**Inhibition of monoamine oxidase by derivatives of piperine, an alkaloid
from the pepper plant *Piper nigrum*, for possible use in Parkinson's
disease**

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Abbreviations

PD: parkinson disease

SN: substantia nigra

SNcp: substantia nigra pars compacta

ARJP: autosomal recessive juvenile parkinsonism

ROS: reactive oxygen species

MAO: monoamine oxidase

MAOA: monoamine oxidase A

MAOB: monoamine oxidase B

FAD: flavin adenine dinucleotide.

ADT: Auto dock tools

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Chapter I: Introduction

1.1. Parkinson Disease (PD)

1.1.1.Etiology

PD is a neurodegenerative disease, and its incidence increases with aging.¹ PD incidence increases at an age range of 55-65 years, and its classified as the second neurodegenerative disorder after Alzheimer disease.² PD affects around six million people globally.³ 10% of PD cases are sporadic and 90% are familial PD.^{4, 5} PD incidence is 1% in patients 65-70 years of age and it starts rising to 4-5% in 85 year old patients.⁶ Sporadic PD account for 95% of patients while familial PD which is caused by gene mutation accounts for 4% of patients.^{7, 8} It has been found that the prevalence of sporadic PD and familial PD is 39% and 36% respectively in Arabs, and the prevalence of sporadic PD and familial PD in Ashenazi jews is 10% and 28% respectively.^{9, 10}

PD etiology is either due to gene mutation which represents 10% of all PD cases or due to idiopathic causes (90% of all PD cases), in which, many risk factors involved in the development of idiopathic PD such as pesticide, bacterial and viral infection, environmental toxins, stroke, head injury, age or genes.^{11, 12} It has shown that many chemicals and drugs increase the risk of PD such as carbon dioxide, reserpine, mefloquine, chlorpromazine, verpamil, and industrial pesticides.¹³ One study showed that many metals such as iron, manganese, mercury, copper, and cadimium contribute and increase the risk of PD.¹⁴ Another study showed that individuals who are in harsh area such as warzone or exposed to chemicals are highly risk in developing PD.¹⁵ One study has found that welding and mining personals have a high chance in developing PD.¹⁶ Head injury increases the risk of developing PD.¹⁷ Atherosclerosis increases the possibility of PD development because many lipoproteins such as LDL, VLDL, and HDL produce an inflammatory response by activation of scavenger receptors which leads to release of cytokine from macrophages.¹⁸ It has been shown that systemic infection and inflammation increase the damage that is associated with PD.^{19, 20} One study showed that systemic infection such as bacterial vaginosis in the prenatal period increase the risk of development of PD.²¹

Several studies showed that tea decreases the risk of developing PD because it has many components such as polyphenol, methylxanthine, caffeine, and volatile substances that have antioxidant, anti-inflammatory, and iron scavenger properties.^{22, 23}

It has been shown that caffeine has adenosine A2A antagonist properties, and theanine, in coffee has the capacity of increase dopamine secretion, flavinoid has anti-inflammatory and the ability to improve circulation in the brain.^{24, 25} . Large study found that chronic consumption of over the counter drugs such as cyclooxygenase enzyme inhibitors leads to less PD development because those inhibitors decrease the production of toxic products from microglia activation and the amount of cyclooxygenase enzyme oxidation of dopamine.^{26, 27} Another study showed that the consumption of milk leads to increase of PD in men while the consumption dairy and non-dairy calcium does not increase risk of PD.²⁸ One study showed that the possible explanation of dairy product causes PD is the presence of neuro-toxic material that causes PD in animal studies.²⁹

The exact mechanism of PD is unknown, and there is different theories explain the etiology of PD. Oxidative stress is one of the proposed mechanisms to this disease.³⁰ Oxidative stress, which results from many environmental factors, causes lipid oxidation, mitochondrial DNA damage, and dopaminergic neuron damage.^{31, 32} Due to reduced level of anti-oxidant mechanism such as glutathion and increased iron content in SN, the dopaminergic neurons in the SN is more susceptible to oxidative stress than other neurons.^{33, 34} Microglia is responsible for production ROS because oxidative stress stimulates microglia activation, hence, ROS causes neuronal damage to dopaminergic neurons and subsequently inflammatory response.³⁵ Microglial activation is responsible for the elevation of iron inside the cell.^{36, 37} The free iron initiate the oxidative stress which promotes the auto-oxidation process to dopamine, thus isoquinoline metabolites and superoxide and H₂O₂ produced.³⁸ Activation of microglia leads to production of

many pro-inflammatory mediators such as TNF-alpha, IL-1Beta, and INF-Gamma.³⁹ Chronic over expression of inflammatory mediators makes the dopaminergic neurons more vulnerable to oxidative stress.⁴⁰ Some studies have shown that PD patients have an iron in the SN, low in free radical protection mechanisms such as decline glutathione production, raise superoxide dismutase activity, and damage to many intracellular organelles such as DNA and mitochondria.^{33, 41-47}

Mutation to α -synuclein results in the development of familial types of PD.³ Another gene that causes ARJP is DJ-1 gene.⁴⁸ Many genes are involved in the etiology of sporadic PD and familial PD such as parkin(PARK2), DJ1 (PARK7), PINK1(PARK6), LRRK2(PARK8), HTRA2(PARK13), and L1.⁴⁸⁻⁵¹ Some PD cases that are caused by the mutation of parkin and LRRK2 genes are not associated by presence of lewy bodies.⁵² Mutation of α -synuclein gene (E46K) leads to development of PD.⁵³ It has been shown that autosomal dominant PD caused by mutation of many genes such as LRRK2 and the gene of α -synculein.^{54, 55} It has been shown that mutation of G2019S gene account for the development of 1% sporadic PD and 4% familial PD.^{9, 10} One study found that the mutation of Parkin gene results in early onset of PD.⁵⁶

Fig (1) illustrates the mechanisms of neural death in SN.⁵⁷ Fig (2) explains summarize the etiology of the familial and sporadic PD.⁵⁸ In PD, the loss of dopamine neurons mostly occurs in SNpc in the brain. Fig. (3) explains the mechanism of dopamine metabolism by different enzymes inside SN presynaptic and postsynaptic part of the striatum.⁶⁰

Figure 1. Mechanisms of neural death in SN

Figure 2. Etiology of the familial and sporadic PD

Figure 3. Mechanism of dopamine metabolism

Many studies showed that α -synuclein is the main causative agent for the development of PD.^{5, 61} The level of α -synuclein in dopaminergic neurons in SNpc is low in normal physiological condition, and this level starts to decline with age, therefore, α -synuclein pathological level starts to increase which leads to the development of PD.^{62, 63}

Increased oxidative stress that is associated with iron level linked to the aggregation of α -synuclein pathology.⁶⁴ It has been shown that the mutation of α -synuclein, presence of oxidative, and the presence of metal cations leads to aggregation of α -synuclein.^{64, 65} Increased iron level within the cell promotes the production of oxygen free radical through Fenton reaction by increase the ratio of $\text{Fe}^{+2}/\text{Fe}^{+3}$ into more oxidized state.⁶⁶ Dopamine overload might leads to cell death because it leads to increase the aggregation of insoluble α -synuclein.⁶⁷ It has been shown that PD development is associated with low level of uric acid since uric acid has antioxidant properties and it reduces the effect of oxidative stress in the nigrostriatal pathway.^{68, 69}

1.1.2. Clinical Manifestation:

Two types of symptoms characterize PD: motor and non-motor. Motor symptoms include rigidity, postural instability, tremor, and bradykinesia.⁷⁰ Motor symptoms of PD start to appear when 60-70% of the dopaminergic neurons are lost.⁷¹ Non-motor symptoms include disturbance in vision, olfactory sensory, constipation, sleep and autonomic nervous system.⁷²

1.1.3. Pathogenesis:

The Braak theory explains that PD progression started at a certain stage before the loss of dopaminergic neurons at SN. Braak theory based on the assumption that PD starts in olfactory bulb area, and it spreads through enteric nervous system of the gut and stomach to vagal nucleus.⁷³ The damage in mitochondria includes impairment in mitochondrial complex I activity, which occurs in SN and in peripheral tissues such as muscles, platelets, and lymphocytes.⁷⁴⁻⁷⁷ However, some studies have shown that there is no alteration in mitochondrial complex I activity in peripheral tissues.^{78, 79}

PD pathological features are loss of dopaminergic neurons in SNpc, presence of lewy bodies, and presence of lewy neuritis.³ Lewy neuritis is defined as inclusions located in intra-cytoplasmic and intra-axonal position, and it composed from fibrillary α -synuclein aggregates.³ The presence of lewy bodies (LB), which can be seen along the progression of the PD, is a neuro-pathological hallmark of PD, and it can be found the area where there is a loss of neurons such as SNc.^{73, 80} α -synuclein has great impact on the pathogenesis of PD, especially, when it presents at the synapses of the neurons.⁵ α -synuclein aggregations mainly located at the presynaptic level of the neurons which lead to dysfunction of synapses and death of neurons.^{4, 5} α -synuclein can move from one cell to other cells by prion-like mechanism, therefore, α -synuclein pathology move to the remaining cell in the advanced stages of PD and it can transform from soluble to insoluble state.^{81, 82} α -synuclein oligomers can trigger the formation of intracellular aggregates of α -synuclein.⁸³ Also, α -synuclein has important role in controlling the release of neurotransmitter and in controlling of synaptic plasticity.^{84, 85}

In addition to SNpc, other areas are involved in the pathology of PD such as locus ceruleus, dorsal motor nucleus of the vagus, amygdala, CA2 of the hippocampus, and the basal nucleus of Meynert.^{3, 86} The loss of dopamine neurons in PD occurs in the ventral midbrain area, which includes the SNpc and nigrostriatal pathway in the striatum, but the loss is not significant in ventral tegmental area as to other two areas.^{87, 88} One hypothesis that explains the loss of dopaminergic neurons in the SN is that the brain consumes 20% of the oxygen through respiration; therefore, many reactive oxygen species produced inside cells. The SN suffered from the highest amount of reactive oxygen species, because the metabolic rate is so high that the high amount of reactive oxygen species cannot be deactivated by normal antioxidant mechanism such as glutathione. In addition, many pro-oxidant factors such as presence of iron, sulfite, peroxynitrate, polyunsaturated fatty acids, and neuromelanin, have the ability to produce reactive oxygen species.^{7, 8}

Reactive oxygen species production comes from many resources such as mitochondria and different cytokines such as TNF, which increase the amount of ROS in the mitochondria. The amount of antioxidant glutathione in the cell is low when compared to the amount of ROS.⁸⁹ PD patients experienced reduced function of mitochondrial complex I and III.⁷⁵ ROS are generated as a byproduct of dopamine metabolism because this metabolism leads to generation of high amount of hydrogen peroxide, which interacts with the iron and produces highly reactive oxygen radicals (OH[•]). Several studies shown the level of glutathione and superoxide dismutase decrease with age, thus patients develop PD when they get old.^{45, 90}

One study suggested that the dysfunction of glial cells, which have a supportive role to CNS, contributes to the pathology of PD.⁹¹ One study showed that the loss of dopaminergic neurons is associated with a change in the activation of microglia; therefore, neuroinflammation develops in early PD.⁹² Because of microglia produces high amount of ROS and their number is high in dopaminergic neurons, dopaminergic neurons are highly sensitive to the actions of microglia.⁹³ α -synuclein aggregates result from dying dopaminergic neurons, and it becomes nitrated by oxidative stress, which facilitates the aggregation of the proteins.⁹⁴ α -synuclein is surrounded by microglia because it is nitrated and oxidized by oxidative stress, thus, more inflammation will occur by the activated microglia.^{92, 95}

One study showed that the SNpc has few numbers of astrocyte cells, which secrete many pro-survival factors such as BDNF, GDNF and bFGF, thus SNpc is vulnerable to degeneration by reactive oxygen species.⁹⁶ Several studies showed that SN and the midbrain areas have low level of glutathione is low, but the levels of Mn, Cu/ZN superoxide dismutase, and mRNA are high.^{43, 44} Several studies showed that several DNA enzymes such as MTH1, adenine DNA glycolyase, and 8-oxoguanine DNA glycosylase level are increased in PD because those enzymes are responsible for repairing DNA which can be affected by oxidative stress in PD.⁹⁷ One study showed that increase Co-enzyme Q10 level in the brain is very important because it reduces the incidence of PD.⁹⁸ Another study showed that eating of dairy product is associated with risk of development of PD in men only.²⁹ However, case control study proved that there is no direct relation between the consumption of dairy product and the development of PD.⁹⁹ It has been

shown that the serotonin system is involved in the pathology of PD because many studies showed that there was a dysfunction in serotonin systems in postpartum samples in animals.¹⁰⁰.

1.2. Monoamine oxidase enzyme:

1.2.1.Function:

Monoamine oxidase enzyme (MAO) is a catalytic enzyme and it is responsible for metabolism of many monoamine neurotransmitters such as norepinephrine, epinephrine, serotonin, dopamine and others by a mechanism known as oxidative deamination.¹⁰¹⁻¹⁰³ The main function of MAO enzymes that are present in peripheral tissue is to protect other tissue from exogenous amines while the main function of MAO enzymes that are present in central nervous and peripheral nervous system is to control the level of amine in neurons.^{60, 104} MAO enzymes act on endogenous neurotransmitters such as norepinephrine, serotonin, and dopamine, and on exogenous amines that come from external diet such as tyramine containing food.¹⁰⁵⁻¹⁰⁷ Other products, which result from the catabolism of neurotransmitter by MAO, are aldehyde and ammonia that are toxic to humans.^{108, 109} MAO enzymes act on either endogenous or exogenous compounds (primary amines, secondary amines and tertiary amines) by the following reaction.¹¹⁰

1.2.2. Classification:

Monoamine oxidase enzymes in humans are classified into two types according to their amino acid sequence : (MAOA) and (MAOB). They are used as targets in many neurological, psychological diseases such as Parkinson, depression, and Alzheimer diseases.¹⁰¹⁻¹⁰³ MAO inhibitor drugs can be classified according to their ability to either reversibly, irreversibly or selectivity toward MAO enzymes: reversible inhibitors such as moclobimide; nonselective MAO inhibitors such as isocarboxazid, tranylcypromine, and phenelzine; selective MAO inhibitors such as selegiline.^{102, 105} Table (1) summarizes MAOB inhibitor drugs that are approved for various indications.

Table 1: MAO inhibitors that are approved for clinical uses

Drug name	Selectivity	Uses
Phenelzine	Non-selective, irreversible	Antidepressant and anxiolytic
Tranylcypromine	Non-selective, irreversible	Antidepressant and anxiolytic
Selegiline (L-deprenyl)	Selective-MAOB, irreversible	Parkinson disease
Rasagiline	Selective-MAOB, irreversible	Parkinson disease
Lazabemide	Selective-MAOB, reversible	Used in Parkinson disease clinical trials
Moclobemide	Selective-MAOA, reversible	Antidepressant and anxiolytic(Not approved in US)
Safinamide	Selective-MAOB, reversible	Antidepressant and anxiolytic-Phase III in clinical trials
Zonisamide	Selective-MAOB, reversible	Antiepileptic, used in Parkinson disease in Japan

1.2.3. Uses

Generally, MAO inhibitor drugs are indicated for depression, PD, bulimia, anorexia nervosa and body dysmorphic diseases.^{105, 111} MAO inhibitors have been used for treatment of depression since 1950 but many of them have been withdrawn from the market because of their side effects.¹⁰⁵

1.2.4. Side effects:

Cheese reaction, which is caused by tyramine containing food such as aged cheese leads to hypertensive crises and it is the main side effect for MAO inhibition.¹⁰⁵⁻¹⁰⁷ However, reversible MAO inhibitors have the advantage that no cheese reaction has been reported.¹⁰⁵⁻¹⁰⁷

1.2.5. Distribution:

MAO enzymes can be found in many sites throughout the human body. MAOB enzyme can be found in brain and liver while MAOA enzyme can be found in peripheral adrenergic nervous system such as intestine, liver, adrenal glands, sympathetic nervous system and arterial vessels.¹⁰⁵⁻¹⁰⁷ MAOB is representing about 80% of the total MAO enzymes in human brain.^{110, 112} MAOA is main type of MAO enzymes in cardiomyocytes although this tissue contains both types of MAO enzymes.^{113, 114} Because 80% of MAO enzymes in the intestine are MAOA type, many MAOA inhibitor drugs cause cheese reaction because those enzyme responsible for catabolism of tyramine.¹⁰⁵⁻¹⁰⁷

The level of MAO enzymes is different throughout human body. MAO enzyme level is high in basal ganglia and hypothalamus while the level is low in cerebellum and neocortex.^{60, 115} Some studies have shown that the level of MAOA enzyme is high in SN while the level of MAOB enzyme is high in dorsal raphe nucleus and astrocyte.^{60, 116} MAOA enzyme level increases with age in the heart while MAOB enzyme level increases in the brain and this leads to the development of cardiovascular diseases and neurodegenerative diseases in old people.^{117, 118} Some studies have shown that MAOB enzyme level increases with age due to the development of gliosis, which is related to aging.¹⁰⁸ MAOA enzyme activity level is high at birth then it starts to fall during the first two years while MAOB enzyme level start to increase significantly.^{110, 119} MAOB enzyme has high affinity to dopamine, phenylethylamine while MAOA enzyme has high affinity toward serotonin, octopamine, epinephrine and norepinphrine. Tyramine has the same affinity to MAOA and MAOB enzymes.

1.2.6. Food interaction:

There are many foods should be avoided during MAO inhibitors drug therapy and two weeks after MAO inhibitor drug therapy discontinuation. These includes aged cheese, aged meat, banana peels, soybean containing products, wine, draft beer, marmite, broad beans sauerkraut, and others.^{105, 109} MAOA enzyme level is inhibited by many compounds such as 1,2,3,4-Tetrahydroisoquinolone which is present in chesse and wind, Eugeol which is present in clove, cinnamon, nutmeg, coumarin, Beta-carbolines.¹²⁰

In addition, MAOA enzyme can be inhibited by many other compounds such as harman and norharman, which are present in tobacco, flavonol part of St John wort, and quercetin glycosides. As a result, smoker individuals have lower MAOA enzyme activity due to presence of Harman.^{121, 122} Some studies have shown that there are endogenous inhibitors to MAO enzymes such as peptide neurocadin which acts on MAOA enzyme and isoquinolium related compounds, phosphatidylserine and quinalolinic acid that act on MAOB enzyme.^{60, 123}

1.2.7. Crystal structure:

MAO enzymes are located in the outer membrane of the mitochondria.¹²⁴ Oxidative deamination of neurotransmitters is a process conducted through FAD cofactor in MAO enzymes.^{101, 124} MAO enzymes use oxygen molecules in the process of oxidative deamination of neurotransmitters.^{124, 125} MAOB structure is illustrated in Fig (3). In general, FAD cofactor is partially embedded within the MAO enzyme protein structure.^{126, 127} FAD cofactor is covalently bound to MAO enzymes at 8- α -S-cysteinyl position of FAD, which is a thioether linkage that is located near the carboxyl end of the MAO enzyme.^{126, 128} The position of this covalent bond in MAOB enzyme is at Cys397 while the position of the covalent bond in MAOA enzyme is at Cys406.^{103, 129}

The position of isoalloxazine part of FAD is bent about 30 degree from plane position in N (5-10) position.^{126, 127} The position of dinucleotide part of FAD cofactor locates near the amino end of MAO enzyme.¹⁰³ The anionic part of FAD cofactor, pyrophosphate

binds to the positive guanidino group of Arg42 residue by a hydrogen bond. Also, Thr45 has no interaction with FAD cofactor^{129, 130}. Fig. (4) illustrates crystal structure of MAOB enzyme. The MAOB crystal structure is adapted from protein data bank website (<http://www.rcsb.org>).

Figure 4. Crystal structure of MAOB enzyme

The ribose ring of FAD cofactor binds by hydrogen bonds to guanidine group of Arg36 residue, carboxyate group of Glu34 residue and to water molecules. The adenine ring of FAD cofactor binds to Val235 residue of MAO enzyme through hydrogen bond and binds to a water molecule through a hydrogen bond.¹³¹ Flavin ring of FAD cofactor is positioned toward the substrate binding cavity which leads to the recognition of the ligand containing amine group, therefore, it leads to catabolism of the ligand. There is a hydrogen bond between 2-carbonyl oxygen in the pyrimidine group of FAD cofactor and N-H group of Met436 residue and a hydrogen bond with a water molecule.¹²⁷ There is a hydrogen bond located between N-3 of the pyrimidine group of FAD cofactor and carbonyl group Tyr60 residue of MAO enzyme and between (N-H of Tyr60 residue, Ser59 residue of MAO enzyme, water molecule) and 4-carbonyl group of FAD cofactor. There is a hydrogen bond between a water molecule and N-5 of position of the flavin ring and 1-amino group of Lys296. The hydrogen bond interaction between the ribose of FAD cofactor and the carboxyl group of Glu34 is essential in maintaining the covalent binding of FAD cofactor to MOA enzyme, therefore, its important to maintain the function of MAO enzymes.¹²⁹ This lead to water molecules play an essential part in the activity due to the hydrogen bonds that are formed between water molecules and other amino acids residues.¹²⁶ Adenin, which is part of FAD cofactor exits as in an extended conformation in comparison with isoalloxazine ring in MAO enzymes.^{126, 129} The FAD cofactor in MAOA enzyme is similar to MAOB enzyme except that there is hydrogen bond between Ser.59 residue of MAOA enzyme (instead of Ala residue in MAOB enzyme) and C-4

carbonyl oxygen of FAD cofactor.^{127, 129} The flavin ring of FAD cofactor is situated in an area that is unavailable to the bulk solvent to enter.¹²⁷

Aromatic cage is very important in facilitating the oxidative deamination of ligand at the substrate cavity of MAOA and MAOB enzymes. It refaced the isoalloxazine ring of FAD cofactor. MAOA enzyme has an aromatic cage between Tyr406 and Tyr444 residues while MAOB enzyme has an aromatic cage between Tyr398 and Tyr435 and the distance between the aromatic rings is 7.8Å.^{126, 127, 132-134} The amide linkage, which is cis-amide linkage between Cys397 and Tyr398 residues of MAO enzyme is energetically unfavorable and provides a good position of the phenolic group of Tyr398 to be in perpendicular position with regard to the flavin ring of FAD cofactor in order to form a cage.^{127, 129, 133} This cage has an essential role in making sure that the substrate in the correct position in order to undergo oxidative deamination process by polarization of the shared electron that are present in the amine group of the substrate in order to make the imine intermediate.¹³³ Due to the steric effect of isoalloxazine group of FAD cofactor, aromatic cage is oriented in a way that is perpendicular to the flavin ring of FAD cofactor, not planner to the FAD, therefore, this steric effect facilitate the formation of flavin adducts which is an intermediate in the catabolism of a neurotransmitter.¹²⁷

The presence of 8alpha methylriboflavin or 7alpha,8alpha-dimethylriboflavin which provides a steric hindrance in MAOB enzyme results in MAOB enzyme has more activity than MAOA enzyme.^{129, 135} One study found that there are many water molecules

inside the active site of MAO enzyme; thus, water molecules can be replaced with hydrophilic groups in a ligand in order to increase the affinity to the MAO enzyme.¹³⁶

1.2.8. MAOA and MAOB:

Human MAOB enzyme has 520 amino acids.^{132, 137} MAO enzymes are an integral protein of the mitochondrial membrane because digestion of the membrane is essential in order to separate the enzyme from the membrane.¹³⁸⁻¹⁴⁰ MAOA enzyme composed from 527 amino acids.^{110, 141} Human MAOA and MAOB enzymes have about 70% similarity in amino acids sequence.^{103, 127, 132} Human MAOA and MAOB enzymes have dimer structure inside the body while MAOA enzyme has monomer crystal structure and MAOB enzyme has dimer crystal structure.^{126, 134} MAOB enzyme active site has two cavities: entrance cavity and substrate cavity while MAOA enzyme active site has entrance cavity only.^{136, 142} The entrance cavity and the substrate cavity include many aromatic and aliphatic amino acids residues which make them hydrophobic area.¹³⁴ The two monomer (dimer) structures of MAOB enzyme are separated by 2095Å, which is equivalent to 15% of the monomer surface area. MAOA enzyme has protein loop that is cover the entrance cavity of the enzyme and it's located at 108-118 amino acid residue, while MAOB enzyme has a protein loop which is 290Å volume that cover the entrance cavity and its located at 99-112 amino acid residue and its partially embedded in the outer membrane of the mitochondria.^{60, 126, 127} In MAOB enzyme, the protein loop must be passed through first in order for the ligand or the substrate to do its action in the enzyme.

¹²⁶ In MAOB enzyme, there is Ile 119 residue which is considered a gate that separates an entrance cavity and substrate cavity and it covers the opening of the entrance cavity.¹²⁶ Tyr326 separates the entrance cavity and the substrate cavity along with Ile119 amino acid residue.^{134, 143} This gate can be either in an open conformation or closed conformation depends on the type of the substrate.^{132, 134} If the substrate is large enough to occupy both the entrance cavity and the entrance cavity, it leads to change the conformation of Ile199 gate from closed to open and forms a fusion cavity about 700Å. If the substrate is small, the Ile199 remains in a closed conformation.¹⁴³ The volume of substrate cavity in human MAOA enzyme is 550Å while the volume of the both entrance cavity and substrate cavity is 700Å.^{126, 134} the volume of MAOB enzyme entrance cavity is 290Å while the volume of MAOB enzyme substrate cavity is 420Å.^{127, 134} MAOB enzyme two cavities become one cavity when ligand binds to the enzyme firmly and specifically.^{126, 134} The substrate cavity of MAOB enzyme shape is a flat long disk and it is located perpendicularly to flavin ring of the FAD cofactor.^{132, 144}

MAOB and MAOA enzymes have hydrophobic environment in their active sites due to the presence of amino acid residues lining the active sites. Additionally, these residues provide the ability to form hydrogen bond with a ligand.^{126, 134} In MAOB enzyme, there are four amino acids, Tyr326, Ile199, Leu171, Phe168 which are located between the entrance cavity and the substrate cavity.⁶⁰ MAOA enzyme has a unique amino acid sequence which is called cavity shaping loop at 210-216 amino acids residues and this sequence is different from MAOB enzyme.¹⁴⁵ The binding site of MAOA enzyme is hydrophobic because it has 5 aromatic and 11 aliphatic amino acids residues.¹⁴⁵ MAOA

enzyme binding site is shorter than MAOB enzyme and wider than the binding site of MAOB enzyme, which is longer and narrower than MAOA enzyme.¹⁴⁵ In MAOB enzyme, the distance between the entrance cavity and the flavin ring of the FAD cofactor is 20Å.¹²⁷

MAO enzyme has three binding domains: FAD binding domain which is located at 4-79, 211-285, 391-453 amino acids residues; Substrate binding domain which is located at 80-210, 286-390, 454-488 amino acids residues; C-terminal membrane binding domain which is located at 489-500 amino acids residues.^{127, 129} FAD binding domain connected to C-terminal alpha-helix through extended loop (461-488) amino acids residues.^{127, 138} There are some amino acids residues (Pro109-Ile110, Trp157) in the substrate binding domain that are exposed to the solvent and near the C-terminal site have important role in binding of MAOB enzyme to the outer membrane of the mitochondria.^{127, 138} MAOA and MAOB enzymes connect to the outer membrane of the mitochondria by their C-terminal through transmembrane alpha-helix structure. The alpha-helix of MAOA enzyme has firm structure while MAOB enzyme has more flexible alpha-helix structure and that is why those the C-terminal in both enzymes are unexchangeable.^{127, 146} Some studies have showed that MAOA and MAOB enzymes surfaces have a negative charge, which allows the surface membrane to form an electrostatic force with negatively charge membrane of the mitochondria. Giving that MAOB enzyme has two monomers, each monomer has a positive dipole moment charge that is positioned toward the negatively charge membrane of the mitochondria.^{126, 127}

1.2.9. MAO oxidation mechanism:

In general, the oxidation reaction involves the cleavage of the α -carbon atom from the substrate (amine) and transfer two hydrogen atoms (reducing equivalent) to the reduced FAD to form two products, Imine and hydroquinone. FAD-Imine undergoes another reaction to form the H_2O_2 . Furthermore, the FAD-Imine undergoes another reaction to form the FAD oxidized and imine as a byproduct. The last undergoes hydrolysis to form aldehyde and NH_4 .^{147, 148} Fig. (5) explains the catalytic reaction by MAOB and MAOA enzymes. Fig(5) was adapted from¹⁴⁹.

Figure 5.MAO enzyme oxidation mechanism.

1.3. Piperine:

Piperine is an extracted from the piper species, the fruit of black pepper (*Piper nigrum* Linn) and long pepper (*Piper Longum* Linn).¹⁵⁰ The fruit of this plant has been used in folk medicine in bronchial disease for many years because it has analgesic effect and carminative effect.¹⁵¹ Piperine has many functions such as central nervous system depression, anti-inflammatory and antipyretic.

Piperine has reversible inhibitory effect on MAOB and MAOB enzymes in mouse.¹⁵¹ Piperine has shown to improve memory impairment and neurodegeneration in the hippocampus of male rats.¹⁵² Another study showed that piperine has muscle relaxant, tranquilizer, sedative and hypnotic actions.¹⁵³ The piperine molecule composed from three parts: part A: methylenedioxyphenyl ring (MDP), part B: side chain with conjugate double bond, and part C: basic piperidine moiety.¹⁵⁴ Fig (6) illustrates piperine structure. The structure of piperine was created using a MarvinSketch program (<http://www.chemaxon.com>).

Figure 6. Piperine structure

Part A

Part B

Part C

Chapter II: Methods

2.1. Test Compounds

The test compounds were chosen using similarity analysis using a software program called software suite vROCS 3.0 (www.openeye.com). The test compounds were purchased from Chembridge (www.hit2lead.com) and piperine was purchased from Sigma Aldrich Company. The 10 test compound structures are summarized in Table (2).

Table 2: Test compounds structures

Compound Number	Compound Identification Number	Chemical Structure
1	5724840
2	5359480
3	5363077
4	5365127
5	5699630
6	5785627
7	5789934
8	5553460
9	5245517
10	Piperine

2.2. MAO enzyme experiment:

2.2.1. MAOB, MAOA enzyme assay:

Purpose: Evaluation of the ability of test compounds to inhibit MAOB and MAOA enzymes. The ability of test compounds to inhibit enzyme activity is determined by measuring the ability of MAOA or MAOB enzymes which bind to a test compound to metabolize the non-fluorescent substrate kynuramine to the fluorescent product 4-hydroxyquinoline through the 3-(2-aminophenyl)-3-oxo-propionaldehyde intermediate. When a test compound binds to the MAO enzyme, its ability to metabolize the non-fluorescent substrate decreases. Fig.(7) explains the mechanism of MAOB assay in which oxidation that kynuramine undergoes by MAOs enzymes.¹⁵⁵

Procedure: MAO activity was performed according to the methods described previously.¹⁵⁶⁻¹⁵⁸ 50 μ L of 0.006mg/ml MAO-A or 0.015mg/ml MAO-B enzymes along with 50 μ L of 20 μ M kynuramine and 100 μ L of the test compounds in DMSO were added into each well of 96 well plates. The compounds were serially diluted across the plate to produce an eight point dose inhibition curve. The 96 well plate was incubated for 20 minutes at 37C°. The final concentration of the chemical compound in the assay is less than 0.5%.

The fluorescence of the 4-hydroxyquinolone product was measured at 300/380nm by using a fluorescence measurement device called BioTek Synergy 4 plate reader. The first well was designated as control because it contained the enzyme and the

substrate without any test compound (inhibitors). The enzyme assay was evaluated for its quality by using a technique called Z-factor, which is illustrated in equation (1). This technique is used in high-throughput screening in drug development.¹⁵⁹ Prism program (www.graphpad.com) was used as a statistical tool to obtain the dose response curve and values of IC₅₀ for each test compound.

In addition, Z-factor statistical method was used by using the following equation:¹⁵⁹

$$Z\text{-factor} = \frac{Avg\ max - Avg\ min - 3(SD\ max - SD\ min)}{Avg\ max - Avg\ min}$$

Figure 7. Mechanism of MAOB assay

2.2.2. Docking studies:

Purpose: To obtain insight of binding of piperine and test compounds with MAOB enzyme.

Procedure: The crystal structure of MAOB enzyme was used in the docking studies is (2V5Z) and it was obtain from protein bank database (<http://www.rcsb.org>). Autodock 4.2 and Autodock tools programs (<http://autodock.scripps.edu>) were used to obtain the binding conformation, free energy, and inhibition constant for the test compounds.¹⁶⁰ The Grid box was selected as 28*28*28 and the rest of the docking parameters were unchanged. The docking steps were followed in the docking studies using instructions provided in the programs website. Marvin Sketch program (<http://www.chemaxon.com>) was used to draw test compounds and convert them into 3D structure with minimum free energy state. LogPlot program(<http://www.ebi.ac.uk>) was used to obtain 2D images of the docking results of test compounds .¹⁶¹ In addition, Pymol (<http://www.pymol.org>) program was used to view the docking result in 3D images of the test compounds.

2.3. Bovine Serum albumin experiment:

In general, albumin in mammalian constitutes about 60% of total protein in the blood, and it binds to most of drugs that enter to the blood.^{162, 163} It considers as a depot for chemical compounds and it's slowly released into the blood and as carrier for most of the drugs through the blood to reach their site of action.^{162, 163} The structure of BSA is different from HAS by the number of binding sites available to a ligand. Previous studies have shown that HAS has two major binding sites I and II while BSA has three major binding sites. Each of those binding sites has two domains, A and B.^{164, 165} BSA has two tryptophan groups, which are responsible for the fluorescence of the protein. One of those amino acids located at the surface of the protein (Trp 134) and one which is (Trp 214) in hydrophobic pocket of BSA like in HAS.¹⁶⁶

The rationality of using BAS instead of HAS is because the sequence of amino acids in both albumins is similar and this similarity reach to 76%.^{167, 168} When a ligand binds to albumin, the intensity of the fluorescence decreases because a ligand quenches the fluorescence of albumin. Most of the quenching effect is result from the amino acids that have aromatic ring in their structure and it includes tyrosine, tryptophan, and phenyl aniline. Most of the fluorescence effect is due to the tryptophan residues.¹⁶⁹

2.3.1. BSA fluorescent HTS assay:

Purpose: To evaluate the ability of the test compounds to bind serum albumin because all the developed drugs must bind to the albumin as a carrier through blood to reach an active site in human body .

Procedure: Falcon 96 well plates was used in this assay. Ten compounds, which were purchased from Sigma Aldrich were used in this assay, 3 μL of 20 mM was used from each compound and those volumes were put in the first row by using a pattern in which each compound had 2 wells. After that, 147 μL DMSO was added into each well that contained the compounds to get a final volume of 150 μL . 100 μL of DMSO was added continuously from the second row until the last row in the well. A Serial dilution was performed in which 50 μL withdrawn from the first row and put in the second row then the another 50 was taken from the second row and put it the third row and so on in order to make series of solution that are diluted by a half in order to make reasonable curve. A BSA solution was prepared by using a BSA powder, which was purchased from Boston BioProducts and a Tris buffer 10mM solution that was prepared previously in a 1mg/1ml ratio. 100 μL of BSA solution was added into each well of 96 well plates. After that, the plate was incubated for 30 minutes in a temperature of 37 C°. After the incubation period was completed, the plate was read by a plate reader called (BioTek Synergy 4 plate reader) at 280nm and 340nm. Prism program (www.graphpad.com) was used as a statistical tool to obtain the dose response curve and values of IC₅₀ for each test compound.

2.3.2. Docking studies:

Purpose: To obtain insight of binding of piperine and test compounds with BSA molecule.

Procedure: The crystal structure (45FS) was used in the docking studies and it was obtained from protein bank database (<http://www.rcsb.org>). Autodock 4.2 and Autodock tools programs (<http://autodock.scripps.edu>) were used to obtain the binding conformation for the test compounds.¹⁶⁰ The Grid box was selected as 28*28*28 and the rest of the docking parameters were unchanged. The steps were followed in the docking using instructions provided in the program website. LogPlot program(<http://www.ebi.ac.uk>) was used to obtain 2D images of the docking results of the test compounds.¹⁶¹ In addition, Pymol (<http://www.pymol.org>) program was used to view the docking result in 3D images of the test compounds.

2.4. Parallel artificial membrane permeability assay (PAMPA):

Brain is a target for many drugs that are used to treat CNS disease such as PD and AD. Brain is protected from any chemical in systemic circulation by two barriers, BBB and cerebrospinal fluid barrier. BBB is composed from cerebral endothelial cells that characterize by having closed junction, lack of fenestration, and little pinocytic vascular transport. Cerebrospinal fluid barrier is composed from continuous layers of polarized epithelial cells.¹⁷⁰ There is another barrier called ependyma that prevent chemicals to

pass to the brain from cerebral spinal fluid.¹⁷¹ Ependyma is composed from a layer of endothelial cell that covers the ventricles of the brain.¹⁷¹ The process of penetration of drugs into the brain cannot complete unless drugs pass blood brain barrier.¹⁷⁰ Hydrophilic drugs have low BBB permeability and low binding ability to brain tissues while hydrophobic drugs have high BBB permeability and high binding ability to brain tissues.¹⁷² BBB has three different kinds of transporters which includes active efflux transport, carrier mediated transport and receptor mediated transport.¹⁷³ Active drug transport is responsible for maintaining concentration gradient across BBB.¹⁷⁴ Drug uptake by the brain is depend on many physicochemical properties of the drug such as molecular size, molecular charge, lipophilicity, hydrogen bonding, number of rotatable bonds in drug molecule.¹⁷⁵

High permeability to BBB and low brain tissue binding are the two important properties in designing a drug that can pass BBB into the brain.¹⁷⁰ P-glycoprotien is considered as efflux drug transport system in BBB that prevent CNS drugs to enter to the brain unless the free concentration of CNS drugs is high enough to reach a desired level of CNS drugs in the brain. This increase in the free concentration outside the brain leads to peripheral side effects due to a high free concentration of CNS drugs outside the brain.¹⁷⁶ Even though P-glycoprotien prevents drugs from entering the brain, most of drugs can pass BBB by passive diffusion.¹⁷⁷ Three limitations that can be encountered when designing a drug to produce CNS effect, passive membrane permeability, facilitated drug transport at the brain, and brain tissue binding with blood.¹⁷⁸ The free concentration of a drug inside the brain is depend on the plasma concentration of the

drug, permeability of the drug, metabolic enzymes that affect the drug, binding of the drug to the brain tissues, and CSF drainage and secretion.^{179, 180} There are many methods used in determine drug permeability into the brain. This includes Caco-2, which is made from colon or kidney endothelial cells. One limitation to this method is that long cell cycle growth and a high cost of operating this method.^{181, 182} Those cells are different from BBB cells in tight junction property of the cells.¹⁷⁷

Another method is called artificial membrane permeability assay (PAMPA). PAMPA assay was the first method that mimics biological membrane properties by measuring the flux of the membrane.¹⁸³ An advantage to this method is that it provides an easy method for screening compounds because using 96-well plate provides an opportunity to add sensitive methods to detect the compounds. The membrane that is used in this method is modifiable in order to use it in a different permeability conditions.¹⁸⁴ Moreover, this method has the ability to add PH gradient across the membrane, which is a property that cannot be used in other methods.¹⁸⁵ PAMPA assay is used as a tool to indicate the permeability of a compound because it measures the permeability measurement based on passive diffusion.¹⁸⁴ Despite the fact that PAMPA assay does not reflect P-glycoprotein properties of BBB and it's the structure of the membrane is not a lipid bilayer, its membrane composed from saturated hydrophobic lipid which is similar to BBB and it can detect small concentration of any drug (less than 0.5mg).¹⁸⁴ Many of highly cost measurement on human can be eliminated by using this method.¹⁸⁶ PAMPA method is used to estimate the passive diffusion in Vitro because

passive diffusion is important factor in determining the transport through many biological barriers such as gastrointestinal tract, BBB, and cell membranes.

Purpose: To provide InVitro model for passive diffusion. Passive diffusion is an important factor in determining transport across blood brain barrier.

Procedure: special 96 well plate which is composed from lid, donor plate, acceptor plate was used this experiment. Into each well, 15 μ L of 5% solution of hexadexane was added, and then the plate left for 45 minutes to one hour to dry in order to make sure that a complete evaporation of hexane. 300 μ L were added of 5% of (PBS/DMSO) solution into acceptor plate at PH 7.4. Donor plate was placed on the acceptor plate making sure the membrane of the donor plate in contact with the buffer. 15 μ L of each compound with the concentration of 500 mM in 5% (PBS/DMSO) was added into each well of acceptor plate. The acceptor plate was covered with the lid and it was incubated at room temperature for 4-6 hours. 100 μ L were transferred into new 96 well plates for fluorescence measurement using a device called BioTek Synergy 4 plate reader.

Standard curve was used in order to obtain the unknown concentration of the chemical compounds in the wells of acceptor plate.¹⁸⁴ Prism program (www.graphpad.com) was used as a statistical tool to obtain a standard curve and values unknown vales for each compound. Theoretical equilibrium concentration the chemical compounds was obtain by combining the volume of the acceptor and donor plate in order to calculate Log P using the following equation:

$$\text{Log } P = \text{Log} \left(C_x - \text{Ln} \left(1 - \frac{[\text{drug}]_{\text{acceptor}}}{[\text{drug}]_{\text{equilibrium}}} \right) \right)$$

$$\text{Where } C_x = \frac{V_D * V_A}{(V_D + V_A) \text{area} * \text{time}}$$

V_D : volume of chemical compound in donor plate.

V_A : volume of chemical compound in acceptor plate.

Area: surface area of the artificial membrane.

Time: Incubation time at room temperature.

Chapter III: Results

3.1. Monoamine oxidase B:

3.1.1. Enzyme assay:

MAOB enzyme ability to metabolize substrates was measured by its ability to metabolize kyunaramine into fluorescent product. Test compounds were competitively bind to MAOB enzyme, therefore, MAOB enzyme ability to metabolize kyunaramine decreases according to binding pattern of test compounds to MAOB enzyme. The results of MAOB assay are summarized in Table (3). The MAO enzyme assay was evaluated for its quality by using a statistical technique called Z-factor which is illustrated in equation (1). This technique was used in high-throughput screening in drug development.¹⁵⁹ The value of Z-factor was (0.88) for MAOA assay and (0.8) for MAOB assay. These values of Z-factor prove that our enzyme assay was an excellent assay because any experiment that has a Z-factor value falls between (0.5) and (1.0), its considered an excellent assay.¹⁵⁹ The 10 compounds showed different level of activity toward MAOA and MAOB enzymes. MAOB enzyme inhibition activity was from ($>100\ \mu\text{M}$) to ($0.483\ \mu\text{M}$) while range of MAOA inhibition activity was from ($>100\ \mu\text{M}$) to ($58.98\ \mu\text{M}$).

Table 3: Effect of test compounds on MAOA, MAOB activities:

Compound Number	Compound Identification Number	Chemical Structure	MAO-A IC ₅₀ , μ M	MAO-B IC ₅₀ , μ M
1	5724840		>100	15.39
2	5359480		>100	0.666
3	5363077		>100	6.39
4	5365127		>100	0.497
5	5699630		>100	4.89
6	5785627		>100	3.18
7	5789934		>100	3.43
8	5553460		>100	1.13
9	5245517		>100	>100
10	Piperine		58.98	0.483

3.1.2. Docking Studies:

MAOB crystal structure was docked with each test compound using Auto dock tools and Autodock 4.2 programs in order to obtain insight understanding of binding between MAOB enzyme and a ligand. The result of docking the test compounds on MAOB enzyme are summarized on Table (4) and Fig. (10-29). Fig. (8) illustrates binding free energy of test compounds. Fig. (9) illustrates inhibition constant of test compounds.

Compound **1** binds to several amino acids which includes aromatic cage amino acids and passed through amino acids of the gating residue that separates the entrance cavity and the substrate cavity. In addition, compound **1** binds to FAD ring. Compound **2** binds to Tyr435 and Tyr398 of the aromatic cage, passes through amino acids of the gating residue and it binds to FAD. Compound **3** binds to FAD ring and also binds to several amino acids involved in the gating residue and aromatic cage. It binds to Tyr435 by a hydrogen bond. Compound **4** binds to Tyr398 but it showed that the position of the ligand as not close to Tyr435 as piperine. In addition, it binds to FAD ring and passes through the gating residue. Compound **5** binds to aromatic cage amino acids and passes through the gating residue amino acids. Piperine binds to several amino acids which are involved in the binding site of the MAOB enzyme including Ile199, Tyr326, Tyr435, and Tyr398 amino acids. Those amino acids are involved in the gating residue and aromatic cage of MAOB enzyme.

Compound **6** binds to several amino acids includes Cyc172 and Tyr188 by hydrogen bonds. Also, it binds to aromatic cage amino acids and passed through gating residue amino acids and it does not bind to FAD ring. Compound **7** binding pattern showed that it binds with Gln206 by hydrogen bond and it does not bind with FAD ring. Compound **8** binds to the aromatic cage, FAD ring and passes the amino acids of the gating residue. It binds by hydrogen bond with Tyr188. Compound **9** binding pattern showed that it binds with Leu171 and Tyr435 by hydrogen bonds and it does not bind with FAD. The K_i predictable values for the compounds are ranged from (2.03 to 591.03). The value of binding energy for the test compounds and piperine is (-7.3 to -9.12).

Table 4: Binding energies and inhibition constants values for the test compounds with MAOB:

Compound Number	Compound Identification Number	Binding Energy (Kcal)	Inhibition constant(μ M)
1	5724840	-7.3	4.43
2	5359480	-9.12	205.22
3	5363077	-7.77	2.03
4	5365127	-8.68	430.81
5	5699630	-7.76	2.06
6	5785627	-7.59	2.71
7	5789934	-7.69	2.33
8	5553460	-8.5	591.03
9	5245517	-7.42	3.62
10	Piperine	-8.78	365.89

Figure 8. Binding free energy of test compounds.

Figure 9. Inhibition constant of test compounds

Figure 10. Compound #1 docking with MAOB enzyme (2D plot)

Figure 11. Compound #1 docking with MAOB enzyme (3D)

Figure 12. Compound #2 docking with MAOB enzyme (2D plot)

Figure 13.Compound #2 docking with MAOB enzyme (3D)

Figure 14. Compound #3 docking with MAOB (2D plot)

Figure 15. Compound #3 docking with MAOB (3D)

Figure 16. Compound #4 docking with MAOB enzyme (2D plot)

Figure 17. Compound #4 docking with MAOB enzyme (3D)

Figure 18. Compound #5 docking with MAOB enzyme (2D plot)

Figure 19. Compound #5 docking with MAOB enzyme (3D)

Figure 20. Compound #6 docking on MAOB enzyme (2D plot)

Figure 21. Compound #6 docking on MAOB enzyme (3D)

Figure 22. Compound #7 docking with MAOB enzyme (2D plot)

Figure 23. Compound #7 docking with MAOB enzyme (3D)

Figure 24. Compound #8 docking with MAOB enzyme (2D plot)

Figure 25. Compound #8 docking with MAOB enzyme (3D)

Figure 26. Compound #9 docking with MAOB (2D plot)

Figure 27. Compound #9 docking with MAOB (3D)

Figure 28. Compound #10(piperine) docking with MAOB enzyme (2D plot)

Figure 29. Compound #10 (piperine) docking with MAOB enzyme (3D)

3.2. Bovine Serum albumin assay:

BSA assay was performed in our lab in order to determine intensity the binding of (10) compounds to BSA.

3.2.1. BSA binding assay:

Binding with BSA is the first step toward reaching the action site. Every test compound must bind with BSA in sigmoid-curve fashion in order to consider the test compound good candidate for further studies. The results of test compound binding with BSA are summarized in Table (5). Test compound binding curve with BSA pattern are illustrated in Figs (30-34).

Table 5: BSA IC₅₀ values for the test compounds:

Compound Number	Compound Identification Number	BSA IC ₅₀ (μM)
1	5724840	160
2	5359480	144
3	5363077	383
4	5365127	16
5	5699630	89.5
6	5785627	87.8
7	5789934	8337
8	5553460	200
9	5245517	1.1
10	Piperine	8.9

Figure 30. Compound #1 and Compound #2 binding pattern with BSA

Figure 31. Compound #3 and compound #4 binding pattern with BSA

Figure 32. Compound #5 and compound #6 binding pattern with BSA.

Figure 33. Compound #7 and compound #8 binding pattern with BSA.

Figure 34. Compound #9 and compound #10 (piperine) binding pattern with BSA.

3.2.2. Docking studies:

BSA was docked with compound **2**, **4** and piperine using Auto dock tools and Autodock 4.2 programs in order to get better understanding of test compounds binding with BSA as compared to piperine. Docking BSA with compound **2**, **4** and piperine on AII and AIII sites are summarized in Fig (35-40).

We found that piperine binds to BSA (AII) in several amino acids including Tyr149, Glu152, Ser191, Arg194, Arg198, Ala290, Ile289, and Leu237. In Fig. (36), docking studies showed that compound **4** binds to several amino acids in BSA (AII) including the same amino acids that are part of the binding site to piperine. In Fig (35), docking studies showed that compound **2** binds to several amino acids in BSA (AII) includes Tyr149, Glu152, Ser191, which are some of the amino acids that have seen in the docking of piperine and BSA. In Fig. (40) ,docking of piperine with BSA(AIII) showed that piperine binds to several amino acids including Ser 488, Arg409, Val432, and Leu429, Leu386, Thr448, Val432, Cys437, Asn390 and Lys413. In Fig. (37), docking of compound **2** with BSA (AIII) showed that it binds to the same three amino acids that piperine binds which are Ser 488, Arg409, Val432. In Fig. (38), docking of compound **4** with BSA (AIII) showed that it binds to the same amino acids that piperine binds.

Figure 35. Compound #2 docking with BSA (AII) site.

Figure 36. Compound #4 docking with BSA (AII) site.

Figure 37. Compound #2 docking with BSA (AIII) site.

Figure 38. Compound #4 docking with BSA (AIII) site.

Figure 39. Compound #10(piperine) docking with BSA (AII) site.

Figure 40. Compound #10 (piperine) docking with BSA (AIII) site.

3.3. Artificial membrane permeability assay:

In order to reach the active site in SNpc in the brain, test compound must pass BBB. PAMPA is in vitro test used to assess ability of test compound to pass a membrane, which has similar characteristics to BBB. LogPe values for the test compounds are summarized in Table (4). Log Pe value for propranolol, which is the standard compound for PAMPA assay was -4.16. This value confirmed that the experiment is valid because the same value that was found in the literature. LogPe values of the 10 compounds are from (-1.21 to -3.87). LogPe of piperine was (-3.87) which is same as seen in the literature. Compounds can be classified into high permeability and low permeability compounds. Compounds with LogPe values more than (-6)(values such as -7, -8, -9, etc) are classified as low permeability while compounds with LogPe less than (-6) (values such as -5,-4,-3, etc) are classified as high permeability compounds.¹⁸⁴

Table 6: Log Pe values for the test compounds:

Compound Number	Compound Identification Number	Log Pe
1	5724840	-1.92
2	5359480	-3.20
3	5363077	-1.71
4	5365127	-3.77
5	5699630	-1.66
6	5785627	-1.42
7	5789934	-1.90
8	5553460	-1.87
9	5245517	-1.21
10	Piperine	-3.87

Chapter IV: Discussion

The fruit of black pepper has been used for many years in folk medicine because it has many therapeutic uses. Piperine, which is the active ingredient of the black pepper has been found to have many therapeutic properties such as memory improvement, sedative, and neuroprotection.

We showed that compounds (**2**, **4**) have IC-50 values are close to piperine which means that they have similar effect on the inhibition on MAOB enzyme as compared to piperine and other compounds. Other compounds have IC-50 values higher than piperine which means those compounds have many side effects because they would lose the selectivity toward MAOB enzyme. Compound **4** has the ability to inhibit 50% of the enzyme at low concentration ($IC_{50}=0.497\ \mu M$) while other compounds have higher IC-50 values than compound **4**. Compound **2** has ($IC_{50}=0.666\ \mu M$) which is acceptable to inhibit MAOB enzyme at this concentration but other factors play in determining whether it's good compound for further testing or not such as BSA binding and the ability to pass BBB. The ability of test compounds to inhibit MAOB enzyme at high concentration is not acceptable parameter in design a drug because the higher concentration of the drug, the more side effects associated with the drug.

All compounds showed poor selectivity toward MAOA enzyme because IC_{50} values are higher than 100 μ M. A compound that showed high IC_{50} (a value more than 0.1 μ M) will have many side effects such as cheese effect because this high concentration leads to losing the selectivity toward MAOB enzyme. Based on MAOB and MAOA activity, we found that there is a structure activity relationship between test compounds and our natural compound, piperine. The activity of chemical compounds showed that modification of the piperidine ring changes the inhibition pattern toward MAO enzyme activity. This result is a confirmation to what it found in the literature.¹⁸⁷

Inclusion of oxygen in the ring of piperidine leads to decrease in the activity, which can be seen when comparing compounds **4** and compound **3**. The replacements of piperidine ring by pyrrolidine leads to decrease the activity by 10 folds, which can be seen when we compare compound **4** and compound **5**. Compound **4** has higher affinity to MAOB than both compound **3** and compound **5**. Compound **4** has similar activity toward MAOB in compared to piperine. When piperidine ring replaced by azopane, this leads to decrease in the activity. When nitrogen atom removed from compound **5**, it leads to slight increase in the activity (compound **8**). This possibly due to decrease in the lipophilicity by introduction of the nitrogen atom which leads to decrease in the activity toward MAOB enzyme.

Compound **2** is more potent than compound **8** because the size of the ring could increase the hydrophobicity of the compound which lead to increase in the activity. The replacement of the oxygen in compound **6** by sulfur leads to decrease in the activity. This

is possibly due to the oxygen in the carbonyl group has important role in the hydrogen bonds with the amino acids or water molecules in the substrate pocket. The change in the length between the nitrogen ring and the carbonyl with 2 carbon atom (-CH₂) leads to slight change in activity when compare compound **6** and **7**. These conclude that the distance between carbonylic oxygen and piperidine ring does not play an important in the activity toward MAOB enzyme inhibition. The replacement of piperidine part of piperine with other substituent leads to decrease the activity of MAOB inhibition as seen in compounds (**1,2,3**). This result is a confirmation to a previous study showed that the replacement of the piperidine group in the piperine by N-methyl phenyl, methoxy or ethoxy group and small amine group leads to inhibit the activity toward MAOB enzyme.¹⁸⁷

We found that compounds (**1, 6, 7, and 9**) lack the conjugated double bond of the piperidine group and this leads to decrease in the inhibition activity toward MAOB enzyme. This result is a confirmation to a previous study, which showed that the removal of the carbonyl of the piperidine group leads to lose in the inhibition activity toward MAOA and MAOB enzymes.¹⁸⁷ This possibly due to conjugated double bond and carbonyl group is essential to the activity toward MAOB inhibition.¹⁸⁷ All 10 compounds have intact part (A) of piperine molecule because changing part A of the piperidine molecule (MDP) leads to decrease in inhibition activity toward MAOB enzyme.¹⁸⁷ This result is similar to the finding in another study.¹⁵⁴

Docking studies using ADT and Autodock programs showed that the piperine binds in the same spot as it was seen in the literature. We found that piperine binds to several amino acids which are involved in the binding site of the MAOB enzyme including Ile199, Tyr326, Tyr435, and Tyr398. Tyr435 and Tyr398 is part of the aromatic cage, which is very important in facilitating the deamination process of MAOB enzyme. The aromatic cage faced isoalloxazine part of FAD, which is essential in the deamination process of MAOB enzyme. Piperine binding site is located at the substrate cavity of MAOB enzyme because it binds to aromatic cage, which located near the substrate cavity of MAOB enzyme.

We found that piperine passed through amino acid, Phe168 that is part of gating residue, which separates the substrate and entrance cavity. Ile199 and Tyr326 is part of gating residue that separates the entrance cavity and the substrate cavity. Therefore, binding piperine to the amino acids of the aromatic cage and passing amino acids of gating residue leads to the conclusion that piperine binding was in the substrate cavity of the MAOB enzyme, therefore it inhibits the activity of MAOB. This binding configuration to substrate cavity of MAOB is similar to what it has been found in the literature. Compounds **1,2,3,4**, and **5** bind to the same amino acids, which include aromatic cage amino acids and they passed through amino acids of the gating residue that separates the entrance cavity and the substrate cavity. In addition, those compounds bind to FAD, which has role of oxidative deamination process and catabolism of the ligand. Therefore, this group of compounds bind to the substrate cavity and has the ability to inhibit MAOB enzyme in a binding pattern is similar to piperine.

Compound **6** binding pattern is not similar to piperine because Cyc172 and Tyr188 binding may limit the number of conformations that the compound shows when binds with MAOB enzyme. This binding is different as compared with piperine.

Compound **7** binding pattern is not similar to piperine because the location of the ligand is far from the aromatic cage and the hydrogen binding with Gln206 leads to limit the number of conformations in binding to MAOB enzyme. Compound **8** binding pattern is not similar to piperine because its binding with .Tyr188 amino acids decreases the number of binding conformations with MAOB enzyme. Compound **9** binding pattern is not similar to piperine because the binding of Leu171 and Tyr435 amino acids limit the number of binding conformations with MAOB.

When binding energy decreases, the possibility of the docking increases. In other words, when the value of free energy is high (in the negative side), the docking of the ligand to the macromolecule is more likely to happen. When the free energy is low (positive side) the possibility of the ligand docks on the macromolecule is low. Piperine has binding energy value of (-8.78). Other compounds (**2**, **4**) have shown binding energy (-9.12, -8.68) respectively which are considered similar to piperine taking in the consideration their binding patterns and their values of IC₅₀ of MAOB enzyme inhibition. This concludes that compound **2** and **4** have similar binding energy to piperine, so they can inhibit MAOB enzyme activity as piperine. Despite the fact that the remaining compounds have acceptable binding energy, their binding pattern and IC₅₀ values are unacceptable when it compared to piperine. When the IC₅₀ increased, the

selectivity toward MAOB enzyme decreased, and this leads to increase in side effects such as cheeses effect.

Inhibition constant (K_i) value represents the affinity of a ligand to a macromolecule. K_i value for the piperine is (365.89) and (205.22, 430.81) for compound **2** and compound **4** respectively, therefore, the affinity of compound **4** and is higher than piperine. Taking into consideration the values of IC_{50} in MAOB enzyme assay for compound (**2**, **4**), compound **4** has more stable binding to MAOB enzyme and it has more affinity to MAOB enzyme than compound **2** and piperine. Despite the fact that compound piperine has slightly higher binding energy value than compound **4**, compound **4** has more affinity than piperine, therefore, compound **4** is better than piperine.

BSA assay was performed in our lab in order to determine intensity of binding of piperine and our (9) compounds to BSA. The IC_{50} for BSA reflects the concentration of free drug that is able to pass the BBB in brain. When the IC_{50} of a compound in BSA assay approximately equal the IC_{50} of MAO inhibition assay, it means that concentration of the free drug that is available to pass the BBB is enough to reach the 50 % inhibition level of the MAO enzyme. High throughput screening of BSA with test compounds showed that IC_{50} values range from (8337-1.1) μM . When the concentration of IC_{50} increases, it means that the amount of free ligand is high, therefore, the ligand is available to interact with biological tissue through the blood stream. Low IC_{50} values means that low amount of the free ligand would available to interact with the body; therefore, the dose of the ligand should be increased in order to reach the desirable concentration inside

the body. The increase in the dose means that high amount of the drug is available which possibly causes many side effects because the ligand starts losing selectivity. Compound **2** has BSA ($IC_{50}=144\mu M$) which means that this amount of the ligand is free from BSA and it's available to pass through BBB taking into consideration that the compound at less concentration than $144\mu M$ has the ability to inhibit 50% of MAOB enzyme inside the brain($MAOB IC_{50}=0.666\mu M$). Compound **4** has BSA($IC_{50}=16\mu M$) which means that the amount of the ligand that is free from BSA binding is enough to inhibit MAOB enzyme by 50% because MAOB ($IC_{50}=0.497\mu M$) taking into consideration that the compound might pass BBB. Because Piperine BSA ($IC_{50}=8.9\mu M$), amount of free ligand is enough to pass BBB and inhibit 50% of MAOB enzyme because ($IC_{50}=0.483$) of MAOB assay.

In Fig.(30,31, 34), piperine, compound **2** and compound **4** binding to BSA curve showed good correlation between BSA and those compounds, therefore, fluoresce of BSA decrease when the concentration of the piperine increases. In addition, the curve of compounds (**2**, **4**) are similar to piperine, therefore, they may binds to BSA in a similar fashion. The binding curve of compounds (**2**, **4**) are smooth and sigmoid in shape which means that when the concentration of the drug increase, the concentration of the BSA decrease and the fluorescence of BSA decreases along with its concentration. In Fig.(34),compound **9** showed that the binding curve of BSA with the ligand is flat, therefore the ligand cannot bind with BSA even when the concentration of the ligand increased. This leads to the fact that the ligand will never reach the BBB because it cannot bind to the carrier, which is BSA. Other compounds showed good correlation

between BSA and the ligand. However, their MAOB enzyme IC₅₀ corresponding values are high and those test compounds are not able to inhibit 50% of MAOB enzyme activity in low acceptable concentration. The binding curve of compounds **2,3,5** and **6** showed their curves are above 100% fluorescence, and this is because the compounds might have auto-fluorescence properties which result in the total fluorescence was above 100% of the curve.

Based on docking studies, we found that piperine and compound **4** binds to the same 10 amino acids in the binding site (AII) of BSA, therefore, the binding pattern of piperine and compound **4** is similar to each other and their IC₅₀ values of BSA experiment is similar to each other. Compound **2** binds to different amino acids and shares only 8 amino acids that are similar to that of piperine binding to BSA, which reflects the difference in the IC₅₀ values of BSA as compared to IC₅₀ value of piperine. Docking of compound **2** with BSA (AIII) is not similar to that of piperine because it binds to only 6 amino acids shared with piperine, therefore, IC₅₀ values of compound **2** is not the same as piperine. Docking of compound **4** with BSA (AIII) showed that it binds the same 8 amino acids that piperine binds. Compound **4** has better binding pattern to BSA then compound **2** when comparing to piperine because compound **4** binds to 10 and 8 amino acids in site (AII) and site (AIII) respectively, while compound **2** binds to only 8 and 6 amino acids in site (AII) and site (AIII) respectively. Therefore, compound **4** has similar binding pattern with BSA in (AII) and (AIII) sites, because it binds to more shared amino acids in site (AII) and site (AIII). This lead to the conclusion that the difference in the IC₅₀ values with BSA between compound **4** and piperine is small.

We used PAMPA method in order to test the ability of test compound to pass BBB. Piperine showed that it has a LogPe value of (-3.87) which put it in high permeability category because the LogPe is lower than (-6). Compound **2** showed that it has high permeability because it has LogPe value equals to (-3.2) which is within the range of the compounds that have high permeability. Compound **4** showed that it has LogPe values of (-3.77), therefore, it is classified as high permeability compound. Other compound showed that they have LogPe values in the high permeability range, which allows the compounds to pass BBB. However, their IC₅₀ values in MAOB enzyme experiment are high and certain BSA IC₅₀ values are unacceptable.

Taking into consideration IC₅₀ values of MAOB enzyme assay, binding energy, Ki values, BSA and PAMPA assay, compound **4** has better profile than compound **2** and other test compounds because compound **4** has better IC₅₀ values, Ki value, and IC₅₀ values of BSA experiment and it can pass BBB at acceptable concentration when compared to compound **2**. Compound **4** has good profile and it can be used as a reference compound in future experiments, or it can be used in drug design as MAOB enzyme inhibitor.

Chapter V: Conclusion

Piperine, which is the active ingredient of a black pepper has many therapeutic advantages such as neuro-protection and anti-depressant. Piperine has inhibitory effect of MAOB enzyme, which plays an important role in PD treatment. We found a compound **4** has acceptable IC_{50} concentration, and it has the ability to inhibit MAOB enzyme activity and passed through a membrane that is similar to BBB with an acceptable concentration. As a conclusion, this compound could be beneficial in designing future potential ligands that are selective to MAOB enzyme. The next step is to test this compound in vivo to measure and determine its activity.

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