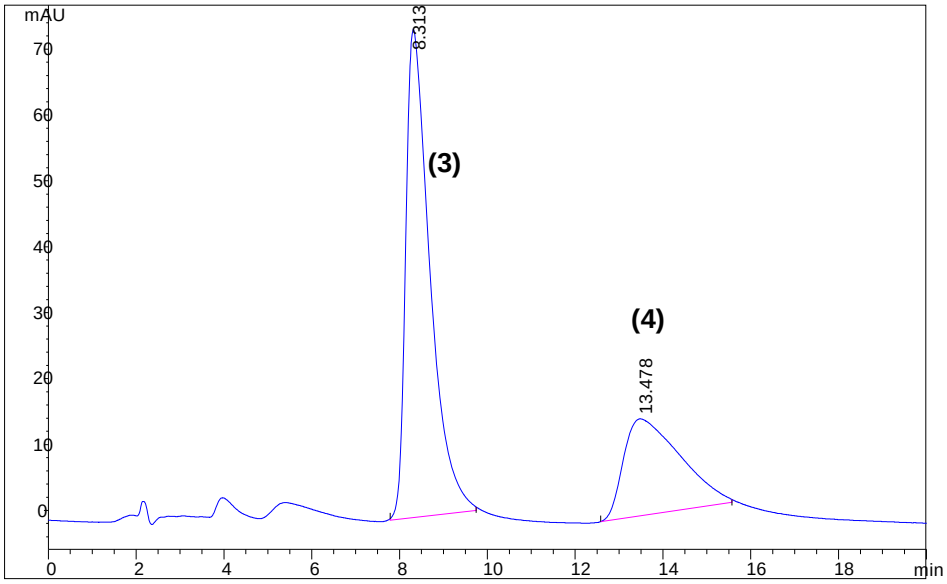


Figure 5.5. Sample chromatogram of harmine fraction (FP 2) (3) harmine, (4) harmaline (Mobile phase flow rate: 1 ml/min, column temperature 30 °C, analytical column)

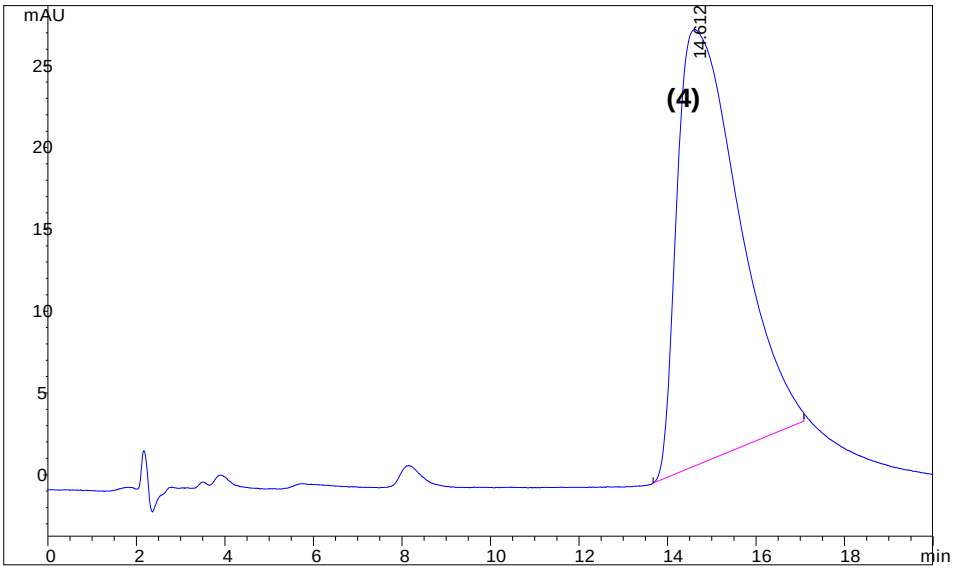


Figure 5.6. Sample chromatogram of harmaline fraction (4) harmaline (Mobile phase flow rate: 1 ml/min, column temperature 30 °C, analytical column)

As it can be seen from the chromatograms of fractions, after fractioning there was a shift in retention times of the 3rd and 4th alkaloids which were determined as harmine and harmaline alkaloids by the crude extract analysis (Figure 5.1). According to this, retention time of harmine peak was delayed to 8.3 minutes from 7.3 minutes, and retention time of harmaline peak shifted to 14 minutes from 10.5 minutes. This

result indicated that the polarities of fractions, of which the purities had been attempted to increase, decreased after fractioning.

Purities of the fractions obtained for the major alkaloids of crude extract were calculated using the calibrations of the standards and the results were given in Table 5.4.

Table 5.4.Characterization results of major alkaloid fractions obtained from harmal seed

Alkaloid Name	Fraction No.		Peak Percentage of Fractions (%)	
	FP 2	FP 3	FP 2	FP 3
Harmine peak	yes	traces	77.0	6.1
Harmaline peak	yes	yes	22.5	93.2

Other than these, HPLC analyses of FP 1 where harmol and harmalol alkaloids were dominant were also performed. However, according to the results, traces of many peaks which were thought to be derivatives of harmala alkaloids were observed. Each alkaloid fraction was collected, dried and stored in appropriate conditions in adequate amounts in order to further use in enzyme inhibition experiments.

5.2.2. Fractionation and Characterization of Olive Leaf Extract

Extraction, characterization and fractionation of polyphenolics in olive leaf were performed based on the studies of Altıok et al., 2008 and Baycin et al., 2007 as it was explained in detail in methods section. As can be seen from the chromatogram of crude extract (Figure 5.3), oleuropein and rutin polyphenols were found as dominant in olive leaf. Other than these, many glycoside derivatives in the crude extract were characterized in the study of Altıok et al., 2008. In the fractioning process performed using silk fibroin packed column, 6 fractions were collected whose elution solvents were given in Table 5.5.

Table 5.5. Elution solvents and contents of fractions obtained from olive leaf extract

Fraction No.	Elution Solvents and Contents
FO 1	1st and 2nd water fraction
FO 2	3rd and 4th water fraction
FO 3	5th and 6th water fraction
FO 4	7th, 8th and 9th water fraction
FO 5	1st and 2nd 70% aqueous ethanol fraction
FO 6	3rd and 4th 70% aqueous ethanol fraction

Chromatograms of olive leaf fractions were given from Figure 5.7 to 5.12 with respect to their order given in Table 5.5. As a results of HPLC analysis of fractions which can also be understood from following chromatograms, it was found that water fractions were characterized as rich in oleuropein, and aqueous ethanol fractions were found rich in rutin and glycosides.

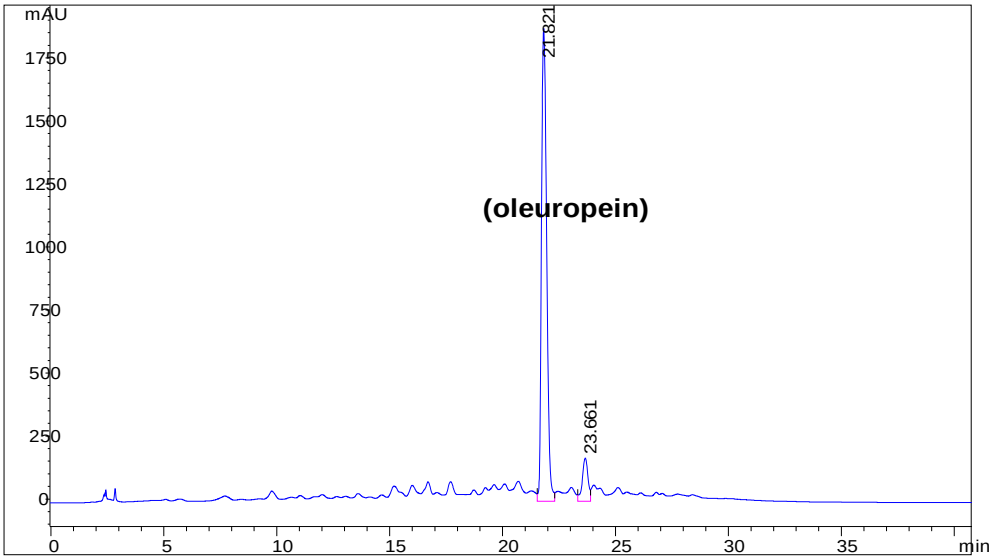


Figure 5.7. Chromatogram of olive leaf fraction coded as FO 1

The purity of oleuropein of which the retention time was 21.6 minutes, was calculated as 91.0 % in the first 2 water fractions (FO 1).

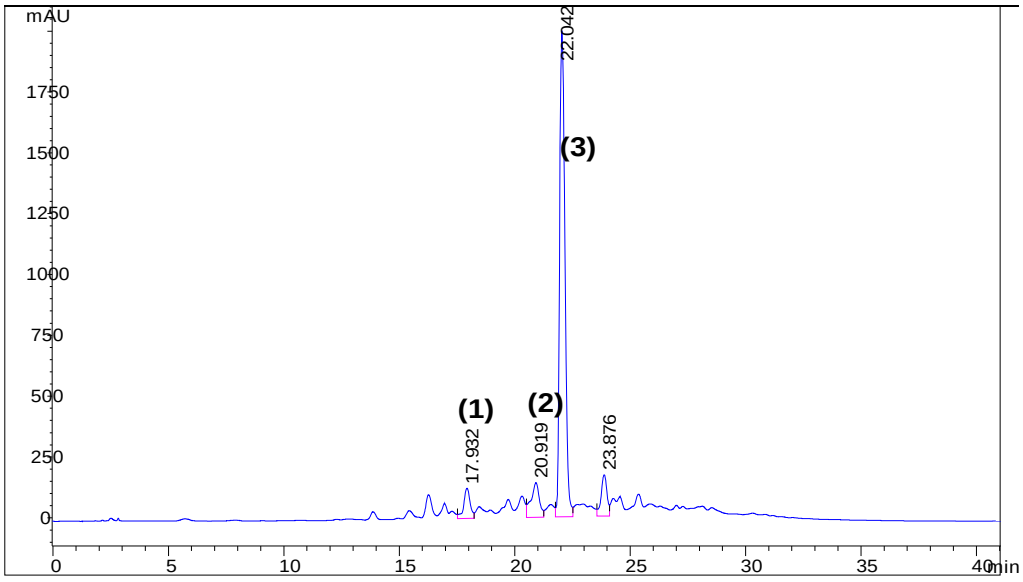


Figure 5.8. Chromatogram of olive leaf fraction coded as FO 2 (1) rutin, (2) verbascoside, (3) oleuropein

As a result of further elution the silk fibroin column with water, oleuropein percentage of olive leaf fraction was reduced while less amounts of polyphenolics such as rutin and verbascoside were observed.

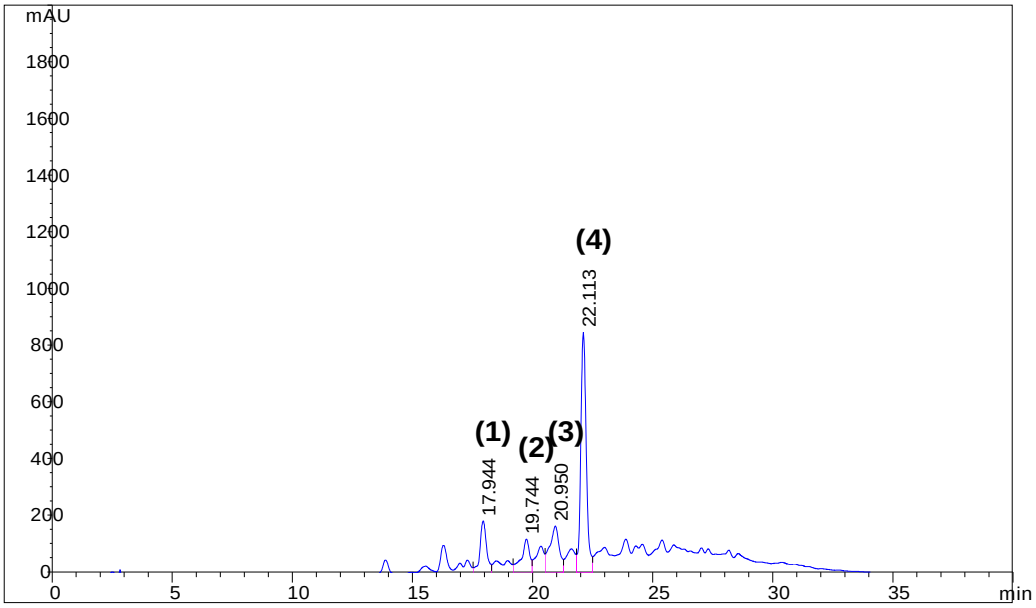


Figure 5.9. Chromatogram of olive leaf fraction coded as FO 3 (1) rutin, (2) luteolin-7-glycoside, (3) verbascoside, (4) oleuropein

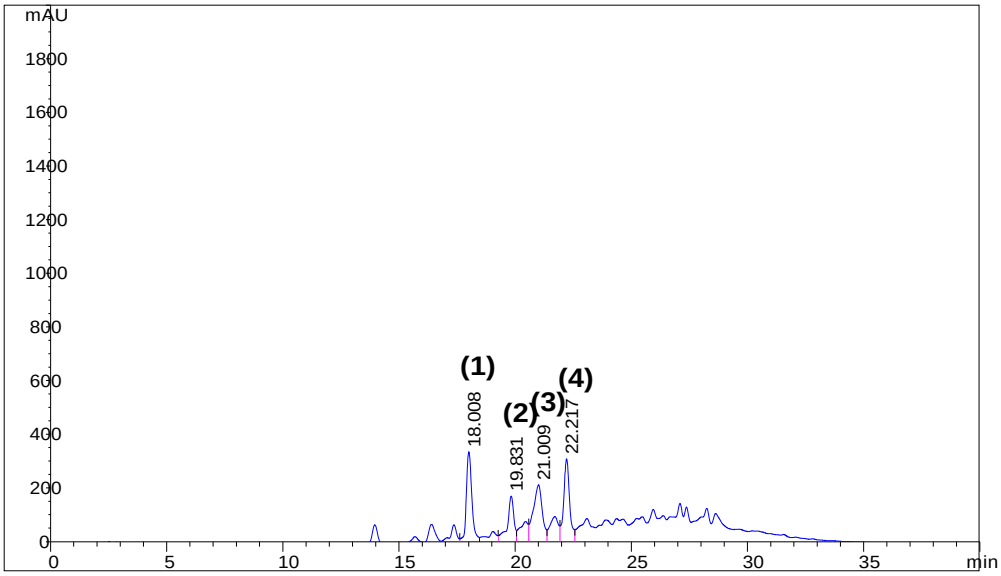


Figure 5.10. Chromatogram of olive leaf fraction coded as FO 4 (1) rutin, (2) luteolin-7-glycoside, (3) verbascoside, (4) oleuropein

As it can be seen from the chromatograms of the water fractions given in Figure 5.9 and 5.10, further water elution reduced the oleuropein content in the fractions to nearly 25%. Moreover, it was observed that the fractions obtained with aqueous ethanol elution, of which the chromatograms were given in Figure 5.11 and 5.12, were found rich in rutin and glycosides.

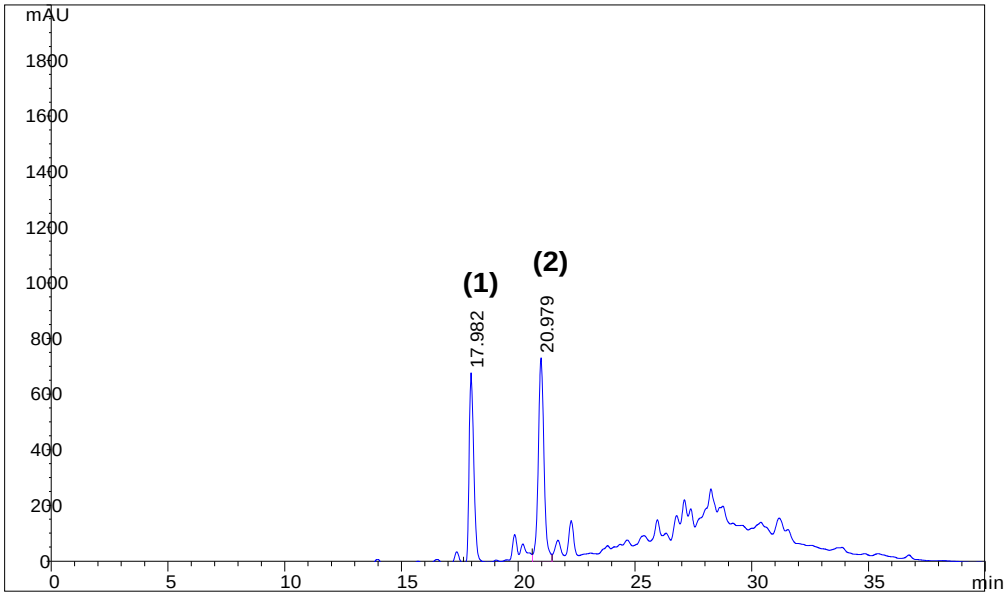


Figure 5.11. Chromatogram of olive leaf fraction coded as FO 5 (1) rutin, (2) verbascoside

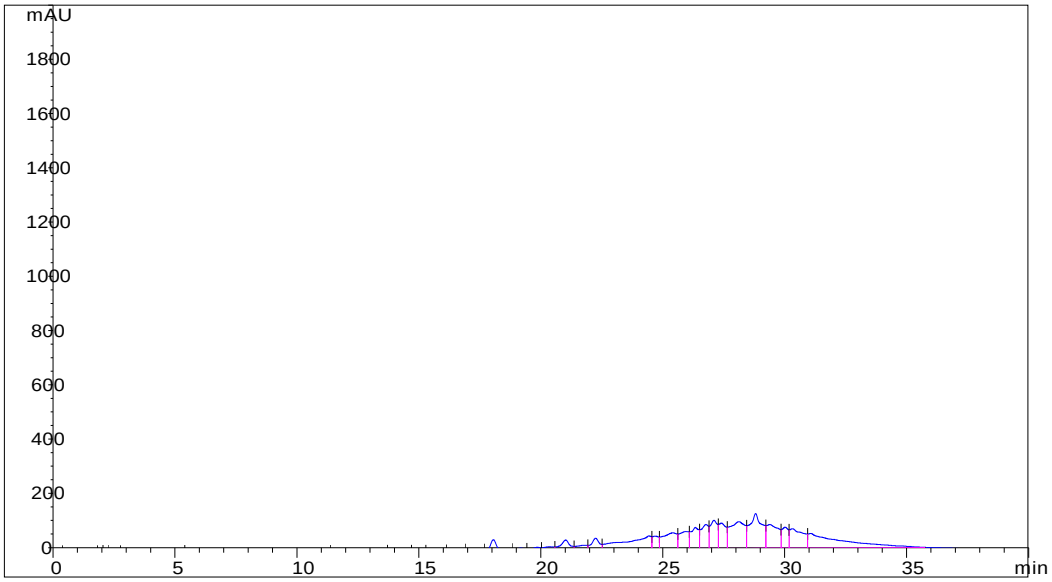


Figure 5.12. Chromatogram of olive leaf fraction coded as FO 6 (other forms of glycosides with low polarity)

It was found that oleuropein contents of fraction coded as FO 5 and FO 6 obtained with aqueous ethanol elution were below detection limits even it was not observed. Despite this, as it can be seen from the chromatograms, fraction coded as FO 5 and FO 6 were characterized as fractions rich in rutin and glycosides. As a result of fractioning of olive leaf extract, oleuropein contents of fractions were calculated as percentage according to calibration curves and the results were summarized in Table 5.6.

Table 5.6. Oleuropein contents of olive leaf fractions

Fraction No.	Time (min.)	Peak Intensity (%)
FO 1	21.8	90.95
FO 2	22.0	78.15
FO 3	22.1	54.70
FO 4	22.2	26.86
FO 5	No oleuropein peak	-
FO 6	No oleuropein peak	-

Accordingly, the purity of oleuropein, the most dominant polyphenol present in the olive leaf extract, could be increased up to 90% by using silk fibroin packed column adsorption process. Fractioning efficiency in terms of oleuropein was decreased as long as water elution was continued. When aqueous ethanol was used as elution solvent, fractions rich in rutin and glycosides which were adsorbed by the column tighter were obtained. Among all of the obtained fractions from olive leaf extract, fractions rich in oleuropein and rutin coded as FO 1 and FO 5 were obtained in adequate quantity to further use in enzyme inhibition studies and stored in appropriate conditions.

5.3. Determination of Protein Amount

As mentioned before, in the enzyme protein amount determination method known as Bradford method, Bovine Serum Albumin (BSA) was used as reference protein and Commassie blue as acidic dye. Results of the spectral scanning obtained in order to observe the effect of the increase in protein concentration on absorbance and to determine the optimum wavelength were given in Figure 5.13. As a result of this scanning, maximum absorbance of unbound Commassie blue (blank) to the protein was found as 465 nm wavelength as given in the literature (Rosenberg, 1996). By adding same amount of different concentrations of BSA to the blank solutions, maximum absorbance value shifted to 595nm from 465 nm.

At the same time, while the increase in the protein concentration affected absorbances inversely between 400-525 nm, it caused an increase in the absorbance values between 525-700 nm. This result which is known characteristics of Bradford method was found in good accordance with the literature (Rosenberg, 1996).

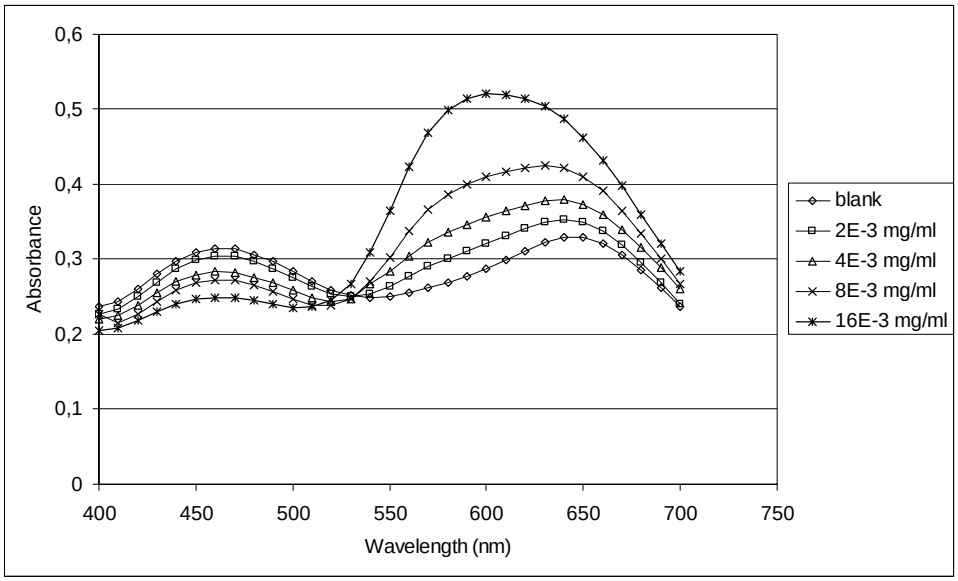


Figure 5.13. Spectral scanning for different BSA concentration

Spectrophotometric measurements were performed at 595 nm which was found as the optimum wavelength in order to determine the protein amount of the COMT enzyme isolated from porcine liver used as the enzyme source of this study. Calibration curves given in Figure 5.14 were obtained by using the measured absorbance values at 595 nm of BSA and COMT solutions prepared in 0-16 µg/ml concentrations.

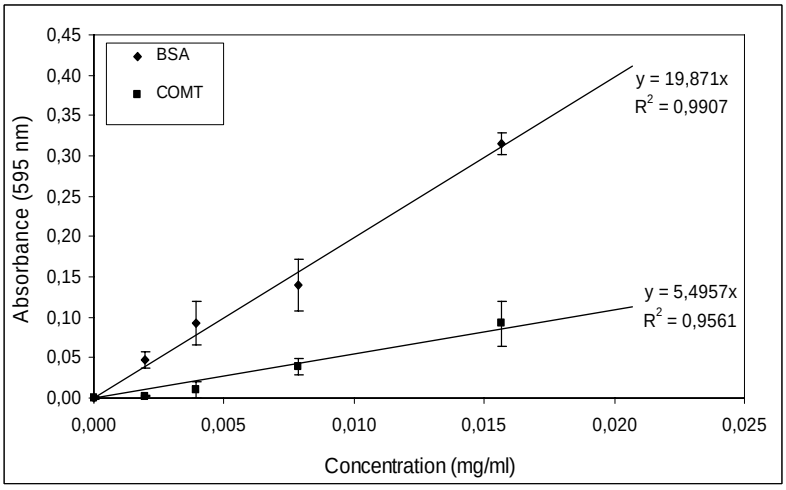


Figure 5.14. Calibration curves obtained for proteins

By taking into consideration of the ratio of estimated slopes given on calibration curves, it was determined that the active protein amount of COMT enzyme was found 27.7% according to BSA reference protein.

5.4. Determination of COMT Activity and Inhibition Kinetics

5.4.1. Activity Determination by Spectrophotometric Method

According to the protocol, by which the COMT enzyme activity was determined spectrophotometrically, absorbances of produced the *O*-methylated forms of DHAP were measured at 344 nm.

$$\text{DHAP} + \text{SAM} \xrightarrow{COMT} \text{SAH} + \text{3H4MAP} + \text{4H3MAP}$$

The possible reaction given above was incubated at pH of 7.6 and 37 °C for one hour. In the reaction performed in a total volume of 500 µl, while DHAP final concentration was fixed at 0.1 mM, varying SAM concentrations were given in Table 5.7. Final concentration of 150-unit COMT standard enzyme in the reaction medium was kept constant at 0.51 mg solid/ml. Absorbances of test and blank solutions measured at 344 nm after the reaction was terminated by adding 500 µl of sodium borate at the end of one hour were also given in Table 5.7.

Table 5.7. Reaction Medium and Absorbances for Spectrophotometric Method

Reaction No.	SAM Concentration (mM)	Absorbance (344 nm)
Blank*	-	0.481
Test 1	0.800	0.512
Test 2	0.160	0.498
Test 3	0.080	0.492
Test 4	0.053	0.490
Test 5	0.040	0.488

* According to the protocol, blank solution absorbance value should be between 0.3-0.5.

Enzyme activity was calculated using the mathematical equations defined in the applied protocol as following;

$$Unit/ml = \frac{(A_{test} - A_{blank})(126.2)(dilutionfactor)}{0.1}; \qquad (5.1)$$

$$Unit / mgprotein = \frac{Unit / ml}{mgprotein / ml} \quad (5.2)$$

where 126.2 was the conversion factor defined for 1 ml reaction volume and 0.1 was the volume of enzyme added to reaction medium (ml). Accordingly, enzyme activities[1] obtained for test solutions were given in Table 5.8.

Table 5.8. Enzyme Activities (spectrophotometric)

Reaction No.	Unit/ml	Unit/mg protein	Unit/mg solid*	*v* (nmol/mg protein. min.)	1/*v* (nmol/mg protein. min.)$^{-1}$
Test 1	195.61	276.93	76.70	4.62 ± 0.10	0.22 ± 0.02
Test 2	107.27	151.86	42.06	2.53 ± 0.16	0.39 ± 0.04
Test 3	69.41	98.26	27.21	1.64 ± 0.11	0.61 ± 0.01
Test 4	56.79	80.39	22.27	1.34 ± 0.10	0.75 ± 0.02
Test 5	44.17	62.53	17.32	1.04 ± 0.13	0.96 ± 0.01

* 27.7 % of one mg solid material was taken as active protein (see Bradford method)

Reaction velocity (v) and inverse reaction velocity (1/v) values in Table 5.8 were analyzed with linear and nonlinear regression analyses performed by using GraphPad Prism 5.0 program as explained in 4.2.6. Enzyme Activity and Inhibition Kinetics Data Analysis section. The obtained Michaelis and Lineweaver-Burk graphs were given in Figure 5.15.

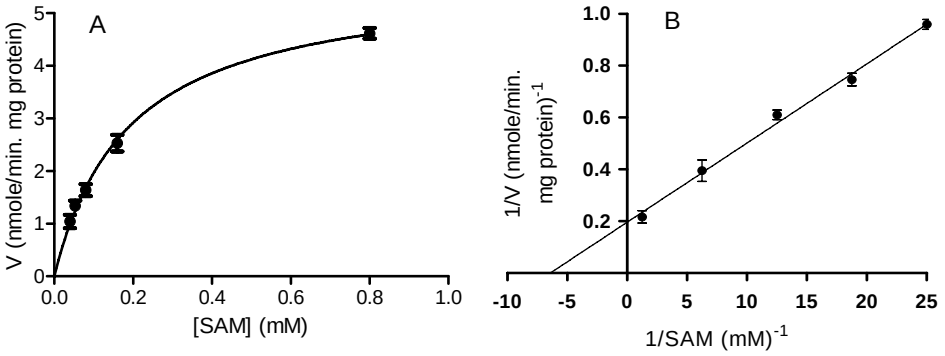


Figure 5.15. A: Michaelis and B: Lineweaver-Burk graphs of spectrophotometrically determined enzyme activity

[1] 1 unit of enzyme activity = 1 nmol of *O*-methylated forms of DHAP produced in one minute per mg of protein.

V_{max} and K_m values for this reaction performed in non-inhibitor medium were found as 5.69 ± 0.15 nmol/min. mg protein and 0.19 ± 0.01 mM, respectively. Due to the fact that, the required amounts of enzyme and substrate in spectrophotometric method were quite high and the protocol was developed for reactions performed in optic cuvettes causing to consume more time, the inhibition study were performed by fluorometric method using 96 well microplates. Since the entire spectrophotometric method was a commercial protocol, it was not possible to compare the obtained results with the literature. However, by performing this protocol, active protein capacity and activity of standard COMT enzyme could be tested.

5.4.2. Determination Activity and Inhibition Kinetics by Fluorometric Method

It was known that the results obtained for enzymatic reactions monitored by fluorometric method were more sensitive than absorption and less interference was observed (Copeland, 2000). Therefore, the protocol modified for determination of COMT activity and inhibition kinetics from the studies employed based on fluorometric method was applied in this study (Kurkela et al., 2004; Veser, 1987).

In this method, transformation rate of aesculetin (ES) to scopoletin by COMT catalyzed *O*-methylation reaction and thereby change in fluorescence was measured at 355 nm excitation and 460 nm emission wavelengths.

$$ES + SAM \xrightarrow{COMT} SAH + Scopoletin$$

Total reaction volumes in activity determination and inhibition studies performed at 37 °C and pH of 7.4 were determined as 200 µl and 250 µl, respectively.

5.4.2.1. Activity Determination by Fluorometric Method

In the activity determination study by fluorometric method, firstly a linear working range for different enzyme and substrate concentrations was found. Concentration changes of enzyme and substrates in these reactions were given in Table 5.9.

Table 5.9. Concentration changes of enzyme and substrates

Enzyme concentrations (µg protein/ml)			
E1	16.5		
E2	11.0		
E3	8.5		
SAM concentrations (µM)		**Aesculetin concentrations (µM)**	
SAM 1	10	ES1	2
SAM 2	50	ES2	3
SAM 3	100	ES3	4
SAM 4	200	ES4	5
SAM 5	300	ES5	7
SAM 6	500	ES6	10
SAM 7	600		

In these reactions, which are known to have a total reaction time of 6-8 hours, activity was determined by taking the change of fluorescence values between two points after reaction progressed at steady state. In order to determine the accurate steady state phase of reactions, fluorescent measurements were recorded for 10 hours and kinetic data of the sample reaction occurred at the condition of E1-ES3-SAM1 concentrations were given in Figure 5.16.

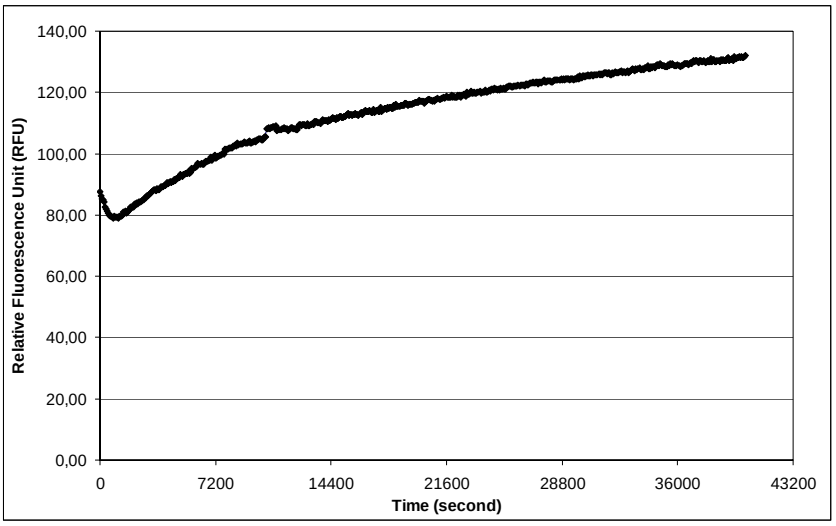


Figure 5.16. Product formation kinetics obtained at E1-ES3-SAM1 concentrations (10 hours)

Based on the kinetic data recorded in 10 hr. time interval given in Figure 5.16, it was found adequate to measure the first two hours of fluorescent change for reactions catalyzed by COMT. Moreover, fluorescent reduction in the first 20 minutes was a characteristic feature of the reaction and the kinetic measurements to be used for activity calculations was composed of the values in the first 30 minutes after this period. For this purpose, a total two hours of fluorescent measurement obtained for the same reaction conditions was given in Figure 5.17.

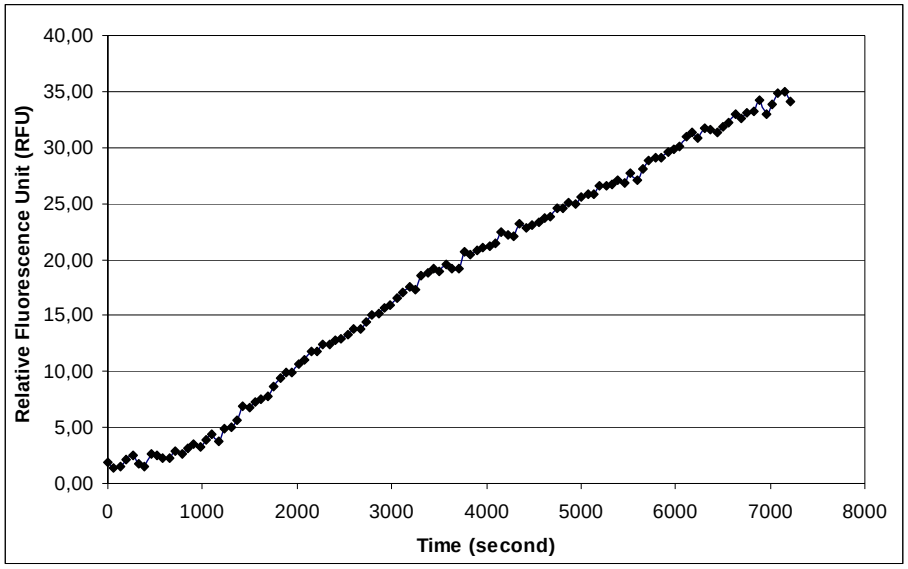


Figure 5.17. Product formation kinetics obtained at E1-ES3-SAM1 (2 hours)

Kinetic data were obtained for all reactions occurred at every concentration values given in Table 5.9, as it was obtained as a result of the reaction given as an example in Figure 5.17. Accordingly, it was determined that the measurements recorded at the first 30 minute after the 1500th second indicated the ones obtained from steady state phase of reactions. Fluorescent change in this 30 min. time interval was transformed into enzyme activity data by using the value indicating the effect of one nanomole of scopoletin formation on the fluorescence obtained as a result of its calibration. Sample calculation of the data transformation and scopoletin calibration curve were also given in Appendix C.

Accordingly, results of the studies, performed in order to determine the linear enzyme-substrate concentration range and where the change of the reaction rate according to SAM and ES substrates was examined, were given as enzyme activity data in Figure 5.18 and 5.19, respectively.

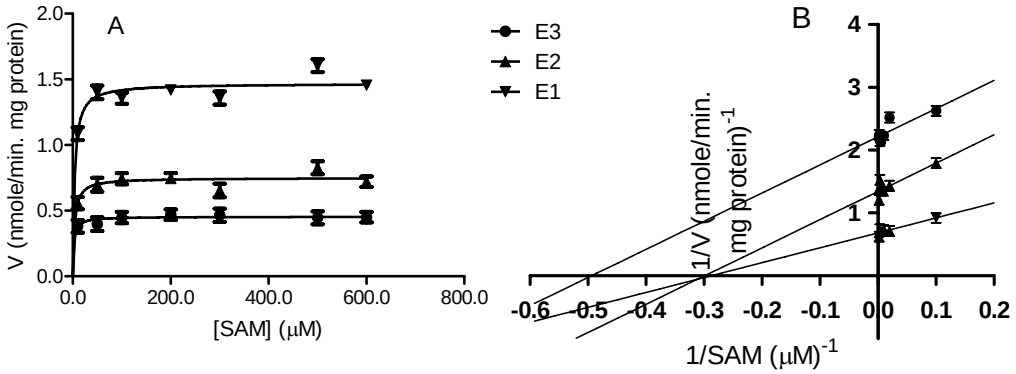


Figure 5.18. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different enzyme concentrations ([ES] was fixed at 4 μM).

It can be seen from Figure 5.18A, increasing enzyme concentration increased reaction rate as expected. However, it is known that in high concentration of SAM caused substrate inhibition takes place (Yassin et al., 1998; Veser, 1987). Therefore it was observed that the reaction rate obtained at the lowest enzyme concentration E3 (8.5 μg protein/ml) deviated from linearity (Figure 5.18B). This deviation which was thought to be arising from substrate inhibition was slightly observed for rates obtained at E2 and E1 concentrations. However, the reaction rate obtained at E2 and E1 concentrations did not deviated from linearity up to 100 μM SAM concentration as given in Figure 5.19.

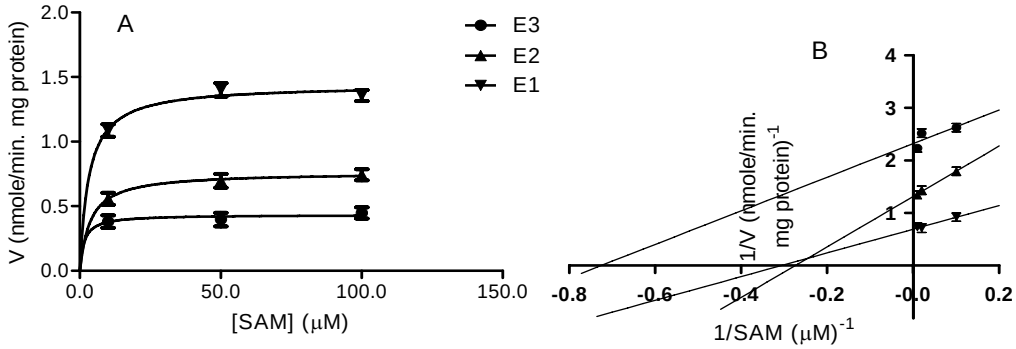


Figure 5.19. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin up to 100 μM SAM concentrations in presence of different enzyme concentrations ([ES] was fixed at 4 μM).

E2 concentration by which it was able to perform reactions in linear concentration range more economically with respect to E1 was chosen as the fixed

enzyme concentration to be used in inhibition study. Moreover, in order to reveal the relationship of substrates, reactions, of which the results were given in Figure 5.20 as reaction rate data, were performed at fixed E2 concentration while both SAM and ES concentrations were varied.

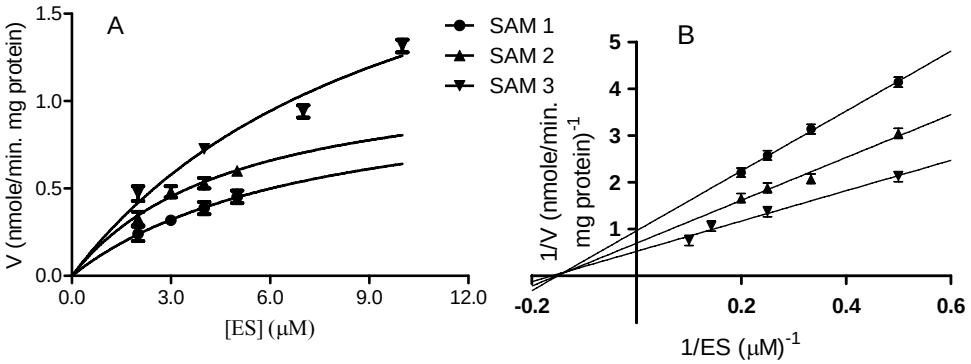


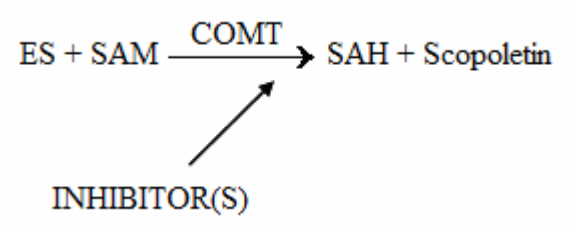
Figure 5.20. A: Michaelis ve B: Lineweaver-Burk graphs of formation rate of scopoletin against various ES concentrations in presence of different SAM concentrations (enzyme concentration was fixed in 11.0 μg protein/ml (E2))

It was observed that the reaction rate increased with the increasing SAM concentration and did not deviate from linearity in this concentration range. As a result of this study performed in order to determine the enzyme-substrate relationship, K_m values for SAM and ES were calculated as 3.5 ± 0.3 μM and 6.4 ± 0.4 μM, respectively. K_m value for aesculetin in Veser, 1987's study was reported as 6.2 μM. K_m values found for SAM in several studies have varied according to the used COMT enzyme source and difference in the linear concentration ranges. However, it was found that SAM which was the methyl donor substrate of COMT catalyzed methylation reaction was the rate limiting reactant.

Based on the results obtained from fluorometric activity tests, it was determined that in inhibition studies, the concentrations of both ES and enzyme should be kept at 4 μM and 11 μg protein/ml, respectively. On the other hand, SAM concentrations were varied between the ranges of 10-300 μM.

5.4.2.2. Inhibition Study by Fluorometric Method

On the basis of the results obtained for fluorometric activity determination, reducing in COMT catalyzed reaction rate was also analyzed fluorometrically by using some plant-derived alkaloids, polyphenolics and their standards as inhibitor.



In inhibition study performed in presence of inhibitors given in Table 5.10, enzyme kinetic measurements were recorded at 355 nm excitation and 460 nm emission wavelengths. Total reaction volume was 250 µl in these experiments performed at 37 °C and pH of 7.4. While the SAM concentrations was changed between 10-100 µM for first 16 inhibitors given in Table 5.10, its concentration was varied between 10-300 µM in studies performed with 17^{th} and 18^{th} inhibitors.

Among the inhibitors given in Table 5.10, 3,5-DNC was used as positive control and the inhibition performances of other inhibitors were compared with that of the positive control. The obtained inhibition performances of alkaloid and polyphenolics based standards, extracts and fractions at 100 µM SAM concentration were given in Figure 5.21 and 5.22. In calculation of inhibition percentages of inhibitors, reaction rate obtained at the medium where any inhibitor was present was used as reference rate.

Table 5.10. The plant extracts, fractions and their standards used as COMT inhibitors in this study

Inhibitor Name	Code	Concentration (µg/ml)	Inhibitor Name	Code	Concentration (µg/ml)
3,5-dinitrocatechol (3,5-DNC)	I1a	0.1	Oleuropein	I10a	10
	I1b	0.02		I10b	5
	I1c	0.005		I10c	2
Harmol	I2a	5	Rutin	I11a	10
	I2b	2		I11b	5
	I2c	0.5		I11c	2
Harmalol	I3a	5	Olive (*O. europaea*) leaf crude extract	I12a	10
	I3b	2		I12b	5
	I3c	0.5		I12c	2
Harmine	I4a	5	Fraction rich in Oleuropein (FO 1)	I13a	10
	I4b	2		I13b	5
	I4c	0.5		I13c	2
Harmaline	I5a	5	Fraction rich in Rutin and glycosides (FO 5)	I14a	10
	I5b	2		I14b	5
	I5c	0.5		I14c	2
Harmal (*P. harmala*) seed crude extract	I6a	5	Vitex (*V. agnus-cactus*) leaf crude extract	I15a	10
	I6b	2		I15b	5
	I6c	0.5		I15c	2
Harmol and Harmalol fraction (FP 1)	I7a	5	Cistus (*C. parviflorus*) leaf crude extract	I16a	10
	I7b	2		I16b	5
	I7c	0.5		I16c	2
Harmine fraction (FP 2)	I8a	5	Terebinth (*P. terebinthus*) leaf crude extract	I17a	10
	I8b	2		I17b	5
	I8c	0.5		I17c	2
Harmaline fraction (FP 3)	I9a	5	Mastic (*P. lentiscus*) leaf crude extract	I18a	10
	I9b	2		I18b	5
	I9c	0.5		I18c	2

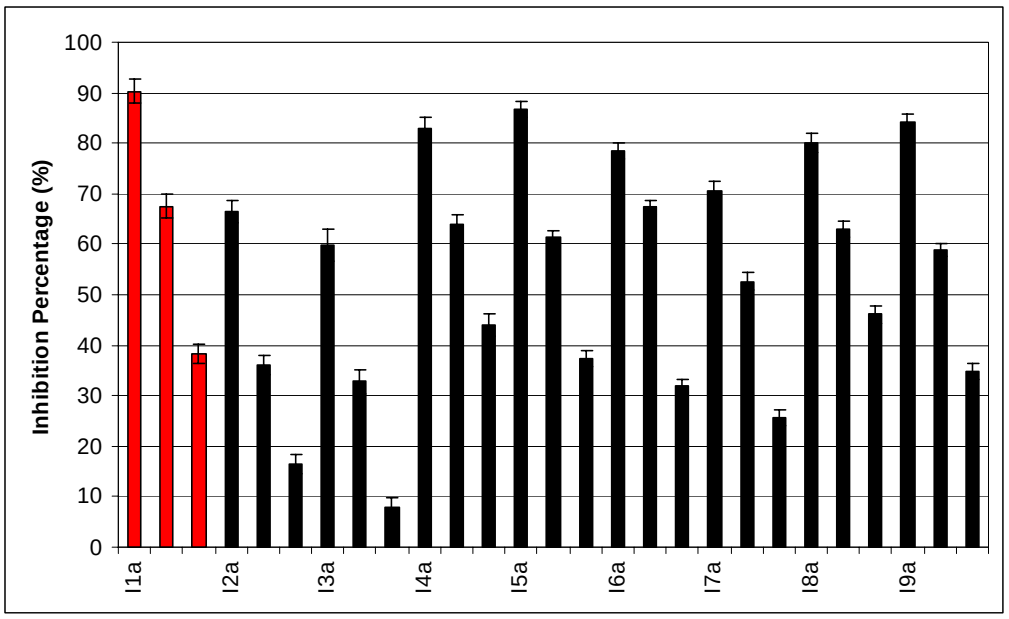


Figure 5.21. Inhibition percentages of alkaloid standards, extracts and fractions

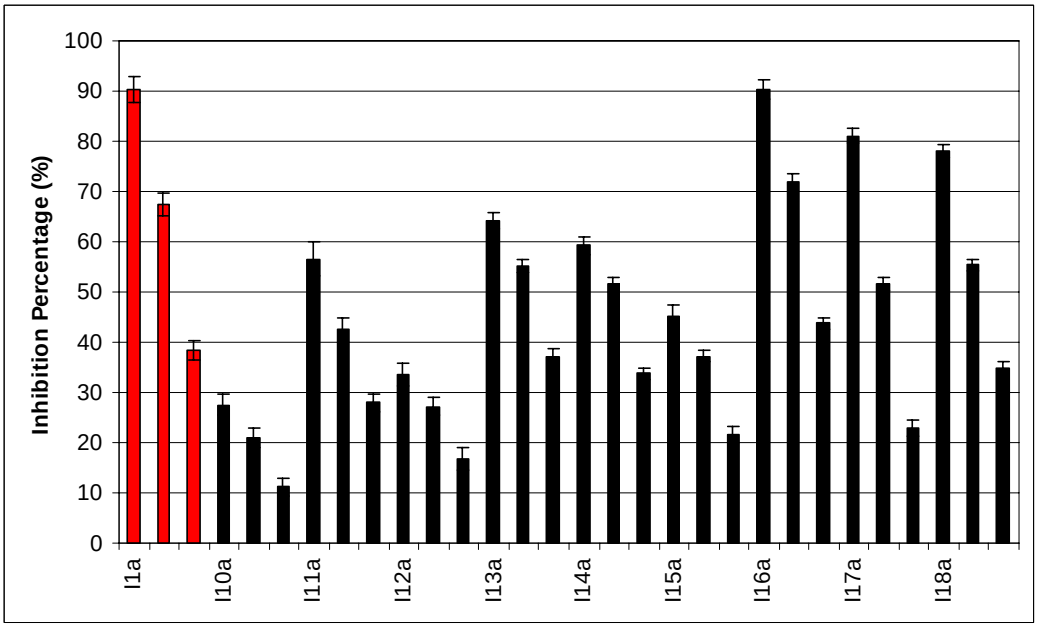


Figure 5.22. Inhibition percentages of polyphenolic standards and extracts and fractions

As it can be seen from Figures 5.21 and 5.22, inhibition performances of alkaloid and polyphenolic standards, extracts and fractions used in this study were compared to that of positive control, 3,5-DNC which is the best known COMT inhibitor. Accordingly, while it was found that inhibition performance of 3,5-DNC was 100 times higher than those of especially harmine and harmaline alkaloid standards and fractions, 3,5-DNC demonstrated about 200 and 300 times higher inhibitory effect than

those of polyphenolics when taking into consideration the concentration ranges of inhibitors.

Although their performances were not found as effective as positive control, it was revealed that inhibitors coded as I6 (*P. harmala*-harmal), I16 (*C. parviflorus*-cistus), I17 (*P. terebinthus*-terebinth) and I18 (*P. lentiscus*-mastic) crude extracts demonstrated quite high reducing effect against the COMT catalyzed reaction rate.

Moreover, it was observed that as a result of the characterization of *P. harmala* seed crude extract, harmine and harmaline alkaloids were the major alkaloids present in crude extract. Therefore, it was thought that these major alkaloids in harmal seed were the compounds responsible for inhibition when similarities in the inhibition performances of crude extract (I6) and its harmine (I8) and harmaline (I9) fractions were also taking into consideration.

In order to explain the inhibition mechanism of each inhibitor used in this study, the mathematically derived enzyme inhibition kinetics model equations were used as mentioned before in experimental section. The results of the most statistically appropriate model, fitted to the experimental enzyme activity data obtained in presence of 18 different inhibitors, were given as graphically from Figure 5.23 to Figure 5.40.

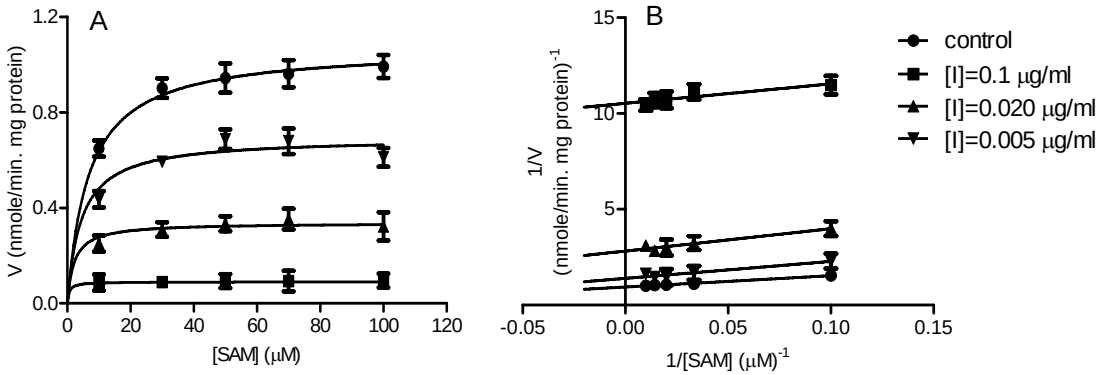


Figure 5.23. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I1 (3,5-DNC) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

As reported in Bonifacio et al., 2007's study, it is known that almost all nitrocatecholics are the tight binding and uncompetitive inhibitors of COMT enzyme. As it can be seen from Figure 5.23B, 3,5-DNC which was used as positive control in this study was found as a COMT inhibitor with uncompetitive behavior in good accordance with literature. The presence of 3,5-DNC even in its low concentrations in

the reaction medium caused a very high reduction in scopoletin formation rate (v). As a result of the experimental data analyzed by GraphPad program, K_m and V_{max} kinetic constants were calculated as 6.83±0.77 μM and 1.07±0.02 nmol/min. mg protein, respectively. Inhibition constant (αK_i) given in rate equation (Equation 2.6) derived for uncompetitive inhibition was found 9.17±0.46 ng/ml (in other words; 45.60±2.29 nM) for 3,5-DNC. Also, the 50% inhibitory concentration (IC_{50}) of 3,5-DNC was found 44.18±0.78 nM which was in good accordance with the value reported as 35 nM in the literature (Kurkela et al., 2004).

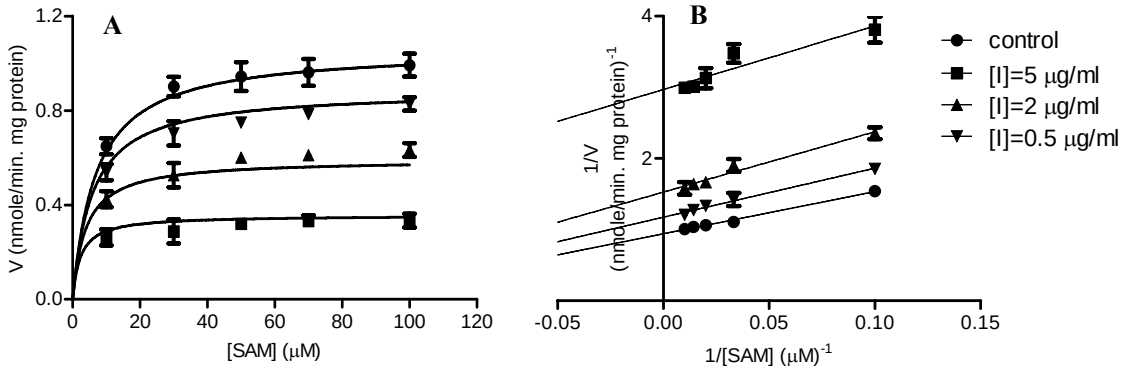


Figure 5.24. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I2 (harmol standard) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

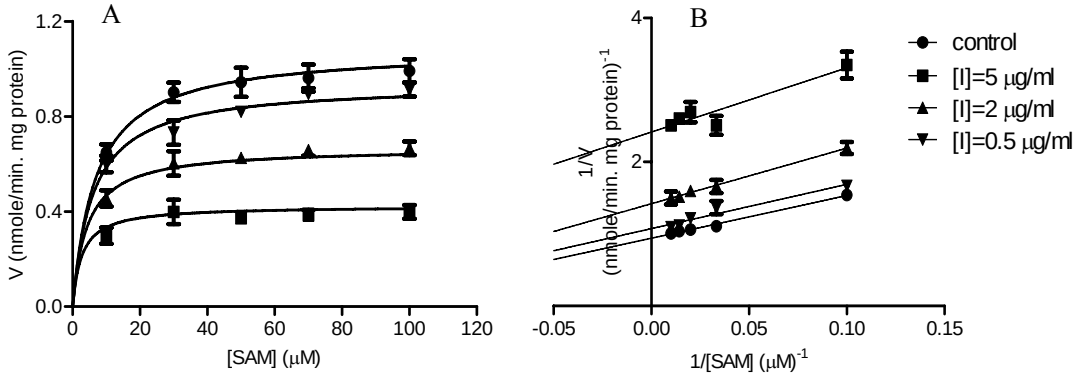


Figure 5.25. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I3 (harmalol standard) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

Graphical demonstrations of the experimental data obtained with standards of harmol and harmalol alkaloids which were the minor alkaloids present in harmal seed

were given in Figures 5.24 and 5.25, respectively. COMT inhibition mechanisms of these two alkaloids which are structurally similar to each other exhibited also similarity with each other and the positive control. As can be seen from Lineweaver-Burk graphs, it was determined that harmol and harmalol alkaloids were uncompetitive inhibitors of COMT.

As a result of the reactions performed in the presence of harmol and harmalol alkaloids, K_m and V_{max} kinetic constants were calculated respectively as 6.97±0.89 μM and 1.06±0.03 nmol/min. mg protein for harmol and 6.93±0.64 μM and 1.08±0.02 nmol/min. mg protein for harmalol. Inhibition constant (αK_i) of harmol was found 2.52±0.16 μg/ml while it was calculated as 3.21±0.16 μg/ml for harmalol. As well as the kinetic constants were very close to each other, the IC_{50} values of harmol and harmalol were found very similar and calculated as 2.79±0.22 μg/ml (=10.31±0.46 μM) and 3.59±0.37 μg/ml (=13.19±0.31 μM), respectively. Accordingly, it was revealed that harmol alkaloid can partially be a better inhibitor than harmalol.

Data analysis graphs obtained for harmine and harmaline alkaloids found dominantly in harmal seed were given in Figure 5.26 and 5.27. Inhibition mechanisms of these alkaloids which are structurally different from harmol and harmalol but similar to each other were also found different from harmol and harmalol alkaloids.

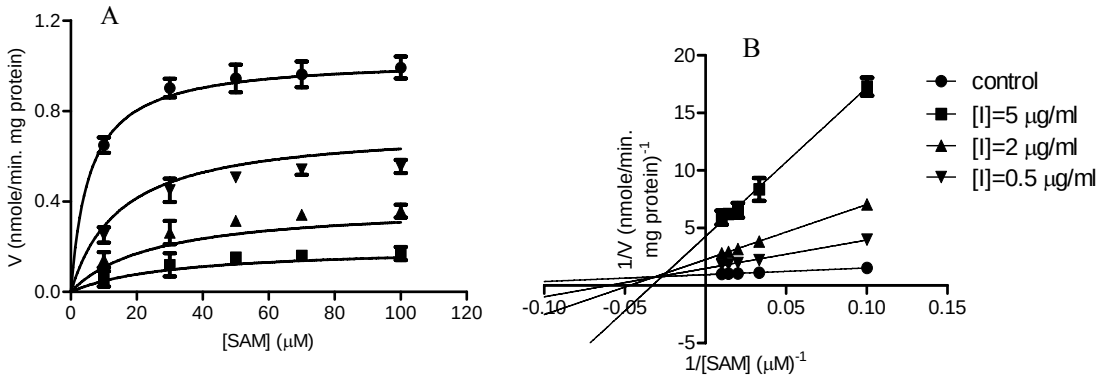


Figure 5.26. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I4 (harmine standard) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

As it can be seen from Figure 5.26B, the data obtained in presence of harmine standard could be explained by mixed type inhibition mechanism. As it was performed for this type of inhibitors, Michaelis graph which was drawn using the kinetic model given in Equation 2.8 was given in Figure 5.26A. Inhibition constants in this equation,

K_i and αK_i values which indicate the competitiveness and noncompetitiveness were found as 0.18±0.07 µg/ml and 1.18±0.08 µg/ml, respectively. Value of α constant was calculated as 6.56±0.81 in the presence of harmine alkaloid. K_m ve V_{max} kinetic constants were also calculated as 5.75±1.21 µM and 1.03±0.04 nmol/min. mg protein, respectively. Besides, IC_{50} value of harmine alkaloid was found 0.75±0.09 µg/ml, or in other words 3.55±0.16 µM.

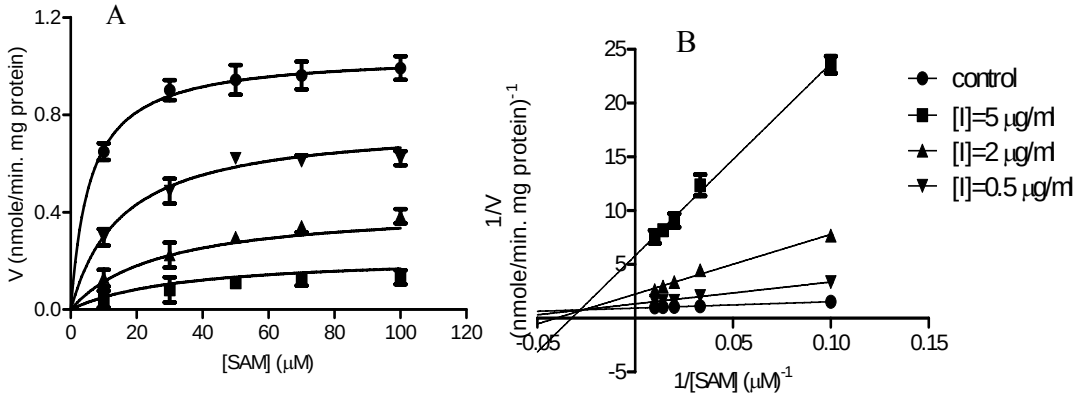


Figure 5.27. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I5 (harmaline standard) concentrations ([E] and [ES] were fixed at 11.0 µg protein/ml and 4 µM, respectively)

Also in the inhibition kinetics data analysis performed for harmaline standard given in Figure 5.27B, mixed type inhibition model was determined as the most appropriate model as it was found for harmine. Inhibition constants of harmaline standard, K_i and αK_i values were calculated respectively as 0.19±0.04 µg/ml and 1.36±0.11 µg/ml. α constant was found somewhat higher than that of found for harmine, as 7.16±0.78. Accordingly, it was revealed that the noncompetitive feature of harmine was higher due to having lower α value. However, harmine and harmaline alkaloids did not demonstrate too different behaviors from each other. Moreover, IC_{50} value was found as 0.98±0.12 µg/ml, in other words, 4.57±0.14 µM for harmaline alkaloids. K_m and V_{max} kinetic constants in presence of harmaline were calculated respectively as 5.92±0.87 µM and 1.05±0.02 nmol/min. mg protein.

As a result of the studies performed with alkaloid standards, the COMT inhibition performances of harmine and harmaline alkaloids was found higher than those of harmol and harmalol. Despite the fact that harmine and harmaline were the inhibitors behaving in very similar mixed type manner, according to results obtained for

standards, COMT inhibitory effect of harmine alkaloid was found the highest. In the light of these results, inhibition performances of harmal seed crude extract and its fractions were analyzed and compared with their corresponding standards. The data obtained in presence of harmal seed crude extract in reaction medium were demonstrated graphically in Figure 5.28.

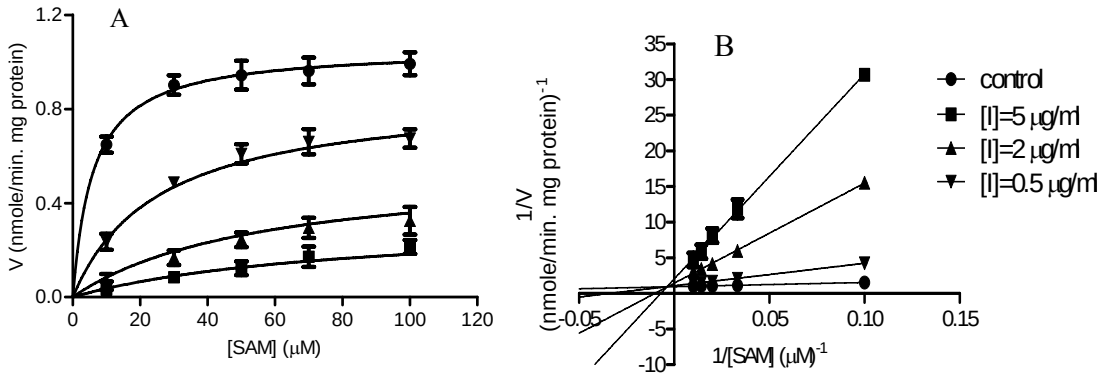


Figure 5.28. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I6 (harmal seed crude extract) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

According to the results, the COMT inhibition performance of the crude alkaloid extract was found as effective as harmine and harmaline which were the major alkaloids exist in the extract, and its mechanism could be explained in a similar manner with that of the standards. As it can be seen from Figure 5.28B, crude extract behaved as mixed type inhibitor and the inhibition constants, K_i and αK_i were calculated respectively as 0.12±0.02 μg/ml and 2.24±0.75 μg/ml having α value of 18.72±2.28. The higher α value of crude extract compared to that of harmine and harmaline alkaloids caused to shift the mechanism to competitive inhibition as it can be seen from the Lineweaver-Burk graph (Figure 5.28B). IC_{50} concentration of crude extract was found 1.09±0.33 μg/ml while K_m and V_{max} were calculated as 6.05±0.69 μM and 1.06±0.02 nmol/min. mg protein, respectively. According to the results obtained in the presence of crude extract, a partial increase in K_m value was observed and it was thought this increase arises from the increase in competitiveness.

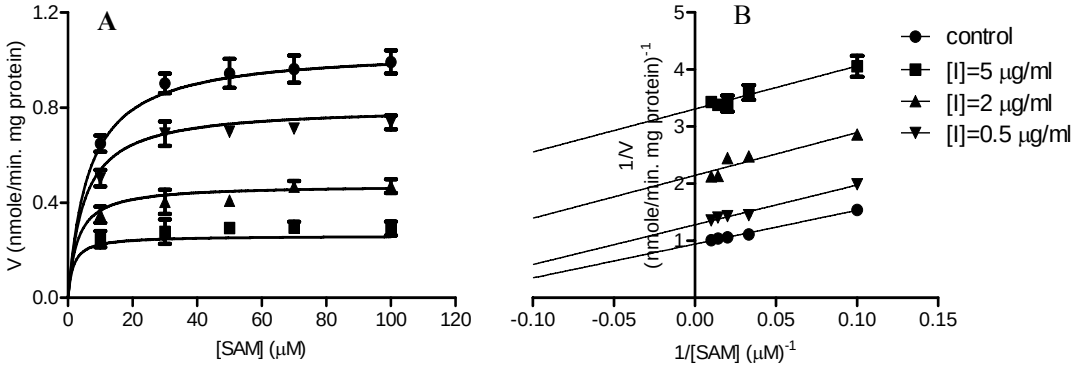


Figure 5.29. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I7 (harmal seed fraction no FP1) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

Results of the inhibition studies performed with fractions of harmal seed alkaloids was found compatible with that of their corresponding standards. Data analysis results obtained for the harmal seed fraction which was characterized as rich in harmol and harmalol alkaloids were given graphically in Figure 5.29. Similar to the results obtained for harmol and harmalol standards given in Figures 5.24 and 5.25, it was observed that harmal fraction coded as FP1 inhibited COMT uncompetitively. The αK_i and IC_{50} values for FP1 fraction were calculated as 1.66±0.09 μg/ml and 1.74±0.13 μg/ml, respectively.

Accordingly, it was thought that the alkaloids being together might have a synergistic effect on the COMT inhibition performance of FP1 fraction which was observed to be more effective compared to harmol and harmalol standards. K_m and V_{max} values in the presence of FP1 fraction was calculated as 6.44±0.82 μM ve 1.05±0.02 nmol/min. mg protein, respectively.

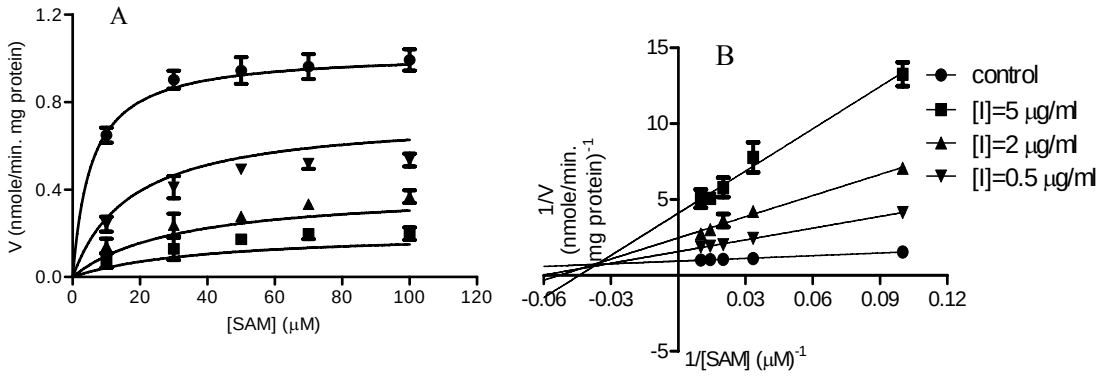


Figure 5.30. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I8 (harmal seed fraction no FP2) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

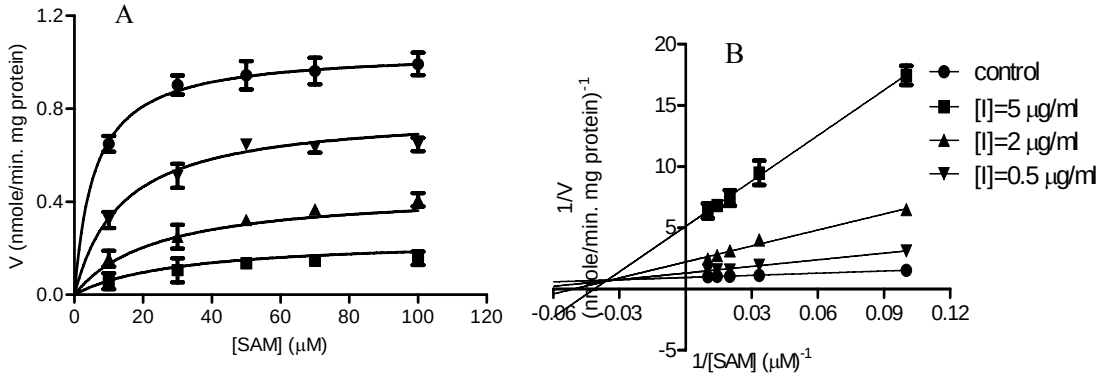


Figure 5.31. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I9 (harmal seed fraction no FP3) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

As a result of the HPLC analyses, it was found that harmal fraction coded as FP2 consisted of 77% of harmine and 23% of harmaline. Therefore, as a result of inhibition experiments performed with the FP2 fraction, it was observed that presence of harmine and harmaline together has no effect on inhibition mechanism and it was defined as mixed type inhibitor (Figure 5.30) like its corresponding standards. However, as it was observed for fraction FP1, presence of harmine and harmaline together provided a partially increasing effect on inhibition performance. K_i and αK_i values, found respectively as 0.18±0.07 μg/ml and 1.18±0.08 μg/ml for harmine standard, were calculated as 0.15±0.07 μg/ml and 1.28±0.06 μg/ml for FP2 fraction which was rich in harmine. The IC_{50} value of FP2 fraction was found lower than the harmine standard, as

0.69±0.03 µg/ml. Also, K_m and V_{max} constants were calculated as 5.61±1.68 µM ve 1.03±0.05 nmol/min. mg protein, respectively.

Data analysis obtained for FP3 fraction having 93.3% harmaline content was graphically demonstrated in Figure 5.31. In accordance with the results obtained for harmine and harmaline standards, inhibition performance of FP3 fraction rich in harmaline was found lower than that of FP2 fraction. However, inhibition mechanism for this fraction was also determined as mixed type, as seen from Figure 5.31B. For the FP3 fraction, K_i and αK_i values were calculated as 0.23±0.05 µg/ml and 1.47±0.08 µg/ml, respectively. The α and IC_{50} values were found to be very close to those of harmaline standard and calculated respectively as 6.39±1.08 and 1.10±0.07 µg/ml.

In inhibition study, it was previously mentioned that the other plant species examined with its fractions and comparing with its corresponding standards was olive leaf rich in polyphenolics. It was observed that the olive leaf polyphenolics of which inhibition performances were found lower than alkaloids when they were compared to that of 3,5-DNC. However, in order to illuminate the inhibition mechanisms of polyphenolics, the experimental data of them were also been attempted to be explained by Michaelis approach.

Oleuropein, as given in the characterization results of this study, was a polyphenol found dominantly in olive leaf. In this respect, COMT inhibition mechanism of oleuropein standard was examined firstly. Results obtained for oleuropein standard were given graphically in Figure 5.32.

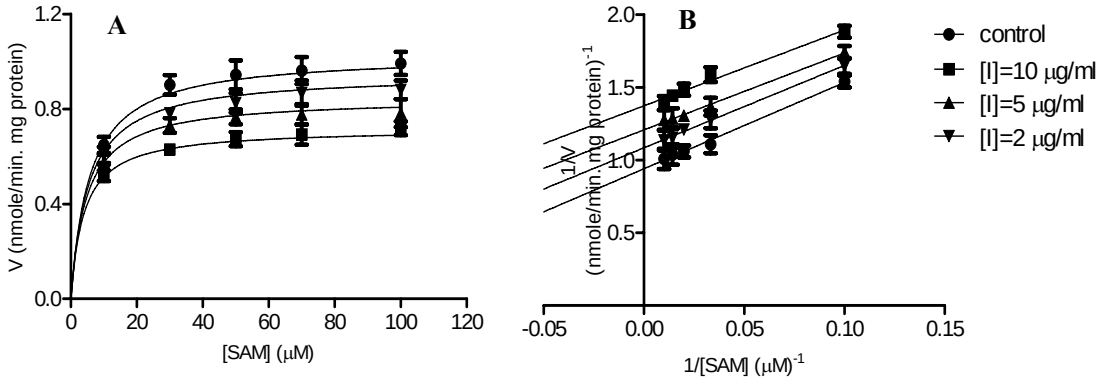


Figure 5.32. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I10 (oleuropein standard) concentrations ([E] and [ES] were fixed at 11.0 µg protein/ml and 4 µM, respectively)

The uncompetitive nature of oleuropein can be seen from Figure 5.32B and inhibition constant (αK_i) was found 22.96±1.39 µg/ml. However as it can be seen from Figure 5.32A, for the used concentration range of oleuropein, 50% inhibition of COMT activity could not achieved. Therefore IC_{50} value could not be calculated for oleuropein standard. K_m and V_{max} kinetic constants in the presence of oleuropein were calculated as 5.74±0.39 µM and 1.03±0.01 nmol/min. mg protein, respectively. Results of the inhibition experiments performed with the standard of rutin which was the secondary dominant polyphenol in olive leaf extract were given in Figure 5.33.

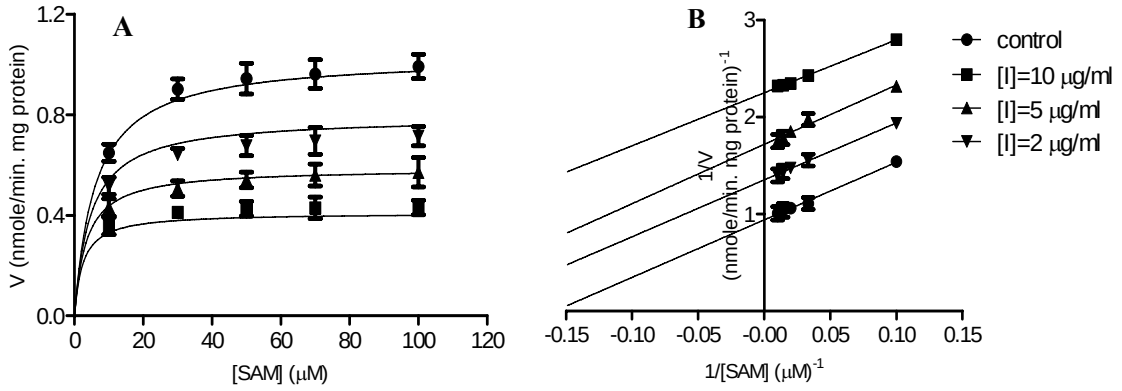


Figure 5.33. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I11 (rutin standard) concentrations ([E] and [ES] were fixed at 11.0 µg protein/ml and 4 µM, respectively)

Oleuropein and rutin which are structurally similar polyphenolics was found as uncompetitive inhibitors of COMT (Figures 5.32B and 5.33B). However rutin standard demonstrated higher inhibitory effect than oleuropein in the same concentration range. Inhibition constant, αK_i was found as 6.58±0.39 µg/ml for rutin standard; in other words, it was 10.78±0.52 µM. In Gugler et al., 1973's study, similar to this study, 19 flavonoids were scanned in order to reveal their COMT enzyme inhibition performances. Among these, quercetin and rutin were reported as the flavonoids with highest inhibitory effect.

Moreover, in the same study inhibition constants for quercetin and rutin were found respectively as 5.3 and 10.8 µM. This value was almost exactly compatible with that of rutin calculated in this study. However, in Gugler et al., 1973's study, it was argued that rutin and quercetin inhibit COMT in competitive manner. It was thought

that the difference in the obtained results arises from the differences in the used methyl acceptor substrates and also in analysis methods.

Furthermore, IC_{50} value for rutin standard was found close to the values calculated for harmol and harmalol alkaloids, as 7.17±0.81 µg/ml (=11.75±0.93 µM). K_m and V_{max} kinetic constants in the presence of rutin were respectively found as 6.05±0.79 µM ve 1.03±0.02 nmol/min. mg protein.

Based on the results of the characterization of olive leaf extract, it was estimated that the results of the inhibition study performed with olive leaf crude extract would be rather similar to that of oleuropein standard. As it can be seen in the graphical demonstration of results obtained for olive leaf crude extract given in Figure 5.34, inhibition mechanism and performance were found much closer to those of oleuropein standard as expected.

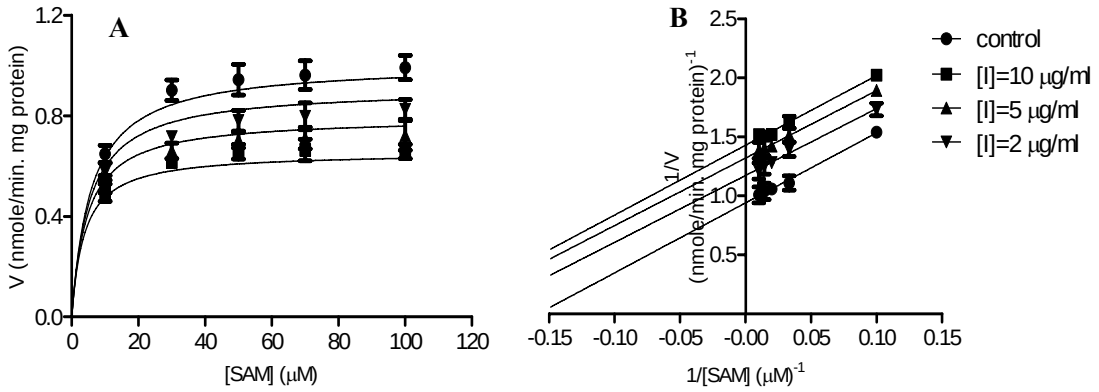


Figure 5.34. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I12 (olive leaf crude extract) concentrations ([E] and [ES] were fixed at 11.0 µg protein/ml and 4 µM, respectively)

Inhibition constant (αK_i) of the olive leaf crude extract, which was observed to inhibit COMT uncompetitively like oleuropein and rutin standards, was found as 18.51±2.05 µg/ml. For the crude extract which demonstrated a more effective inhibition character than oleuropein but lower rutin, IC_{50} value could not be calculated as for oleuropein standard. In the presence of crude extract, K_m and V_{max} kinetic constants were found 5.83±0.83 µM and 1.01±0.03 nmol/min. mg protein, respectively.

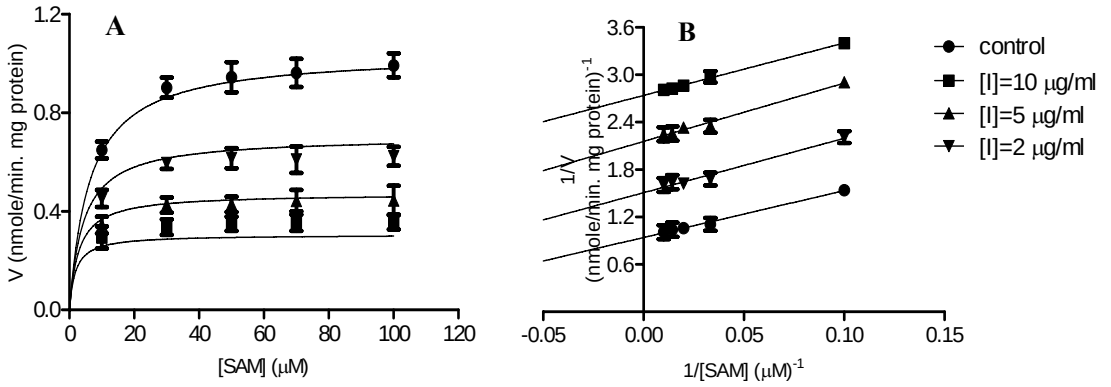


Figure 5.35. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I13 (olive leaf fraction no FO1) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

As a result of the HPLC analyses of the fraction numbered FO1 obtained from olive leaf extract, it was found that FO1 fraction was consisted of 91% of oleuropein and a little amount of an unidentified polyphenol which was less polar than oleuropein. As it can be seen from Figure 5.35B, oleuropein rich FO1 fraction exhibited uncompetitive inhibition behavior similar to oleuropein and rutin standards and also olive leaf raw extract. However, contrary to expected, FO1 fraction demonstrated much higher inhibitory effect than oleuropein and crude extract. This result was thought to be arising from the possibility that the presence of unidentified polyphenol present and oleuropein together in the fraction FO1 can create positive outcomes regarding their inhibitory activities. Inhibition constant (αK_i) and IC_{50} value of FO1 fraction was calculated as 4.14±0.28 μg/ml and 4.09±0.16 μg/ml, respectively. Also, in presence FO1 fraction, K_m and V_{max} kinetic constants were found as 6.29±1.07 μM and 1.04±0.03 nmol/min. mg protein, respectively.

Furthermore, inhibition kinetic data obtained for the rutin and other glycosides especially verbascoside rich fraction of olive leaf extract coded as fraction FO5 were given in Figure 5.36.

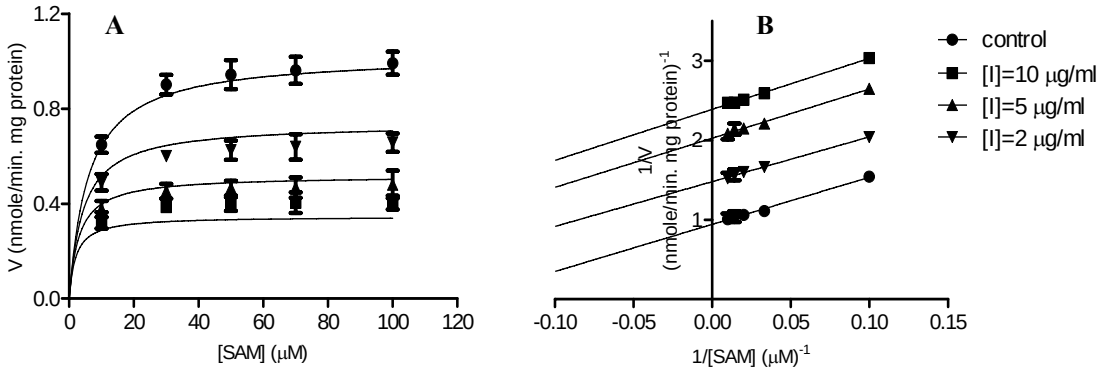


Figure 5.36. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I14 (olive leaf fraction no FO5) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

Inhibition mechanism of polyphenolic FO5 fraction could be explained with uncompetitive behavior as in other polyphenolic standard, extract and fractions of olive leaf (Figure 5.36B). This fraction which dominantly included rutin and verbascoside showed higher inhibition effect than the rutin standard. Inhibition constant for this fraction (αK_i) and IC_{50} values were calculated as 5.08±0.43 μg/ml and 5.21±0.26 μg/ml, respectively. K_m and V_{max} kinetic constants were found respectively as 5.98±1.21 μM and 1.03±0.04 nmol/min. mg protein in the presence of FO5 fraction.

Despite the COMT inhibitory potentials of crude extracts obtained from olive leaf and harmal seeds were increased via fractioning, it was revealed that some compounds present in the fractions may create synergistic effect on each other and thereby on their inhibitory activities. The crude extracts of other plant species were also used in inhibition study without fractioning. Inhibition kinetics data obtained for crude extract of vitex leaf among these plants were graphically given in Figure 5.37.

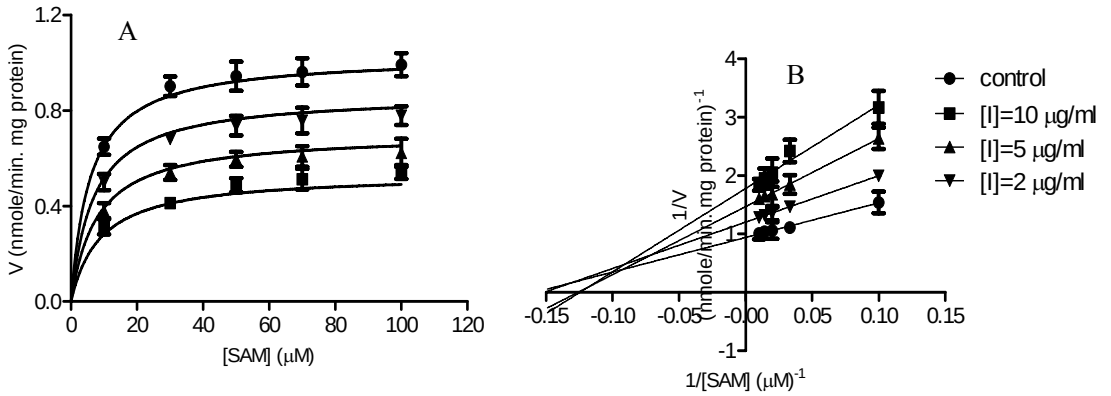


Figure 5.37. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I15 (vitex leaf crude extract) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

As it can be seen from Figure 5.37B, vitex leaf crude extract demonstrated a mixed type inhibition behavior like harmal seed crude extract and its harmine and harmaline fractions. K_i and αK_i values obtained for vitex leaf crude extract were calculated as 4.43±0.89 μg/ml and 9.48±0.58 μg/ml, respectively. For this extract, a lower α value was found than those of harmine and harmaline fractions and their corresponding standards. Vitex leaf crude extract was found an inhibitor of which the noncompetitiveness was higher than that of alkaloids due to having α value of 2.14±0.23. However, IC_{50} value was calculated higher than those of alkaloids, as 12.96±0.29 μg/ml. Therefore, despite noncompetitiveness of vitex leaf crude extract was higher, it was revealed that inhibition performance of it were lower than those of harmal seed crude extract and its fractions.

It is known that the vitex leaf crude extract includes some first generation COMT inhibitors such as gallic acid, hydroxybenzoic acid, rutin and ferulic acid and also some estrogenic compounds like linoleic acid and halimane, labdane type diterpenes (Ono et al., 2008; Proestos et al., 2006). In this respect, it was thought that it is very critical to inhibit the COMT activity of which an adverse effect on estrogenic metabolism is known by plant extracts which consists of estrogenic compounds such as vitex leaf extract.

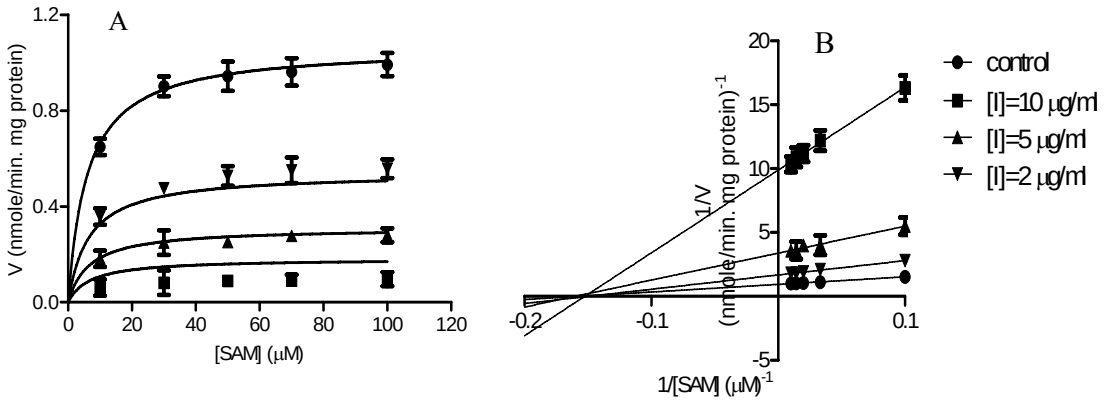


Figure 5.38. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I16 (cistus leaf crude extract) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

COMT inhibition by the cistus leaf crude extract, which is known to have high terpenoids and flavonoids content as it is vitex leaves, could be observed in the concentration range used in this study (Somoza et al., 1996). As it can be seen from the graphical results obtained for cistus leaf crude extract and given in Figure 5.38, COMT enzyme activity inhibited by mixed type inhibition mechanism in the presence of cistus leaf crude extract. Inhibition constants indicating competitiveness and noncompetitiveness, (K_i and αK_i) of cistus leaf crude extract were calculated as 2.51±0.54 μg/ml and 1.99±0.35 μg/ml, respectively. As it was found for this extract vitex leaf crude extract, a lower α value was also found for this extract than those of alkaloids. It was revealed that the noncompetitiveness of cistus leaf crude extract was the highest due to having α value of 0.79±0.32 which is the closest value to 1. Moreover, IC_{50} value for cistus leaf crude extract was found 2.44±0.09 μg/ml and was very close to those of alkaloids. In presence of cistus leaf crude extract, K_m and V_{max} constants were calculated as 6.02±1.29 μM and 1.06±0.04 nmol/min. mg protein, respectively.

It is also known that the cistus leaf crude extract, which demonstrated a comparable COMT inhibition performance with those of alkaloids, has vasodilator and relaxant effects due to its high terpenoids and flavonoids contents like vitex leaves (Somoza et al., 1996). Additionally, it is known that the cistus leaf includes some terpenoid volatile oils such as carvacrol, thymol, manoil oxides and esters such as benzyl benzoate (Angelopoulou et al., 2001).

In this study, COMT enzyme inhibition studies were also performed with crude extracts of two *Pistacia* species known as terebinth and mastic, and the obtained results were respectively given in Figures 5.39 and 5.40.

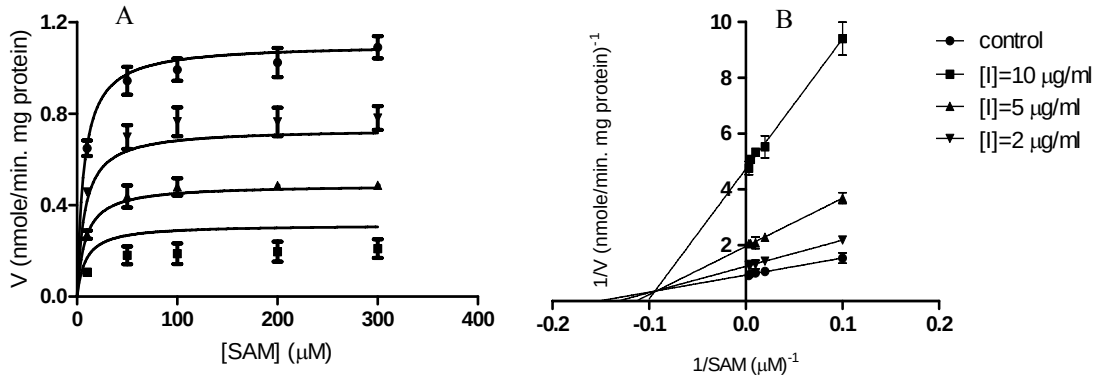


Figure 5.39. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I17 (terebinth leaf crude extract) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

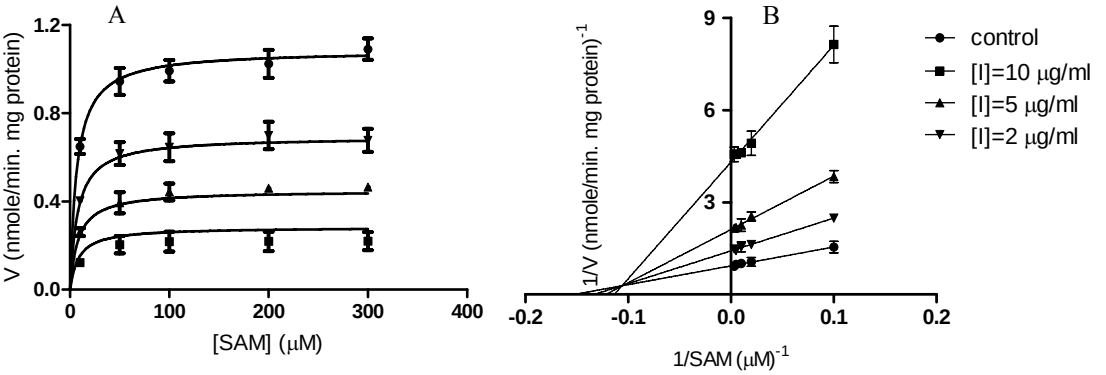


Figure 5.40. A: Michaelis and B: Lineweaver-Burk graphs of formation rate of scopoletin against various SAM concentrations in presence of different I18 (mastic leaf crude extract) concentrations ([E] and [ES] were fixed at 11.0 μg protein/ml and 4 μM, respectively)

As it can be seen from Figures 5.39B and 5.40B, extracts obtained from terebinth and mastic leaves demonstrated mixed type inhibition character as in other polyphenolic plant leaves and harmal seed extracts except olive leaf. The αK_i values were calculated respectively as 3.95±0.37 μg/ml and 3.52±0.61 μg/ml for terebinth and mastic crude extracts having respectively α values of 1.37±0.49 and 1.35±0.54 which were found to be very close to one and each other. IC_{50} values for these two *Pistacia* species, of which indicate lower inhibition character than cistus leaf crude extract, were

also found very effective and close to each other. These were calculated as 4.40±0.28 μg/ml and 3.69±0.32 μg/ml for terebinth and mastic leaf crude extracts, respectively. In presence of terebinth leaf crude extract, while K_m and V_{max} kinetic constants were respectively found as 6.79±0.86 μM and 1.10±0.04 nmol/min. mg protein, kinetic constants in the presence of mastic leaf crude extract were calculated as 6.76±0.93 μM and 1.08±0.02 nmol/min. mg protein, respectively.

As for cistus leaf crude extract, COMT inhibitory effects of terebinth and mastic leaf crude extracts were observed as comparable as with those of alkaloids. It has been reported in the literature that in the leaves of these two *Pistacia* species, apigenin, luteolin, quercetin, gallic acid and and some anthocyanidins are present (Topcu et al., 2007; Benhammou et al., 2008). Therefore, it was thought that the high COMT inhibitory potentials of *Pistacia* species can be arise from the fact that they include these compounds which are also known as the first generation COMT inhibitors.

CHAPTER 6

CONCLUSIONS

As a result of this study, it was first observed that harmal seed and olive leaf demonstrated to have inhibition potencies against COMT enzyme activity with the help of their alkaloids and polyphenolics content. In this study, it was mainly focused on COMT inhibition performances of harmol, harmalol, harmine and harmaline alkaloids found in harmal seed, and oleuropein and rutin polyphenols present in olive leaf. By means of extraction and fractionation, it was tried to obtain high yield extracts from plant sources and their purities were attempted to be increased in order to determine the compounds that were responsible for COMT inhibition. Additionally, COMT enzyme inhibition performances of extracts obtained from vitex, cistus, terebinth and mastic leaves were examined.

Firstly, harmal seed was subjected to different extraction methods. By means of HPLC analyses, effects of parameters such as temperature, pH, extraction time and solvent type on alkaloid extraction efficiency were investigated. Accordingly, extraction process performed with Soxhlet apparatus was determined as the method resulted with the highest efficiency. In this method, powdered seeds were extracted with Soxhlet apparatus for 7.5 hours in 70°C in which methanol was used as extraction solvent. The extract obtained in methanol was dissolved in 2 % of hydrochloric acid solution after removal of methanol. Acidic part of the extract, which was subjected twice to liquid-liquid extraction with petroleum ether, was collected. pH value of the collected solution was adjusted to 10 by adding ammonium hydroxide. The extract solution at elevated pH values was subjected to liquid-liquid extraction with chloroform for four times. Finally, crude alkaloid extract was obtained by collecting the chloroform parts. As a result of the HPLC analytical column analysis, trace amount of harmol and harmalol alkaloids were found in the crude alkaloid extract while harmine and harmaline contents of the extract were calculated as 41% and 58%, respectively. Alkaloid extraction efficiency was found also compatible with the literature, as 6.63 %.

Solvent extraction method was used in order to obtain olive leaf extract. Extraction process, in which 70 % of aqueous ethanol solution was used as solvent, was

performed in 25°C and 250 rpm for 2 hours using solid/liquid ratio of 1/20. According to the HPLC analytical column analysis results of the obtained extract, it was observed that one gram of olive leaf dominantly includes 92.50 mg of oleuropein and 9.12 mg of rutin. Moreover, it was also observed that minor amounts of polyphenolics such as verbascoside, luteolin 7-glycoside are present in olive leaf extract. Besides, leaves of plants such as vitex, cistus, terebinth and mastic used as inhibitors in this study were also extracted by applying the process optimized for olive leaf.

Crude alkaloid extract obtained from harmal seed was collected into 3 fractions by using semipreparative column in HPLC system and with the help of fractionation unit compatible to this system. The purities of 2 major fractions of these, harmine and harmaline fractions were determined by HPLC analytical column. Accordingly, 77% of the part collected as harmine fraction was found as harmine and nearly 23% of it was characterized as harmaline. It was also found that the harmaline fraction includes 93% of harmaline and nearly 7% of harmine.

Fractionation of the olive leaf extract was performed by using silk fibroin packed column. Among the parts collected as a total of six fractions, only 2 fractions of them, one of which eluted with water was rich in oleuropein and other eluted with ethanol was found rich in rutin and other glycosides, were used in inhibition studies. Oleuropein content of the water fraction was determined as 91% according to the HPLC analyses. In the ethanol fraction, it was revealed that rutin and verbascoside content has great majority.

Before the inhibition studies, the active protein amount of COMT enzyme was determined. In this study, which was performed according to the Bradford protein determination method, active protein amount of COMT enzyme was found as 27.7 % with respect to BSA that was reference protein. COMT enzyme activity was examined both spectrophotometrically and fluorometrically and it was revealed that the fluorometric activity determination method was more accurate and economical compared to absorbance. In the fluorometric activity determination study, formation kinetics of scopoletin was followed and firstly, linear operation concentration ranges for COMT enzyme and for both substrates SAM and aesculetin was tried to be determined. According to the results of this study, enzyme and aesculetin concentrations were fixed at 11 μg protein/ml and 4 μM, respectively in the inhibition experiments. It was revealed that SAM which is the rate limiting substrate demonstrated inhibitory feature

at its concentrations of higher than 100 μM. K_m values for SAM and aesculetin were calculated as 3.5 ± 0.3 μM and 6.4 ± 0.4 μM, respectively.

In the study, where basically inhibition performances of extracts and fractions obtained from harmal seed and olive leaf on COMT enzyme were examined, 3,5-DNC was used as reference inhibitor. Apart from that, performances of 17 inhibitors including alkaloid and polyphenolic standards, extracts and fractions were tested. While it was found that inhibition performance of 3,5-DNC, which is the best known uncompetitive inhibitor of COMT, was 100 times higher than especially harmine and harmaline alkaloid standards and fractions, it demonstrated 200 and 300 times inhibitory effect than polyphenolics.

Taking into consideration the concentration ranges of inhibitors, despite it was observed that their performances were not as effective as positive control, the inhibition percentages of extracts obtained from harmal seed and from vitex, terebinth and mastic leaves were found quite high. While the inhibition mechanisms for major alkaloids present in harmal seed were explained with the mixed type model, it was determined that in the presence of standards, extracts and fractions of olive leaf polyphenolics, uncompetitive inhibition occurred. On contrary to that, polyphenolic extracts of the other four plants behaved as mixed type inhibitors similar to major alkaloids.

In this study, crude extract of the harmal seed, which was one the plant source of which the inhibition performances of both extract and their fractions were examined together, and its harmine and harmaline rich fractions demonstrated the highest inhibition among the other plant species and were found very close to each other. This result obtained was found in good accordance with HPLC analysis results and it was revealed that the high inhibitory effect of harmal seed extract arises from its harmine and harmaline content. Concentration values (IC_{50}) of the alkaloids where 50 % inhibition occurs were found between 0.69 and 1.10 μg/ml. Among these, harmine fraction showed the highest inhibition percentage compared to those of other alkaloid fractions, crude alkaloid extract and its own standard. Competitiveness and noncompetitiveness inhibition constants (K_i and αK_i) of the harmine fraction which was found to be a mixed type inhibitor were calculated as 0.15±0.07 μg/ml and 1.28±0.06 μg/ml, respectively.

According to the results of the inhibition experiments performed with standards, crude extract and fractions of olive leaf polyphenolics, water fraction rich in oleuropein was found to have the highest inhibitory effect. Inhibition constant (αK_i) of this fraction,

which was determined as an uncompetitive inhibitor of COMT, and its IC_{50} value were calculated as 4.14±0.28 μg/ml and 4.09±0.16 μg/ml, respectively. This result, contrary to expected, was found very different from those of oleuropein standard and olive leaf crude extract. It was thought that the presence of compounds together may have both positive and sometimes negative effects on their specific biological activities. Thus, the importance of fractioning had been understood.

As a more general conclusion, it is known that today synthetic COMT inhibitors are still used in the treatment of Parkinson disease. It was determined that plant derived compounds have great potential to replace with their synthetic derivatives. Especially, it was revealed that harmal seed alkaloids and olive leaf fractions are promising natural COMT inhibitors. Moreover, it is known that these alkaloid and polyphenolics based natural compounds have the possibility to be functionally used in food and cosmetics industries because of their antioxidant and antimicrobial features.

REFERENCES

Akalın S. H., 'Elemtere Fis, Anadolu'da Buyu ve Inanclar', İstanbul, "Uzerlik", **2003**. http://turkoloji.cu.edu.tr/CUKUROVA/sempozyum/semp_2/ akalin.pdf

Akyol O., 'Şizofrenide Oksidatif Stres', *Kocatepe Tıp Dergisi*, **2004**, 5, 15-25.

Al-Azzawie H. F., Saeed A. M. S., 'Hypoglycemic and Antioxidant Effect of Oleuropein in Alloxan-Diabetic Rabbits', *Life Sciences*, **2006**, 78, 1371-1377.

Altıok E., Baycin D., Bayraktar O., Ülkü S., 'Isolation of Polyphenols from the Extracts of Olive Leaves (*Olea europaea L.*) by Adsorption on Silk Fibroin', *Separation and Purification Technology*, **2008**, 62, 342-348.

Angelopoulou D., Demetzos C., Perdetzoglou D., 'An interpopulation study of the essential oils of *Cistus parviflorus* L. growing in Crete (Greece)', *Biochemical Systematics and Ecology*, **2001**, 29, 405-415.

Aoyama N., Tsunoda M., Imai K., 'Improved Assay for Catechol-*O*-Methyltransferase Activity Utilizing Norepinephrine as an Enzymatic Substrate and Reversed-Phase High-Performance Liquid Chromatography with Fluorescence Detection', *Journal of Chromatography A*, **2005**, 1074, 47-51.

Bäckstrom R., Honkanen E., Pippuri A., Kairisalo P., Pystynen J., Heinola K., Nissinen E., Linden I. B., Mlinnisto P. T., Kaakkola S., Pohto P., 'Synthesis of Some Novel Potent and Selective Catechol-*O*-Methyltransferase Inhibitors', *Journal of Medicinal Chemistry*, **1989**, 32, 841-846.

Bai H. W., Shim J. Y., Yu J., Zhu B. T., 'Biochemical and Molecular Modeling Studies of the *O*-Methylation of Various Endogenous and Exogenous Catechol Substrates Catalyzed by Recombinant Human Soluble and Membrane-Bound Catechol-*O*-Methyltransferases', *Chemical Research in Toxicology*, **2007**, 20, 1409-1425.

Bailey K., Cowling R., Tan E. W., Webb D., 'A Colormetric Assay for Catechol-*O*-Methyltransferase', *Bioorganic & Medicinal Chemistry*, **2004**, 12, 595-601.

Bailey K., Tan E. W., 'Synthesis and Evaluation of Bifunctional Nitrocatechol Inhibitors of Pig Liver Catechol-*O*-Methyltransferase', *Bioorganic & Medicinal Chemistry*, **2005**, 13, 5740-5749.

Baycin D., Altıok E., Ülkü S., Bayraktar O., 'Adsorption of Olive Leaf (*Olea europaea L.*) Antioxidants on Silk Fibroin', *Journal of Agriculture and Food Chemistry*, **2007**, 55, 1227-1236.

Benhammou N., Atik B. F., Kadifkova P. T., 'Antioxidant and antimicrobial activities of the *Pistacia lentiscus* and *Pistacia atlantica* extracts', *African Journal of Pharmacy and Pharmacology*, **2008**, 2, 22-28.

Bertolini F., Novaroli L, Carrupt P. A., Reist M., 'Novel Screening Assay for Antioxidant Protection against Peroxyl Radical-Induced Loss of Protein Function', *Journal of Pharmaceutical Sciences*, **2007**, 96, 2931-2944.

Bonifacio M. J., Palma P. N., Almeida L., Soares-da-Silva P., 'Catechol-*O*-Methyltransferase and Its Inhibitors in Parkinson's Disease', *CNS Drug Reviews*, **2007**, 13, 352-379.

Bonifati V., Meco G., 'New, Selective Catechol-*O*-Methyltransferase Inhibitors as Therapeutic Agents in Parkinson's Disease', *Pharmacology & Therapeutics*, **1999**, 81, 1-36.

Bonilla M., Salido S., Beekb T., Linares-Palomino P. J., Altarejos J., Nogueras M., S´anchez A., 'Isolation and Identification of Radical Scavengers in Olive Tree (*Olea europaea*) Wood', *Journal of Chromatography A*, **2006**, 1112, 311-318.

Cavalli A., Bolognesi M. L., Minarini A., Rosini M., Tumiatti V., Recanatini M., Melchiorre C., 'Multi-Target-Directed Ligands to Combat Neurodegenerative Diseases', *Journal of Medicinal Chemistry*, **2008**, 51, 347-372.

Chatterjee S. S., Noldner M., Koch M., Erdelmeier C., 'Antidepressant Activity of *Hypericum Perforatum* and Hyperforin, the Neglected Possibility', *Pharmacopsychiatry*, **1998**, 31, 7-15.

Chen D., Wang C. Y., Lambert J. D., Ai N., Welsh W. J., Yang C. S., 'Inhibition of Human Liver Catechol-*O*-Methyltransferase by Tea Catechins and Their Metabolites: Structure-Activity Relationship and Molecular-Modeling Studies', *Biochemical Pharmacology,* **2005**, 69, 1523-1531.

Chen S., Le W., 'Neuroprotective Therapy for Parkinson's Disease', *American Journal of Therapeutics*, **2006**, 13, 445-457.

Cheng D. H., Ren H, Tang X. C., 'Huperzine A, a Novel Promising Acetylcholinesterase Inhibitor', *Neuroreport*, **1996**, 8, 97-101.

Cheng D. H., Tang X. C., 'Comparative Studies of Huperzine A, E2020, and Tacrine on Behavior and Cholinesterase Activities', *Pharmacology Biochemistry & Behavior*, **1998**, 60, 377-386.

Chiueh C. C., Andoh T., Lai A. R., Lai E., Krishna G., 'Neuroprotective Strategies in Parkinson's Disease: Protection Against Progressive Nigral Damage Induced by Free Radicals', *Neurotoxicity Research*, **2000**, 2, 293-310.

Chung Y. K., Heo H. J., Kim E. K., 'Inhibitory Effect of Ursolic Acid Purified from *Origanum Majorana* L. on the Acetylcholinesterase', *Molecules and Cells*, **2001**, 11, 137-143.

Copeland R. A., 'Enzymes: A Practical Introduction to Structure, Mechanism and Data Analysis', 2nd ed., Wiley, New York, **2000**.

De Boer A. G., Gaillard P. J., 'Drug Targeting to the Brain', *Annual Review of Pharmacology and Toxicology*, **2007**, 47, 323–55.

De Santi C., Giulianotti P. C., Pietrabissa A., Mosca F., Pacifici G. M., 'Catechol-*O*-Methyltransferase: Variation in Enzyme Activity and Inhibition by Entacapone and Tolcapone', *European Journal of Clinical Pharmacology*, **1998**, 54, 215-219.

Dhar K., Rosazza J. P. N., 'Purification and Characterization of *Streptomyces Griseus* Catechol-*O*-Methyltransferase', *Applied and Environmental Microbiology*, **2000**, 66, 4877-4882.

Dingemanse J., 'Catechol-*O*-Methyltransferase Inhibitors: Clinical Potential in the Treatment of Parkinson's Disease', *Drug Development Research*, **1997**, 42, 1-25.

Erdal N., Erdal M. E., Çamdeviren H., Gökdoğan T., Herken H., 'Bir Grup Sağlıklı Gönüllüde Kateşol-*O*-Metiltransferaz (KOMT) Gen Polimorfizmi', *Bulletin of Clinical Psychopharmacology*, **2002**, 12, 174-178.

Esposito E., Rotilio D., Di Matteo V., Di Giulio C., Cacchio M., Algeri S., 'A Review of Specific Dietary Antioxidants and the Effects on Biochemical Mechanisms Related to Neurodegenerative Processes', *Neurobiology of Aging*, **2002**, 23, 719-735.

Garcia O. B., Castillo J., Lorente J., Ortuno A., Del-Rio J. A., 'Antioxidant Activity of Phenolics Extracted From *Olea Europaea* L. Leaves', *Food Chemistry*, **2000**, 68, 457-462.

Ghica M. E., Brett A. M. O., 'Electrochemical Oxidation of Rutin', *Electroanalysis*, **2005**, 17, 313-318.

Gogos J. A., Morgan M., Luine V., Santha M., Ogawa S., Pfaff D., Karayiorgou M., 'Catechol-*O*-Methyltransferase Deficient Mice Exhibit Sexually Dimorphic Changes in Catecholamine Levels and Behavior', *Proceedings of the National Academy of Sciences*, **1998**, 95, 9991-9996.

Grella B., Dukat M., Young R., Teitler M., Herrick-Davis K., Gauthier C.B.,Glennon R. A., 'Investigation of Hallucinogenic and Related *Beta*-Carbolines', *Drug and Alcohol Dependence*, **1998**, 50, 99-107.

Gugler R., Dengler H. J., 'Inhibition of Human Liver Catechol-*O*-methyltransferase by Flavonoids', *Naunyn-Schmiedeberg's Archives of Pharmacology*, **1973**, 276, 223 – 233.

Guldberg H. C., Marsden C. A., 'Catechol-*O*-Methyl Transferase: Pharmacological Aspects and Physiological Role', *Pharmacological Reviews*, **1975**, 27, 135-206.

Halpern J. H., 'Hallucinogens and Dissociative Agents Naturally Growing in the United States', *Pharmacology & Therapeutics*, **2004**, 102, 131-138.

Hirano Y., Tsunoda M., Shimosawa T., Fujita T., Funatsu T., 'Measurement of Catechol-*O*-Methyltransferase Activity in the Brain of Dahl Salt-Sensitive Rats', *Biological and Pharmaceutical Bulletin*, **2007**, 30, 2178-2180.

Hou W. C., Lin R. D., Chen C. T., Lee M. H., 'Monoamine Oxidase B (MAO-B) Inhibition by Active Principles from *Uncaria Rhynchophylla*', *Journal of Ethnopharmacology,* **2005**, 100, 216-220.

Houghton P. J., Houghton Howes M. J., 'Natural Products and Derivatives Affecting Neurotransmission Relevant to Alzheimer's and Parkinson's Disease', *Neurosignals*, **2005**, 14, 6-22.

Howes M. J. R., Perry N. S. L., Houghton P. J., 'Plants with Traditional Uses and Activities, Relevant to the Management of Alzheimer's Disease and Other Cognitive Disorders', *Phytotherapy Research*, **2003**, 17, 1-18.

Institute of Neurodegenerative Diseases, University of California, http://ind.university-ofcalifornia.edu/diseases, **2009**.

Joy J. E., Watson J. H. J., Benson J. A., 'Marijuana and Medicine; Assessing the Science Base', National Academy Press, Washington, DC, **1999**.

Juma G. M., '*Peganum Harmala* Production', *European Journal of Scientific Research***, 2005**, 11, 6-12.

Kadowaki M., Ootani E., Sugihara N., Furuno K., 'Inhibitory Effects of Catechin Gallates on *O*-Methyltranslation of Protocatechuic Acid in Rat Liver Cytosolic Preparations and Cultured Hepatocytes', *Biological and Pharmaceutical Bulletin*, **2005**, 28, 1509-1513.

Kartal M., Altun M. L., Kurucu S., 'HPLC Method for the Analysis of Harmol, Harmalol, Harmine and Harmaline in the Seeds of *Peganum Harmala* L.', *Journal of Pharmaceutical and Biomedical Analysis,* **2003**, 31, 263-269.

Khan O. Y., 'Studies in the Chemical Constituents of *Peganum Harmala*', Ph. D. Dissertation, University of Karachi, Pakistan, **1990.**

Kong L. D., Christopher H. K., Cheng H. K., Tan R. X., 'Inhibition of MAO-A and B by Some Plant-Derived Alkaloids, Phenols and Anthraquinones', *Journal of Ethnopharmacology*, **2004**, 91, 351-355.

Köse E., Sarsılmaz M., Ögetürk M., Kub İ., Kavaklı A., Zararsız İ., 'Öğrenme Davranışlarında Gül Esans Yağ Aromasının Rolü: Deneysel Bir Çalışma', *Fırat Tıp Dergisi*, **2007**, 12, 159-162.

Kurisawa M., Chung, J. E., Uyama, H., Kobayashi, S., 'Enzymatic Synthesis and Antioxidant Properties of Poly(rutin)', *Biomacromolecules*, **2003**, 4, 1394- 1399.

Kurkela M., Siiskonen A., Finel M., Tammela P., Taskinen J., Vuorela P., 'Microplate Screening Assay to Identify Inhibitors of Human Catechol-*O*-Methyltransferase', *Analytical Biochemistry*, **2004**, 331, 198-200.

Lautala P., Ulmanen I., Taskinen J., 'Molecular Mechanisms Controlling the Rate and Specificity of Catechol-*O*-Methylation by Human Soluble Catechol-*O*-Methyltransferase', *Molecular Pharmacology*, **2001**, 59, 393-402.

Lautala P., 'Catechol *O*-Methyltransferase: Glucuronidation of Inhibitors and Methylation of Substrates', Academic Dissertation, University of Helsinki, Finland, **2000**.

Lautala P., Ulmanen I., Taskinen J., 'Radiochemical High-Performance Liquid Chromatographic Assay for the Determination of Catechol-*O*-Methyltransferase Activity Towards Various Substrates', *Journal of Chromatography B*, **1999**, 736, 143-151.

Learmonth D. A., Bonifacio, M. J. Soares-da-Silva P., 'Synthesis and Biological Evaluation of a Novel Series of "*Ortho*-Nitrated" Inhibitors of Catechol-*O*-Methyltransferase,' *Medicinal Chemistry*, **2005**, 48, 8070-8078.

Learmonth D. A., Freitas A. P., 'Chemical Synthesis and Characterization of Conjugates of a Novel Catechol-*O*-Methyltransferase Inhibitor', *Bioconjugate Chemistry*, **2002**, 13, 1112-1118.

Lee-Huang S., Zhang L., Huang P., Chang Y., Huang P. L., 'Anti-HIV Activity of Olive Leaf Extract (OLE) and Modulation of Host Cell Gene Expression by HIV-1 Infection and OLE Treatment', *Biochemical and Biophysical Research Communications*, **2003**, 307, 1029-1037.

Lerner C., Masjost B., Ruf A., Gramlich V., Jakob-Roetne R., Zürcher G., Borroni E., Diederich F., 'Bisubstrate Inhibitors for the Enzyme Catechol-*O*-Methyltransferase (COMT): Influence of Inhibitor Preorganisation and Linker Length Between The Two Substrate Moieties on Binding Affinity', *Organic and Biomoecular Chemistry*, **2003**, 1, 42-49.

Lerner C., Siegrist R., Schweizer E., Diederich F., 'Bisubstrate Inhibitors for the Enzyme Catechol *O*-Methyltransferase (COMT): Dramatic Effects of Ribose Modifications on Binding Affinity and Binding Mode', *Helvetica Chimica Acta*, **2003**, 86, 1045-1062.

Leskovac V., 'Comprehensive Enzyme Kinetics', Kluwer Academic Publisher, New York, **2003.**

Lin R. D., Hou W. C., Yen K. Y., Lee M. H., 'Inhibition of Monoamine Oxidase B (MAO-B) by Chinese Herbal Medicines', *Phytomedicine*, **2003**, 10, 650–656.

Lotta T., Vidgren J., Tilgmann C., Ulmanen I., Melen K., Julkunen I., Taskinentn J., 'Kinetics of Human Soluble and Membrane-Bound Catechol-*O*-Methyltransferase: A Revised Mechanism and Description of the Thermolabile Variant of the Enzyme', *Biochemistry*, **1995**, 34, 4202-4210.

Loureiro A. I., Bonifácio M. J., Fernandes-Lopes C., Almeida L., Wright L. C., Soares-da-Silva P., 'Human Metabolism of Nebicapone (BIA 3-202), A Novel COMT Inhibitor: Characterization of in Vitro Glucuronidation', *Drug Metabolism and Disposition*, **2006**, 34, 1856-1862.

Lozano A. M., Lang A. E., Hutchison W. D., Dostrovsky J. O., 'New Developments in Understanding the Etiology of Parkinson's Disease and in its Treatment', *Current Opinion in Neurobiology*, **1998**, 8, 783-790.

Mamedov N., 'Adaptogenic, Geriatric, Stimulant and Antidepressant Plants of Russian Far East', *Journal of Cell and Molecular Biology*, **2005**, 4, 71-75.

Mannistö P. T., Kaakkola S., 'Catechol-*O*-methyltransferase (COMT): Biochemistry, Molecular Biology, Pharmacology, and Clinical Efficacy of the New Selective COMT Inhibitors', *Pharmacological Reviews*, **1999**, 51, 593-628.

Mannistö P. T., Tuomainen P., Tuominen R. K., 'Different in Vivo Properties of Three New Inhibitors of Catechol-*O*-Methyltransferase in the Rat', *British Journal of Pharmacology*, **1992**, 105, 569-574.

Manyam B. V., Dhanasekaran M., Hare T. A., 'Effect of Antiparkinson Drug HP-200 (*Mucuna pruriens*) on the Central Monoaminergic Neuro-Transmitters', *Phytotherapy Research*, **2004**, 18, 97-101.

Molchanov L. V., Plugar V. N., D'yakonov A. L., Aripov K. N., 'Isolation of Peganine and Deoxypeganine from the Total Alkaloids of *Peganum Harmala* Through Their Complex Salts', *Chemistry of Natural Compounds*, **1996**, 32, 56-58.

Nadeau S. E., 'Parkinson's Disease', *Journal of American Geriatrics Society*, **1997**, 45, 233-240.

Nagai M., Conney A. H., Zhu B. T., 'Strong Inhibitory Effects of Common Tea Catechins and Bioflavonoids on the *O*-Methylation of Catechol Estrogens Catalyzed by Human Liver Cytosolic Catechol-*O*-Methyltransferase', *Drug Metabolism and Disposition*, **2004**, 32, 497-504.

National Institute of Neurological Disorders and Stroke, http://www.ninds.nih.gov/disorders/parkinsons_disease/parkinsons_disease.htm, **2009**.

National Parkinson Foundation, http://www.parkinson.org, **2009**.

Nissinen E., Lindén I. B., Schultz E., Pohto P., 'Biochemical and Pharmacological Properties of a Peripherally Acting Catechol-*O*-Methyltransferase Inhibitor Entacapone', *Naunyn-Schmiedeberg's Archives of Pharmacology*, **1992**, 346, 262-266.

Nissinen E., Linden I. B., Schultz E., Kaakkola S., Mannisto P. T., Pohto P., 'Inhibition of Catechol-*O*-Methyltransferase Activity by Two Novel Disubstituted Catechols in the Rat', *European Journal of Pharmacology*, **1988**, 153, 263-269.

Olin J., Schneider L., 'Galantamine of Alzheimer's disease', *Cochrane Database of Systematic Reviews*, **2001a**, 1.

Olin J., Schneider L., 'Galantamine for Alzheimer's disease: Adult and Geriatric Treatment and Preventative Interventions Branch', *Cochrane Database of Systematic Reviews*, **2001b**, 4.

Ono M., Yamasaki T., Konoshita M., Ikeda T., Okawa M., Kinjo J., Yoshimitsu H., Nohara T., 'Five New Diterpenoids, Viteagnusins A - E, from the Fruit of *Vitex agnus-castus*', *Chemical and Pharmaceutical Bulletin*, **2008**, 56, 1621-1624.

Ott, J., 'Entheogenic Drugs, Their Plant Sources and History', *Pharmacotheon*, **1996**, 205.

Page L., 'Olive Leaf Extract', in Healthy Healing', edited by Page L. G. (Traditional Wisdom, USA), **2002**, 107.

Palma P. N., Rodrigues M. L., Archer M., Bonifacio M. J., Loureiro A. I., Learmonth D. A., Carrondo M. A., Soares-da-Silva P., 'Comparative Study of *ortho*- and *meta*-Nitrated Inhibitors of Catechol-*O*-methyltransferase: Interactions with the Active Site and Regioselectivity of *O*-Methylation', *Molecular Pharmacology*, **2006**, 70, 143-153.

Parkinson's Disease Society, www.parkinsons.org.uk, **2009**.

Paulini R., Lerner C., Diederich F., Jakob-Roetne R., Zürcherb G., Borroni E., 'Synthesis and Biological Evaluation of Potent Bisubstrate Inhibitors of the Enzyme Catechol *O*-Methyltransferase (COMT) Lacking a Nitro Group', *Helvetica Chimica Acta*, **2006**, 89, 1856-1887.

Pekin B., 'Bazı Kompleks Enzim Reaksiyonları ile İnhibisyon Mekanizmalarının Matemetiksel Analizi', Ege Üniversitesi Fen Fakültesi İlmi Raporlar Serisi, **1970**, 85.

Perry E. K., Pickering A. T., Wang W. W., Houghton P. J., Perry N. S., 'Medicinal Plants and Alzheimer's Disease: From Ethnobotany to Phytotherapy', *Journal of Pharmacy and Pharmacology*, **1999**, 51, 527-34.

Perry N. S. L., Houghton P. J., Jenner P., '*Salvia Lavandulaefolia* Essential Oil Inhibits Cholinesterase in Vivo', *Phytomedicine*, **2002**, 9, 48-51.

Pierce Biotechnology, Inc., Coomassie Plus – The Better Bradford™ Assay Kit, http://www.piercenet.com, **2009**.

Polymeropoulos M. H., Higgins J. J., Golbe L. I., Nussbaum R. L., 'Mapping of a Gene for Parkinson's Disease to Chromosome 4q21-q23', *Science*, **1996**, 274, 1197-1198.

Proestos C., Komaitis M., 'Ultrasonically Assisted Extraction of Phenolic Compounds from Aromatic Plants Comparison with Conventional Extraction Techniques', *Journal of Food Quality*, **2006**, 29, 567-582.

Ranalli A., Contento, S., Lucera, L., Febo, M., Archegiani, D., Fonzo V., 'Factors Affecting the Contents of Iridoid Oleuropein in Olive Leaves (*Olea europaea L.*)', *Journal of Agricultural Food Chemistry*, **2006**, 54, 434-440.

Reenilä I., 'Catechol-*O*-Methyltransferase Activity: Assay, Distribution and Pharmacological Modification', Academic Dissertation, University of Helsinki, Finland, **1999**.

Rosenberg I.M., Protein Analysis and Purification: Benchtop Techniques, Birkhäuser, Boston, **1996**, 110-117.

Ruottinen H. M., Rinne U. K., 'COMT Inhibition in the Treatment of Parkinson's Disease', *Journal of Neurology*, **1998**, 245, 25-34.

Salyan M. E. K., Pedicord D. L., Bergeron L., Mintier G. A., Hunihan L., Kuit K., Balanda L. A., Robertson B. J., Feder J. N., Westphal R., Shipkova P. A., Blat Y., 'A General Liquid Chromatography/Mass Spectroscopy-Based Assay for Detection and Quantitation of Methyltransferase Activity', A*nalytical Biochemistry,* **2006**, 349, 112-117.

Savournin C., Baghdikian B., Elias R., Dargouth-Kesraoui F., Boukef K., Balansard G., 'Rapid High-Performance Liquid Chromatography Analysis for the Quantitative Determination of Oleuropein in *Olea europaea* Leaves', *Journal of Agricultural Food Chemistry,* **2001**, 49, 618-621.

Schliebs R., Liebmann A., Bhattacharya S. K., Kumar A., Ghosal S., Bigl V. R., 'Systemic Administration of Defined Extracts from *Withania Somnifera* (Indian Ginseng) and Shilajit Differentially Affects Cholinergic but not Glutamatergic and Gabaergic Markers in Rat Brain', *Neurochemistry International*, **1997**, 30, 181-190.

Schwarz M. J., Houghton P. J., Rose S., Jenner P., Lees A. D., 'Activities of Extract and Constituents of *Banisteriopsis Caapi* Relevant Parkinsonism', *Pharmacology Biochemistry and Behavior*, **2003**, 75, 627-633.

Sharaf M., El-Ansari M. A., Matlın S. A., Sale N. A. M., 'Four Flavonoid Glycosides from *Peganum Harmala*', *Phytochemisty*, **1997**, 44, 533-536.

Shen X., Puthran N., Weissman A., 'A Refined HPLC Method to Measure Catecholamine-*O*-Methyltransferase Activity in Selected Brain Regions, *Journal of Neuroscience Methods*, **2005**, 144, 137-142.

Sheu J. R., Hsiao G., Chou P. H., Shen M. Y., Chou D. S., 'Mechanisms Involved in the Antiplatelet Activity of Rutin, Glycoside of the Flavonol Quercetin, in Human Platelets', *Journal of Agricultural and Food Chemistry*, **2004**, 52, 4414-4418.

Shuler M. L., Kargı F., 'Bioprocess Engineering: Basic Concepts', 2nd ed., Prentice Hall PTR, **2001**.

Singh N., Pillay V, Choonara Y. E., 'Advances in the Treatment of Parkinson's Disease', *Progress in Neurobiology*, **2007**, 81, 29-44.

Sirnonian N. A., Coyle J. T., 'Oxidative Stress in Neurodegenerative Diseases', *Annual Review of Pharmacology and Toxicology*, **1996**, 36, 83-106.

Sloley B. D., Urichuk L. J., Morley P., Durkin J., Shan J. J., Pang P. K., Coutts R. T., 'Identification of Kaempferol as a Monoamine Oxidase Inhibitor and Potential Neuroprotectant in Extracts of *Ginkgo Biloba* Leaves', *Journal of Pharmacy and Pharmacology*, **2000**, 52, 451-459.

Somoza B., Sanchez de Rojas V. R., Ortega T., Villar A. M., 'Vasodilator effects of the extract of the leaves of *Cistus populifolius* on rat thoracic aorta', *Phytotherapy Research*, **1996**, 10, 304-308.

Srivastava S., Verma R. K., Gupta M. M., Singh S. C., Kumar S., 'HPLC Determination of Vasicine and Vasicinone in *Adhatoda Vasica* with Photo Diode Array Detection', *Journal of Liquid Chromatography & Related Technologies*, **2001**, 24, 153-159.

Sudha K., Rao A. V., Rao S., Rao A., 'Free Radical Toxicity and Antioxidants in Parkinson's Disease', *Neurology India*, **2003**, 51, 60-62.

SukYin A., Catechol-*O*-Methyltransferase (COMT) Gene and Breast Cancer, Technical Report, National Office of Public Health Genomics, **2002**.

Sumio O., 'The Physiological Function of the Blood-Brain Barrier Transporters as the CNS Supporting and Protecting System', *Journal of the Pharmaceutical Society of Japan*, **2004**, 124, 791-802.

Theodore L. S., '"Rational Hope" in the Early Treatment of Parkinson's Disease', *Canadian Journal of Physiology and Pharmacology*, **1999**, 77, 375-382.

Topcu G., Ay M., Bilici A., Sarıkürkçü C., Öztürk M., Ulubelen A., 'A new flavone from antioxidant extracts of *Pistacia terebinthus*', *Food Chemistry*, **2007**, 103, 816–822.

Tsunoda T., Takezawa K., Masuda M., Imai K., 'Rat Liver and Kidney Catechol-*O*-Methyltransferase Activity Measured by High-Performance Liquid Chromatography with Fluorescence Detection', *Biomedical Chromatography*, **2002**, 16, 536-541.

Van Duursen M. B. M., Sanderson J. T., de Jong P. C., Marlies K., Van den Berg M., 'Phytochemicals Inhibit Catechol-*O*-Methyltransferase Activity in Cytosolic Fractions from Healthy Human Mammary Tissues: Implications for Catechol Estrogen-Induced DNA Damage', *Toxicological Sciences*, **2004**, 81, 316-324.

Veser J., 'Kinetics and Inhibition Studies of Catechol *O*-Methyltransferase from the Yeast *Candida tropicalis*', *Journal of Bacteriology*, **1987**, 169, 3696-3700.

Vieria-Coelho M. A., Soares-da-Silva P., 'Ontogenic Aspects of Liver and Kidney Catechol-*O*-Methyltransferase Sensitivity to Tolcapone', *British Journal of Pharmacology*, **1996**, 117, 516-520.

Yassin M. S., Cheng H., Ekblom J., Oreland L., 'Inhibitors of catecholamine metabolizing enzymes cause changes S-adenosylmethionine and S-adenosylhomocysteine in the rat brain', *Neurochemistry International*, **1998**, 32, 53-59.

Waidmeier P. C., Baumann P. A., Feldtrauer J. J., Hauser K., Bittiger H., Bischoff S., Sprecher G., 'CGP 28014 a New Inhibitor of Cerebral Catechol-*O*-Methylation with a Non-Catechol Structure', *Naunyn-Schmiedeberg's Archives of Pharmacology*, **1990**, 342, 305-311.

Wake G., Court J., Pickering A., 'CNS Acetylcholine Receptor Actvity', *Ethnopharmacology*, **2000**, 69, 105-14.

Weinreb O., Mandel S., Amit T., Youdim M. B. H., 'Neurological Mechanisms of Green Tea Polyphenols in Alzheimer's and Parkinson's Diseases', *Journal of Nutritional Biochemistry,* **2004**, 15, 506-516.

Zhang R. W., Tang X. C., Han Y. Y., 'Drug Evaluation of Huperzine A in the Treatment of Senile Memory Disorders', *Chung Kuo Yao Li Hsueh Pao*, **1991**, 12, 250-252.

Zheng W., Chodobski A., 'The Blood-Cerebrospinal Fluid Barrier', CRC Press, Florida, **2004**.

Zhu B. T., Shim J. Y., Nagai M., Bai H. W., 'Molecular Modelling Study of the Mechanism of High-Potency Inhibition of Human Catechol-*O*-Methyltransferase by (-)-Epigallocatechin-3-*O*-Gallate', *Xenobiotica*, **2008**, 38, 130-146.

Zhu B. T., 'CNS Dopamine Oxidation and Catechol-*O*-Methyltransferase: Importance in the Etiology, Pharmacotherapy, and Dietary Prevention of Parkinson's Disease', *International Journal of Molecular Medicine*, **2004**, 13, 343-353.

Zhu B. T., Patel U. K., Cai M. X., Lee A. J., Conney A. H., 'Rapid Conversion of Tea Catechins to Monomethylated Products by Rat Liver Cytosolic Catechol-*O*-Methyltransferase', *Xenobiotica,* **2001**, 31, 879-890.

APPENDIX A

CALIBRATION CURVES OF ALKALOIDS AND SAMPLE CALCULATION

It has been determined with High Performance Liquid Chromatography (HPLC) analyses that harmol, harmalol, harmine and harmaline are the major alkaloids present in the extract obtained from harmal seed. Calibration curves have been obtained with their standards in order to determine the quantifications of these alkaloids. For calibration of each standard alkaloid, concentrations given in section 5.1.1.-Table 5-3 have been used. Peak areas in the chromatograms obtained with HPLC analyses performed in these concentrations have been modeled as a linear function of the concentration.

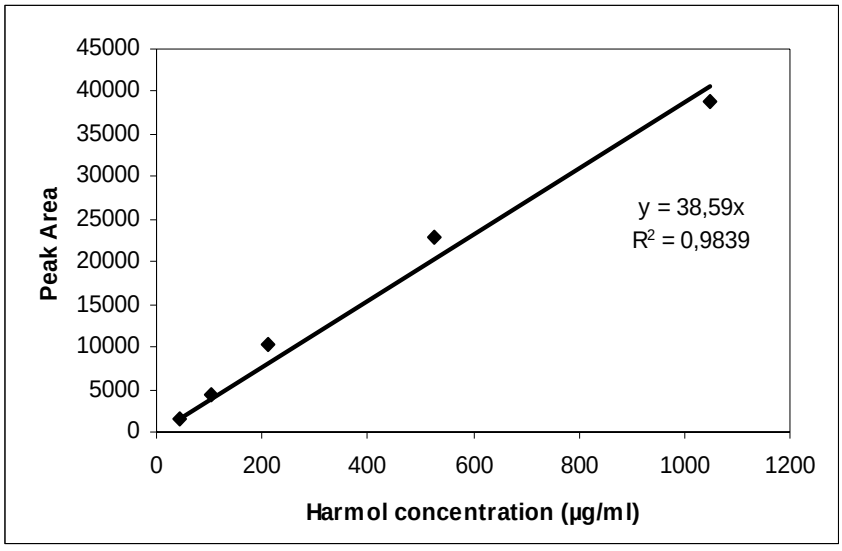


Figure A1. Harmol calibration curve

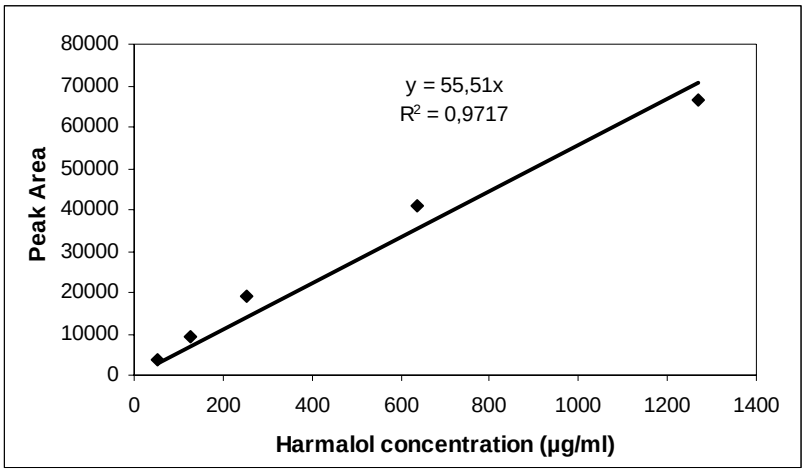


Figure A2. Harmalol calibration curve

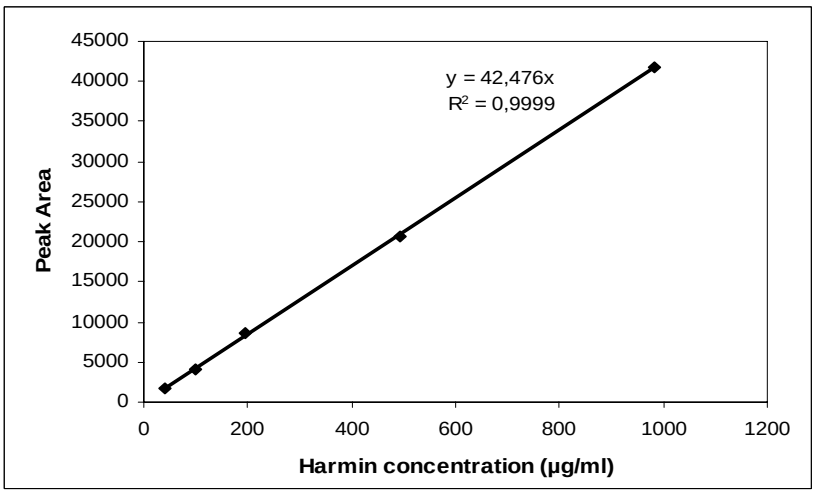


Figure A3. Harmine calibration curve

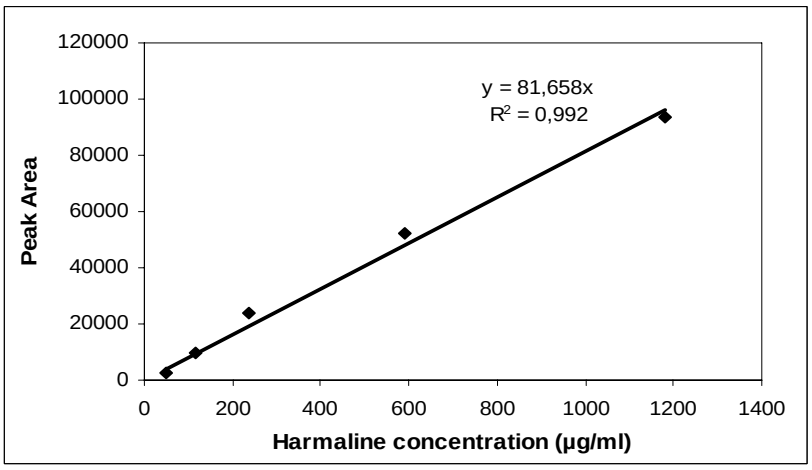


Figure A4. Harmaline calibration curve

Chromatogram obtained with HPLC analysis performed for the extract diluted 7 times is given in Section 5.1.1., in Figure 5-1. Accordingly, quantification calculation for alkaloids was performed and parameters such as extraction efficiency, alkaloid amount in the extract and fractions have been determined as a result of these calculations.

Sample Calculation: A total of 165.76 mg extract was solved in 20 ml mobile phase and diluted 7 times. After the diluted sample was filtered, it was injected to HPLC system with 20 µl of injection volume. As a result of the analysis performed;

The harmine peak intensity on the chromatogram is integrated and the peak area is found as 20829. By using calibration curve equation y=42.476x given in A3, value x (concentration, µg/ml) has been calculated.

$x = \frac{20829}{42.476} = 490.4$ µg/ml. This value is multiplied by total volume*dilution factor and harmine alkaloid amount in the crude extract has been calculated in terms of µg.

Harmine content (mg harmine/165.76 mg extract) $= \frac{490.4 * 20 * 7}{1000} = 68.7$ mg harmine. As a result of this calculation it has been calculated that the extract includes nearly 41% of harmine. Amounts of harmaline, harmol and harmalol alkaloids in the extract were determined with the same calculation method. Accordingly, the peak area of harmol and harmalol alkaloids in the extract could not be calculated since they were below the detection limits. Harmaline content was found as 97.1 mg. Harmaline content of crude alkaloid extract was found as 58% in percentage. It is thought that the remaining part consists of harmol, harmalol and other unidentified alkaloid derivatives.

In this extraction method, initially 2500 mg of powdered harmal seed was used. At the end of the extraction process, 165.76 mg crude alkaloid extract was obtained. Therefore;

Extraction efficiency was calculated as, mg alkaloid/mg seed (%) = $\frac{165.76 * 100}{2500} = 6.63$ %.

APPENDIX B

CALIBRATION CURVES OF OLEUROPEIN AND RUTIN AND SAMPLE CALCULATION

As in defining alkaloid amounts in the alkaloid extract, amounts of oleuropein and rutin in the olive leaf extract were calculated using peak area values obtained from HPLC analysis results.

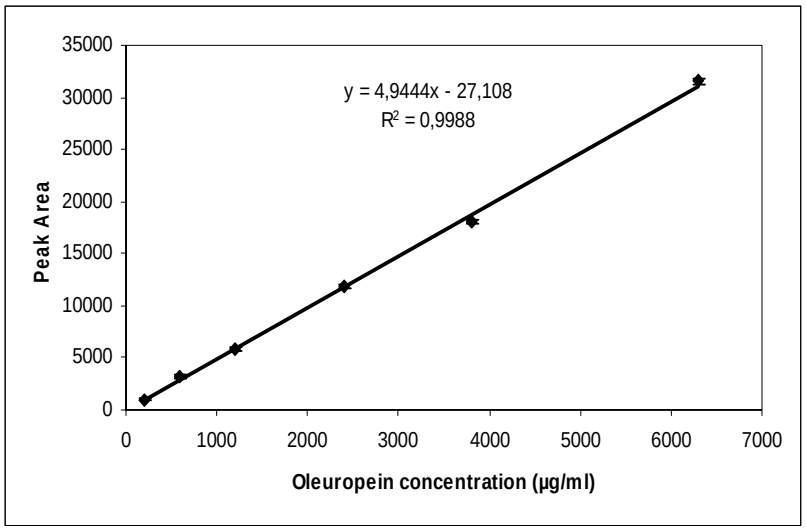


Figure B1. Oleuropein calibration curve

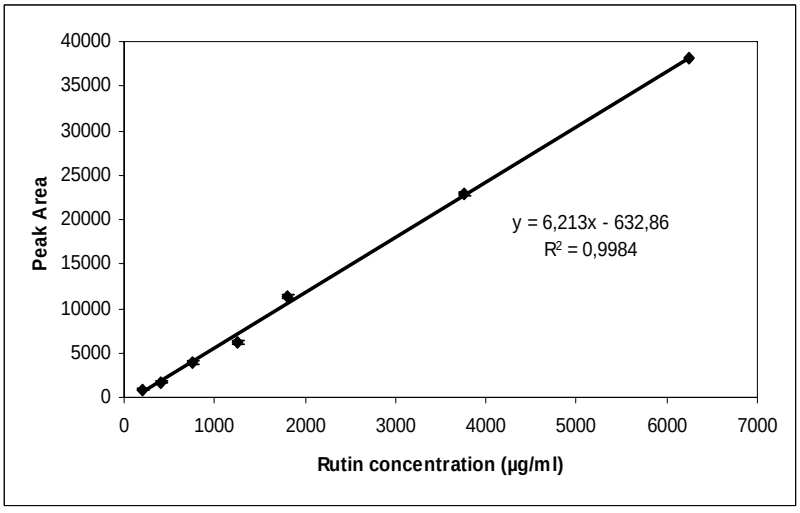


Figure B2. Rutin calibration curve

Sample Calculation:

One gram of olive leaf powder is extracted at a solid/liquid ratio of 1/20. According to the result of the HPLC analysis of the crude extract obtained with the extraction process, oleuropein peak area was calculated as 22815. Using the calibration curve equation y=4.9444x-27.108 given in Figure B1, x value (concentration, µg/ml) was calculated.

$x = \frac{22815 + 27{,}108}{4.9444} = 4624$ µg/ml. This value was multiplied by total extraction volume (20 ml) and the amount of oleuropein in one gram of olive leaf has been calculated. Accordingly, it was calculated that one gram of olive leaf includes 92.5 mg oleuropein and according to the similar calculations for rutin, 9.12 mg of rutin.

APPENDIX C

CALIBRATION CURVE OF SCOPOLETIN AND SAMPLE CALCULATION

According to fluorometric enzyme activity determination method, aesculetin is transformed to scopoletin as a result of COMT catalyzed *O*-methylation reaction. Production rate of scopoletin which has fluorescence feature also by itself, as a result of the reaction was measured at 355 nm excitation and 460 nm emission wavelengths. By adding different amounts of scopoletin into the reaction medium, an increase in fluorescence was achieved and therefore calibration curve was obtained (Figure C1).

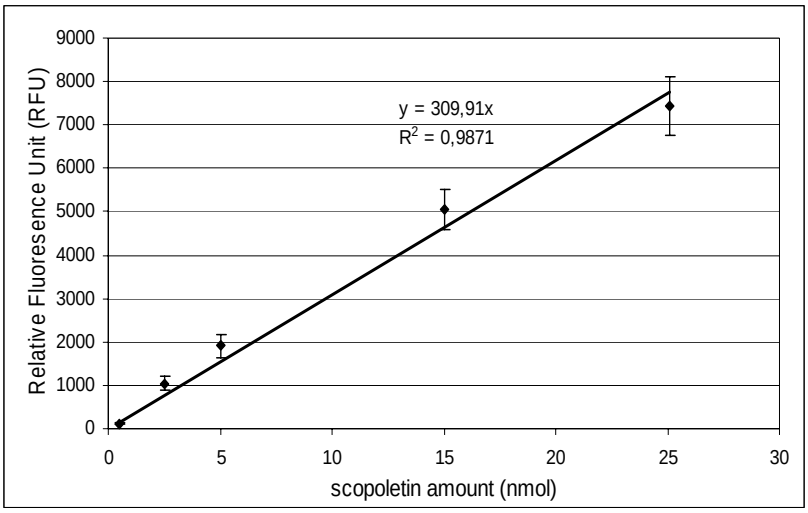


Figure C1: Scopoletin calibration curve

Slope of the calibration curve given in Figure C1 is calculated as 309.91. This value indicated that a nanomole scopoletin is identical to 309.91 units of fluorescence and it was used in activity calculations.

Sample Calculation:

For instance, in inhibition experiments performed with fluorometric method, blank solution fluorescence change (Δ_{RFB}) was found 20.282 for 30 minutes reaction

time interval. In a reaction medium of total 250 µl volume performed in a 96-well plate, there is 2.2 µg of protein. Therefore, reaction rate is calculated as;

$$v = \frac{\Delta_{RFB}}{(309.91)(30)(0.0022)} = \frac{20.282}{(309.91)(30)(0.0022)} = 0.9916 \text{ nmole scopoletin /min. mg protein}$$