FEBRUARY 2023 M.Sc. in Biochemistry Science and Technology SAFAA BASSOUT

REPUBLIC OF TÜRKİYE

GAZİANTEP UNIVERSITY

GRADUATE SCHOOL OF NATURAL & APPLIED SCIENCES

THE USE OF CUCURBITACIN I IN THE INHIBITION OF ENZYMES IN THE INFLAMMATORY MECHANISM THAT CAUSES THE ACCUMULATION OF TAU FIBRILS IN AN ALZHEIMER ANIMAL MODEL, AND THE INVESTIGATION OF ITS EFFECT ON MEMORY ACQUISITION BY MEASURING THE EXPRESSION OF THE RELEVANT GENES

M.Sc. THESIS

IN

BIOCHEMISTRY SCIENCE AND TECHNOLOGY

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February 2023

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ABSTRACT

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M.Sc. in Biochemistry Science and Technology
Supervisor: Assoc. Prof. Şenay GÖRÜCÜ YILMAZ
February 2023
68 pages

Alzheimer's disease (AD) is a progressive neurodegenerative disease. Phytochemicals have potential effective in the treatment of neurological diseases. Neurofibrils cause neuronal losses and contribution to the inflammation process in AD. In this thesis, the effect of cucurbitacin I (CuI) on hippocampal TAU accumulation, *TDO2,* and *Nf-κB* genes expressions and gain of cognitive functions were investigated in an okadaic acid induced AD rat model. 36 *Sprague Dawley* female rats (200-250 g) were divided into 6 groups (6 animals in each group). Groups; Control, artificial cerebrospinal fluid (aCSF), stereotaxic, stereotaxic+aCSF, stereotaxic+aCSF+CuI (2mg/kg), stereotaxic+aCSF+CuI (4mg/kg). Morris water maze (MWM) and the elevated plus maze test (EPM) were used for cognitive and anxiety analysis. Detection of hippocampal deposition of TAU fibrils was performed by immunohistochemical analysis. Expressions of two genes were analyzed by quantitative real-time PCR (q-PCR) and protein expressions by western blotting. One-way ANOVA was used for statistical analyses. The results suggest that CuI may have therapeutic potential in the hippocampal reduction of TAU with the contribution of *TDO2* and *NF-κB* genes and acquisition of cognitive functions.

Key Words: Alzheimer’s Disease, Cucurbitacin I, NF-κB, Phytochemicals.

ÖZET

CUCURBITACIN I'İN ALZHEIMER HAYVAN MODELİNDE TAU FİBRİL BİRİKİMİNE NEDEN OLAN ENZİMLERİN ENGELLENMESİNDE KULLANILMASI VE İLGİLİ GENLERİN EKSPRESİNİN ÖLÇÜLMESİYLE HAFIZA EDİNİMİ ÜZERİNDEKİ ETKİSİNİN İNCELENMESİ

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Yüksek Lisans Tezi, Biyokimya Bilimi ve Teknolojisi
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Şubat 2023
68 sayfa

Alzheimer hastalığı (AD), ilerleyici bir nörodejeneratif hastalıktır. Fitokimyasallar nörolojik hastalıkların tedavisinde etkili potansiyele sahiptir. Nörofibriller, AD'de nöronal kayıplara ve inflamasyon sürecine katkıda bulunur. Bu tezde, okadaik asit indklü AD sıçan modelinde hipokampal TAU birikimi, *TDO2* ve *Nf-κB* gen ekspresyonları ve bilişsel işlevlerin kazanımı üzerine cucurbitacin I (CuI)'nin etkisi araştırıldı. 36 adet Sprague Dawley dişi sıçan (200-250 g) 6 gruba ayrıldı (her grupta 6 hayvan). Gruplar; Kontrol, yapay beyin omurilik sıvısı (aCSF), stereotaksik, stereotaksik+aCSF, stereotaksik+aCSF+CuI (2mg/kg), stereotaksik+aCSF+CuI (4mg/kg). Bilişsel ve kaygı analizi için Morris su labirenti (MWM) ve yükseltilmiş artı labirent testi (EPM) kullanıldı. TAU fibrillerinin hipokampal birikiminin tespiti, immünohistokimyasal analiz ile yapıldı. İki genin ifadeleri kantitatif gerçek zamanlı PCR (q-PCR) ile ve protein ifadeleri western blotlama ile analiz edildi. İstatistiksel analizler için tek yönlü ANOVA kullanıldı. Sonuçlar, CuI'nin, *TDO2* ve *NF-κB* genlerinin katkısı ve bilişsel işlevlerin kazanılması ile TAU'nun hipokampal azalmasında terapötik potansiyele sahip olabileceğini düşündürmektedir.

Anahtar Kelimeler: Alzheimer's Disease, Cucurbitacin I, NF-κB, Phytochemical

"Dedicated to my family"

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Assoc. Prof. Dr. Şenay GÖRÜCÜ YILMAZ for his guidance and support throughout the study. I am thankful for his encouragement and motivation.

I would like to express my love and gratitude to my family for their support, always best wishes.

This thesis is supported by Gaziantep University, through project Scientific Research Projects Unit (Project number**: SBF.YLT.21.03**)

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LIST OF SYMBOLS

α	Alfa
β	Beta
Δ	Delta
μ	Mikro
κ	Kappa
γ	Gamma

LIST OF ABBREVATIONS

AD	Alzheimer's disease
ICV	Intracerebroventricularly
CuI	Cucurbitacin I
aCSF	artificial Cerebrospinal Fluid
OKA	Ocadaic acid
EOAD	Early-Onset Alzheimer's disease
Aβ	beta- amyloid
AHA	Arachidonic acid
PGs	Prostaglandins
PSEN1	Presenilin genes 1
PSEN2	Presenilin genes 2
APP	Amyloid Precursor Protein
LOAD	Late-Onset AD
APOE	Apolipoprotein E gene
DS	Down's syndrome
CAA	Congophilic Amyloid Angiopathy
CSF	Cerebrospinal Fluid
NINCDS-ADRDA	National Institute of Neurological and Communicative Disorders and Stroke
MMSE	Mini-Mental State Examination
BBB	Blood-brain barrier
PC	Prostate Cancer
PC12	Pheochromocytoma
EPM	Elevated plus maze test
STAT3	Signal Transducers and Activators of Transcription-3 inhibitory activity
CGC	Cucurbitacin Glucoside Combination

SAR	Structure-activity relationship
PBS	Phosphate Buffer Saline
Bsa	Bovine Serum Albumin

CHAPTER I

INTRODUCTION

1.1 Motivation of study

The most common type of dementia, Alzheimer's disease (AD), is largely defined by a neurobehavioral defect that becomes more severe over time (Karantzoulis & Galvin, 2011). Neuropathologically, the two main mechanisms of the disease are the development of extracellular plaques of amyloid-type AD and intracellular neurofibrillary tangles (NFT) (Dong, Duan, Hu, & Zhao, 2012). Since the onset of AD, there have been multiple fibrous lesions arising in the limbic and cerebral cortices by intra-neuronal and extracellular pathways, and perhaps an insidious progressive memory loss, cognition, and behavioral instability in the elderly individuals (Karpas & Disease, 1907). The unstable 20-nm cytoplasmic fibers bundles in the form of paired helical, have been discovered in neurons as well as axons, dendrites, and neuronal cell bodies (including neurofibrillary tangles) (Selkoe, 1996).

Aging, genetics, brain traumas, diseases of the vascular system, infections, and environmental factors are just a few AD risk factors, which is thought to be multifaceted illness. Several conceptions have been put forth to interpret the underlying causes of pathological changes (Aβ, NFTs, and synaptic loss) in AD. However, most of the theories confirmed that either cholinergic dysfunction or changes in the production and processing of the protein named amyloid are the prime initiating factors for AD disease development (Breijyeh & Karaman, 2020; Querfurth, 2010). The studies' primary point is to limit and dismantle AD (Schelle et al., 2019).

Diagnosis may be delayed due to difficult early diagnosis symptoms and slow progression. In this thesis, we focused on cellular TAU to prevent or minimize TAU-induced neuronal losses. Compared to TAU-induced neuronal losses, Aβ-induced neural conduction blockade is a more pressing issue. The accumulation of TAU signals the onset of cognitive decline. (Wood, 2019).

Considering that TAU also exhibits the proliferation of neurons in the normal aging brain, it now causes a threat to AD. We think that phytochemicals are advantageous to bring TAU to its normal levels. Plant-derived phytochemicals are a new field being investigated as an alternative to the use of chemicals. They can be a treatment option with the specific targets and appropriate analysis.

We hypothesized that Cucurbitacin I (CuI) contained in the plant *Ecballium elaterium* would be a TAU fibril target in AD. CuI can be a solution in the treatment of AD by managing the inflammation mechanism (Arel-Dubeau et al., 2014). There is limited research on brain disorders such as AD in living individuals. At this point, the best source for mimicking the disease is animal models. Therefore, we constructed a okadaic acid induced AD model to examine the effect of CuI on molecular and cognitive processes. Insight into the expression of CuI target proteins and prediction of therapeutic use can be obtained by analyzing important genes in molecular pathways. The Morris Water Maze and Elevated T-tests, which are part of cognitive assessments, are effective methods of detecting TAU-induced cognitive impairment in CuI-treated AD animals. CuI is reported to have neuroprotective properties. Since this molecule can easily cross the blood-brain barrier without a carrier or electrical stimulus, it will be able to demonstrate its effectiveness at a high level. The treatment regime of CuI will be revealed by demonstrating hippocampal TAU status in histological analyses. The effect of CuI on AD in all its manifestations will thus be demonstrated and the efficacy of the treatment clarified.

1.2 Alzheimer's Disease

AD markedly affects the brain, along with a sustained decline in a person's memory and cognitive abilities, as well as a decrease in their ability to maintain basic functions (Schneider, 2020). Most individuals begin to have late-onset type symptoms (LOAD) around their mid-60s (Dubois et al., 2021). Detection of the onset of AD is extremely rare and typically occurs between the ages of 30 and 60 (Andrews et al., 2020). Among the elderly, AD is one of the common causes of dementia (Tiwari et al., 2019). Alois Alzheimer, who first described Alzheimer's, is honored with the disease's name. In 1906, a woman passed due to an old mental disease, underwent the examination of the brain tissues and he discovered anomalies throughout his research (Schneider, 2020). According to Andrews et al. (2020), some of the symptoms of AD include memory

loss, language problems, aggression, hallucinations, delusions, and sun downing. He looked at post-mortem brain tissue and found clusters called Aβ and neurofibrillary filaments called TAU.

With the development of technology, we now recognize that another feature of AD is the loss of neuromuscular connections (Arel-Dubeau et al.) in the brain. Neurons communicate with and from the brain to the muscles and organs of the body (Liu et al., 2019). Various other complex brain abnormalities involving neurons are thought to be associated with Alzheimer's disease (Schneider, 2020). The hippocampus and entorhinal cortex are the two main brain regions that are damaged in AD. The cerebral cortex is the behind. Language, reason, and social interaction are very important aspects of this field. In the later stages, more areas of the brain are also compromised (Dubois et al., 2021).

1.2.1 Causes

AD is an important public health issue and there is still much we do not understand about it. Amyloid and tau proteins in the brain, which accumulate over the years and eventually cause memory and thought loss, are believed to be the primary cause of AD's neuronal deterioration (Tiwari, Atluri, Kaushik, Yndart, & Nair, 2019). Impairment of the immune system, exposure to toxins, and inflammation are factors that contribute to the onset of AD. Since women are more likely to get AD than males, hormonal changes are likely to affect how the disease develops. Alzheimer's disease is often caused by a combination of aging, genetic, environmental, and lifestyle factors. The exception to this rule is early-onset AD (EOAD).

1.2.2 Disease etiology

The factor attributed to dementia is the deposition of beta-amyloid (A beta) (Cummings & Cotman, 1995; Näslund et al., 2000), consisting of plaques and fibrils, in the extracellular space of the brain in AD. Aggregation of TAU fibrils accumulates in the brain and results in intracellular neurofibrillary tangles (Dalton, Krishnan, Parker, Catanese, & Hooker, 2020). However, increasing data suggest that neuroinflammation cascades play a crucial role in AD pathogenesis and progression (Meraz R, et al., 2013). Inflammation is still hard to ascertain as to whether it is a cause, contributor, or outcome of the condition. However, scientists studying the function of neuroinflammation in AD have discovered that intraneuronal amyloid-

beta(A) storage stimulates the production of pro-inflammatory genes (McManus, Heneka, & therapy, 2017; Wyss-Coray & Rogers, 2012), resulting in high amounts of eicosanoids, arachidonic acid (AHA), as well as cytokines and prostaglandin (PGs) factors as AHA acid derivatives (Currais, Fischer, Maher, & Schubert, 2017; Welikovitch et al., 2020). It appeared to have a neuroprotective effect at first, but could then trigger additional neuron degradation in a dose-dependent manner (Glass, Saijo, Winner, Marchetto, & Gage, 2010). Neuro-inflammation, which can facilitate the development of tau fibrils and their clusters, is ultimately observed in dystrophic neurites (Lathe, Sapronova, & Kotelevtsev, 2014). However, the maximum number of patients acquire signs and symptoms later in life at the age of 65, termed old-age onset of AD, with 1% to 2% of people developing symptoms early in life (presenile or early-onset AD). The genes responsible for the disease, the amyloid precursor protein gene (*APP*) and the presenilin genes 1 and 2 (*PSEN1* and *PSEN2*) have been found to have highly penetrating modifications (Cruts & vanBroeckhoven, 1998). Another important contributing factor, the apolipoprotein E gene (*APOE*), was also discovered in late-onset AD families (Farrer et al., 1997).

1.2.3 Amyloid precursor protein gene (APP)

The first gene identified in autosomal dominant early-onset AD families was *APP*, which appeared to be located on chromosome 21 (Goate et al., 1991). The inspection of AD pathology in patients with Down's syndrome (DS) (Wisniewski, Wisniewski, Wen, & Society, 1985), the separation and succession of Ab peptide in senile plaques (Masters et al., 1985), and the placement of its precursor gene *APP* (Kang et al., 1987) to the AD-linked chromosomal band on 21 chromosomes were all crucial (P. St George-Hyslop et al., 1990; St George-Hyslop et al., 1987).

The findings of correlation with APP (Van Broeckhoven et al., 1990) and a variant mutation in the sequence of Aβ in congenital cerebral hemorrhages with the amyloidosis-Dutch type (HCHWA-D) triggered *APP* mutation research in AD (Frangione & Ghiso, 2006). The HCHWA-D is disrupted by the small cerebral blood vessels which is the main lead of the re-occurring cerebral hemorrhages as it is caused by congophilic amyloid angiopathy (CAA).

1.2.4 Presenilin 1 and 2 genes (PSEN1 and PSEN2)

Previous studies have shown that a genetic abnormality on the 21 chromosome (P. H. St George-Hyslop et al., 1990) could not explain all AD families, whereas mutational studies have revealed that most AD families do not accumulate an APP alteration (Schellenberg et al., 1991; Tanzi et al., 1987; Van Duijn et al., 1991). In conclusion, the inherited heterogeneity of familial AD was also studied and a strong association was found across multiple AD families, suggesting that chromosome 14 (14q24.3) is a prominent EOAD locus. Positional cloning eventually found the chromosome 14 gene called *PSEN1* (Sherrington et al., 1995) after missense mutations were discovered in numerous related origins (Clark et al., 1995; Cruts et al., 1995). Genome-wide research found a connection to chromosome 1q3142 in the extensive Volga-German AD family (Bird et al., 1989; Levy-Lahad, Wijsman, et al., 1995). Following the reveal of PSEN1, homology mapping revealed the presence of a PSEN2, in the linked region (Levy-Lahad, Wasco, et al., 1995; Rogaev et al., 1995) where a missense mutation (N141l) was found to be associated with AD in seven Volga-German kindreds. In an Italian family, an M239V (second missense mutation) in *PSEN2* was also discovered (Rogaev et al., 1995). Mutations in *PSEN2* was found in eighteen families so far, while one hundred and 164 mutant *PSEN1* mutations have been found in 361 families (Cruts & Van Broeckhoven, 1998). AD Mutation Database stated that the estimated overall modification abundance of *PSEN1* and *PSEN2* be 6% and 1%, respectively, in an epidemiological sample of early-onset AD (Cruts et al., 1998).

Most *PSEN* mutations result in commonly occurring AD that is pathologically and clinically identical to sporadic AD, unlike early-onset and intense and indeterminate disease development. However, PSEN mutations have sometimes been associated with AD in adults over 65 (Cruts et al., 1998) (AD Mutation Database). Two more *PSEN2* mutations (V148I and Q228L) were highlighted in patients with an onset of 65 years (Rogaev et al., 1995; Żekanowski et al., 2003) Some *PSEN2* N141l carriers in the Volga-German kinship exhibited late-onset AD (LOAD) (Levy-Lahad, Wasco, et al., 1995; Rogaev et al., 1995) Furthermore, patients with LOAD tend to have *PSEN1* polymorphisms A79V and R269H, which are peer-to-peer interactions with EOAD (Kauwe et al., 2007; Larner, Ray, & Doran, 2007). The three AD patients were determined in Belgian who was diagnosed with the Missense mutations of *PSEN1*

(C263F) and *PSEN2* (R62C and R71W) especially at the onset age 65 years (Brouwers et al., 2006).

1.2.5 Novel locus on chromosome 7

The explanation of variation in the APP and PSEN genes has made an important contribution to the congenital etiology of familial AD. However, the genetic origin of familial AD is largely unknown (Daw et al., 2000). The disease is transmitted predominantly autosomal in families of early-onset or late-onset AD (Rademakers et al., 2005). A large genome scan of a Dutch pedigree with a mean age of onset of 66.8 (range 4777 years) discovered a new region on chromosome 7q36 (C. Van Duijn et al., 1994). The responsible gene was not definitively identified after mutation analysis of all 29 known genes in the 5.44 Mb linked domain.

1.2.6 Apolipoprotein E gene (APOE)

In LOAD families, a genome-wide linkage investigation has discovered a new gene on chromosome 19 (19q13.119q13.3) (Pericak-Vance et al., 1991). Apolipoprotein E (APOE) was discovered to react via the cerebrospinal fluid at approximately the same time as the Aβ peptide found in CSF, and the gene (*APOE*) was discovered near the linked domain of chromosome 19 (Strittmatter, Saunders, et al., 1993). In addition, APOE has been associated with senile plaques and tangles of neurofibrillary structures (Namba & Ikeda, 1991), and APOE transcription is upregulated in AD patients' brains. Following this, patients suffering from the LOAD had an excessive accumulation of APOE4, one of the isoforms of APOE, as compared with elderly healthy controls. The association of APOE4 has been widely confirmed in patients with EOAD (C. M. van Duijn et al., 1994) and LOAD from a variety of familial and sporadic ethnicities (E. H. Corder et al., 1993; Farrer et al., 1997; Namba & Ikeda, 1991).

APOE 4 increases the risk between heterozygotes and homozygotes by 3-fold and 1-fold, respectively, through lower age of onset, mostly in a dose-dependent manner (E. H. Corder et al., 1993). Still, the other isoform called O2 has been shown to produce a protective effect, although not consistently (E. Corder et al., 1994). It has been shown for APP mutations that in carriers of causative AD mutations, the APOE genotype also affects the age of onset (Hardy et al., 1993; Sorbi et al., 1995) as well as many PSEN2 mutations (Wijsman et al., 2005). No effects were detected for PSEN1 mutations when

compared to a large Colombian PSEN1 pedigree (Christine Van Broeckhoven et al., 1994). All primary protein isoforms, as well as four promoter polymorphisms that affect the expression level of APOE, have been associated with an increased risk of Alzheimer's disease in several studies (Bullido et al., 1998; Laws, Hone, Gandy, & Martins, 2003). Although only one mutation (491A/T) seems to have an APOE O4-independent effect, this finding is interesting because APOE O4 greatly increases the risk and effect of AD relative to the dose given to such patients.

In vitro studies show that the O4 isoform of APOE has a high affinity for binding with the binding Aβ peptides, compared to the affinity of the APOE O3 isoform (Strittmatter, Weisgraber, et al., 1993). There is also a clear link between having an APOE O4 allele and increased Ab abundance in the brains of Alzheimer's patients (Rebeck, Reiter, Strickland, & Hyman, 1993; Schmechel et al., 1993), implying that APOE reacts with Ab to increase plaque formation. The fact that APOE (APOE/+) mice are homozygous knockout of Alzheimer's genetics update 571, with a less and more extensive accumulation of non-fibrillary Ab supports this theory (Bales et al., 1997).

Some, but not all, studies have found that the O4 isoform causes enhanced Aβ aggregation in vitro when different APOE isoforms have been tested for their effects on Ab fibrillation (LaDu et al., 1994; Ma, Yee, Brewer, Das, & Potter, 1994). In vivo studies in Apoe/ mice also showed that O4-APOE increased fibrillation of Ab and plaque formation instead of O3-APOE (Holtzman et al., 2000; Holtzman et al., 1999). Still, it's reasonable for the APOE to have alternative courses of action. For example, APOE is an important cholesterol transporter, and high cholesterol levels have been associated with higher Aβ load among animal models (Sparks et al., 1994) and changes in APP processing. In conclusion, changes in cholesterol binding and transport in the brain caused by APOE isoforms may affect plaque formation in the brains of AD patients.

The most important mutation in the C-terminal region of the Aβ sequence (London mutation) and double mutation (Aβ sequence in the N-terminus, APP KM670/671NL named Swedish mutation) were analyzed. The next Flemish APP mutation (A692G), which is close to the APP mutation, called the Dutch mutation and is dependent on AD and CAA, was identified within the Aβ sequence.

1.2.7 Other Factor

There is evidence that vitamins associated with alcohol, lipids, oxidative stress, and homocysteine play a role in the etiology of AD. A paper on vitamin E in the control of AD has been published, and several important epidemiological studies have examined the relationships between diet and AD. According to some studies, a low risk of AD has been associated with high use of vitamins C, E, B6, B12, and folate, as well as unsaturated fatty acids and fish, but the evidence is conflicting (Luchsinger and Mayeux, 2004).

Twin studies have shown 80% expected susceptibility to the important hereditary component of AD (Gatz et al., 2006). Age, illness history, male, lack of any physical illness, poor mental function, depression, and misidentification syndromes are thought to be associated with intentionally lower survival rates. Early death was predicted by apraxia rather than aphasia or dysmnesia (Burns, Lewis, Jacoby, & Levy, 1991).

1.2.8 Signs and symptoms

Because of the progressive nature of the disease, AD presents with typical symptoms as it develops, gradually worsening over time. The most common things like accessories, names, or asking multiple questions are first forgotten, then the memory is fully formed (Vaz & Silvestre, 2020). As a result of the death of essential brain cells, patients may face problems such as the inability to remember, communicate, process information, or perform routine daily activities, as well as emotional and behavioral problems (Dubois et al., 2021). For both patients and caregivers, learning about AD in its early stages can be a life-changing, distressing and stressful event (Tiwari et al., 2019). Although there is no known cure for AD, you still can fight it. However, it is necessary to understand that memory loss is not a symptom of Alzheimer's or other types of dementia. It is necessary to distinguish between normal procedures and signs of progressive disease with signs of degenerative aging (Schneider, 2020).

1.2.9 Prevalence

According to the fact and figures of the Alzheimer's Association in 2021, in 2050, this disease will jump from 6 million to 13 million in America, while the cost to the country due to Alzheimer's and other dementias is up to 355 billion dollars, these expenditures may rise to 1.1 trillion dollars. It is recognized that these caregivers provided 15.3

billion hours of care in 2020, worth approximately $257 billion. According to a European population-based study, the prevalence of AD is 5% in people aged 65 and over, and rises to 22% in people aged 95 and over (Lobo et al., 2000). According to studies, the incidence of the disease in Istanbul is comparable to that in Turkey and European countries (Gurvit et al., 2008).

1.2.10 Frequency

AD International commissioned an international committee of professionals in 2005 and provided a consensus on the prevalence and estimated incidence of dementia in 14 WHO regions based on epidemiological data collected from previous years. It is estimated that 24.2 million people suffer from dementia once in their lifetime, while approximately 4.6 million new patients are diagnosed each year (Ferri et al., 2005).

1.2.11 Criteria for diagnosis

Representatives from the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS-ADRDA) developed a unified set of criteria in 1984 to assist practitioners and researchers with specific emphasis judgments. Medical history, clinical examination, cognitive testing, and laboratory analysis were all components of the method (G. McKhann et al., 1984). The suggested criteria remain valid for thirty years (Galasko et al., 1994). The NINCDS-ADRDA criteria were modified only recently (G. M. McKhann et al., 2011). Revisions were much needed to make significant progress in neuropsychological screening, neuroimaging, and the neuropathological, biochemical, and genetic understanding of this disease. The phenotype of AD disease is more common in the population than originally anticipated. The Mini-Mental State Examination (MMSE) used the Cambridge Cognition Examination (CAMCOG), a rapid mini-mental test to assess the development of dementias such as AD (Burns et al., 1991; Galasko et al., 1990).

Depression and dementia are common consequences of aging. It is common in the normal elderly, but it is not clear whether depression is a risk for dementia. A study shows that mid- or late-age depression in AD and vascular AD is associated with the risk of developing dementia (Barnes et al., 2012).

1.2.12 The drugs in the management of AD

The various therapies that are recommended to treat AD include acetylcholinesterase, acetylcholinesterase, and memantine combination therapy. Antipsychotic agents and NSAIDs and vitamin E, statins and hormones (testosterone, estrogen, and insulin), and the following drugs are often prescribed. The mentioned groups are given below.

1.2.12.1 Acetylcholinesterase inhibitors

The acetylcholinesterase enzyme is inhibited and inactivated by inhibitors, which reversibly destroys memory-located acetylcholine. The drug in this group is Donepezil, which inhibits acetylcholinesterase centrally and non-competitively (Birks, 2006). The other component included in this group is Galantamine, which competitively inhibits acetylcholinesterase. The 3rd member of this category is Rivastigmine, which causes only partial blockade (N.-C. Li et al., 2010).

1.2.12.2 NMDA receptor antagonist

Memantine is a member of the glutamine receptor inhibitor (NMDA) receptor antagonist family (Porsteinsson, Grossberg, Mintzer, & Olin, 2008).

1.2.12.3 Monoamine oxidase type B inhibitor

Selegiline (Eldepryl) chemically has both monoamine oxidase type B inhibitor and minor anticholinergic properties (J. Birks & Flicker, 2003). Some antipsychotic agents such as olanzapine are included in the treatment of AD, although not validated by U.S. FDA (Wilcock, Ballard, Cooper, & Loft, 2008). Certain drugs such as vitamin E (Isaac, Quinn, & Tabet, 2008), NSAIDs (Feldman et al., 2010), insulin sensitizers (Sato et al., 2011), and statins are ineffective therapies while testosterone and ginkgo are conflicting therapeutics (Tan & Pu, 2003).

Despite the uncertain link between AD with inflammation, anti-inflammatory drugs have been shown to reduce AD complaints (Imbimbo, Solfrizzi, & Panza, 2010). AD is using inflammatory markers for the prevention, diagnosis, and treatment to achieve the target. As we know, some herbal remedies containing natural and active ingredients have anti-inflammatory qualities, including the ability to treat AD by influencing various molecular pathways of neuro-inflammation (Akram & Nawaz, 2017).

1.2.13 Role of the biological membranes

The lack of new therapeutics for AD disease is due solely to the blood-brain barrier (BBB). The important point is that there is no BBB drug delivery technology, so almost 98 percent of all small molecule therapies and not all biologic drugs pass the BBB. Second, it can be used in human studies. A Pubmed search of the literature reveals that BBB drug delivery techniques are significantly underdeveloped in October 2020, Pubmed has 9837 citations for the topic "AD disease pharmaceutical development". A Pubmed search for 'AD disease drug development and blood-brain barrier drug transplant' yields 191 citations, accounting for 1.9 percent of all results. More than 95% of all AD pharmaceutical development efforts target nanoparticles that have proven to be stressful to assign to human neurotherapeutics (Pardridge, 2005).

1.3 Potential beneficial plants

Widespread use of plants for different ailments is very common worldwide. Medicinal plants used in the treatment of AD are as follows; *Salvia officinalis*, *Melissa officinalis*, Chinese community favor *Ginkgo biloba* (Howes, Perry, Houghton, & Derivatives, 2003), and other plants in this section are Hypericum (*Hypericum perforatum*), Maca (*Lepidium meyenii*), *Prunella vulgaris*, *Cyperus rotundus*, *Zizyphus jujube, Lavandula officinalis*, Ginseng (Jia, Zhao, & Liang, 2009), *Panax notoginseng* (Ng, 2006), *Morinda citrifolia, Lycopodium serratum, P.tenuifolia,* and *C. paniculatus.*

1.3.1 *Ecballium elaterium*

We present one of the potential plants in Turkey, *Ecballium elaterium* containing CuI, as a candidate to reduce the symptoms related to AD. General information about the plant is given in Table 1.3.1 (Souilah, Amrouni, Bendif, Daoud, & Laredj, 2020).

Table 1.3.1. Details of plant *Ecballium elaterium*.

Plant	Details
Botanical Name	*Ecballium elaterium*
Family	Cucurbitaceae
Part used	Fruit, seed,
Location	Mediterranean region
Common name	
English	squirting cucumber
German	spritzgurke
French	concombre sauvage

Tunisia	Feggous elhemir
Turkish	Bitter Melon, Donkey cucumber

The coordinates where the plant can be collected are given in Figure 1.3.1.

Figure 1.3.1 *Ecballium elaterium*. Coordinates: 36°37'00.2"N 4°19'33.9"E/ 36.616718, 34.326084, Region: Alata, Erdemli, Mersin, Turkey.

1.3.2 Traditional uses

This herb has traditional use as a treatment for fever or flu in the form of an infusion, fruit mix, or aerosol form (Bizid et al., 2015) and has naturally been therapeutic in a variety of foodborne infections. The juice of this fruit type has long been used in mild to severe idiopathic jaundice, rhinosinusitis, and sinusitis (Salhab, 2013; Sargın, et al, 2013). The *Ecballium elaterium* is also traditionally used for its cathartic, diuretic, and antitumor activity. Other uses included are anti-inflammatory. The plant has potential in the treatment of AD as an antioxidant and by inhibiting the accumulation of TAU protein.

1.3.3 Phytochemistry

It is a perennial flowering plant native to the Mediterranean Basin, cultivated in central Europe and England. Hairy vines and hairy leaves are characteristic of this plant. The fruit is oval and fleshy, about 4 cm long (Rust, Vaissière, & Westrich, 2003). "De Materia Medica"2, which is considered the oldest scientifically documented treatise on botanical and pharmacological subjects, includes this plant (Caiozzi, Cabrera,

Mardónez, & Saldías, 2002). The secondary metabolites present in the fruit of *Ecballium elaterium* are elaterins (AR, 1995; Budavari & biologicals. Merck) and cucurbitacin (Bauer & Wagner, 1983) the effects of plants are due to the latter compound. cucurbitacin B is present in its seed (Favel et al., 1989; LE-NGUYEN, NALIS, CASTRO, & research, 1989).

Cucurbitacins are a class of triterpenoid, tetracyclic compounds it is famous for their bitterness and toxic nature. They are chemically identified as 19-(10→9β)-abeo-10α-lanost-5-ene (also known as 9β-methyl-19-nor lanosta-5-ene (Saboo, Thorat, Tapadiya, & Khadabadi, 2013). These are arbitrarily divided into twelve groups, including the cucurbitacins from A to T. The oxidative functions of some cucurbitacins vary in different locations (A, C, B, and D) (Yesilada, Tanaka, Sezik, & Tabata, 1988). It has been tried to increase the yield of CuB obtained from various structures of the plant by tissue culture for treatment performance (Yesilada et al., 1988).

1.3.4 Pharmacological activities

1.3.4.1 Remedy of acute rhino sinusitis

The juice from the fruit of *E. elaterium* has often been used by traditional people to treat rhino sinusitis because of its effective anti-inflammatory contents (Kloutsos et al., 2001; Sezik & Yeşilada, 1995; Yesilada et al., 1988). It is also used in Turkey by mixing with milk to relieve long-lasting headaches. (Patel, Lavasanifar, & Choi, 2009). The use of this juice has been linked to a variety of toxicity and allergic reactions (Kloutsos et al., 2001).

1.3.5 Antimicrobial activities

The antibacterial potential of the ethanolic extract of *E. elaterium* fruits was investigated in vitro against S. aureus, C. albicans, and S. aureus when used alone or in combination with penicillin. The presence of sub-inhibitory amounts increased the efficacy of the antibiotic against S. aureus. Most clinical strains of S. aureus are resistant to penicillin, and a large percentage of these strains are also methicillin-resistant. MIC values for antibiotics were used as a reference point to characterize interactions to analyze the effect of ethanolic extract and antibiotic combinations (Vacher, Pellegrin, Leblanc, Fourche, & Maugein, 1999). According to the

observations, the ethanolic extract of *E. elaterium* fruits is particularly beneficial in combating MRSA-related problems and may also be useful in treating C. Albicans-related ailments.

1.3.6 Immunomodulation

The cytotoxic amplification of lymphocytes against PC-3 (prostate cancer) and ZR-75-1 (Breast carcinoma) was evaluated. Both cell lines were less pronounced in the control than in the treated cultures. Cells treated with the PHA/CuI combination gave a better result. The effects of PHA and CuI were accompanied in PC-3 cells, whereas it was coadjuvant in ZR-75-1 cells. PC-3 cells were found to be resistant to lymphocyte-mediated cancer (Borsellino et al., 1999).

1.3.7 Hepatoprotection

The antihepatotoxic activity of the dry juice of the plant fruit and cuB (isolated from water) was investigated in carbon tetrachloride-induced hepatotoxicity. Pre- and post-treatment with Elaterium and CuB reduced the effect, as evidenced by a reduction in abnormally high SGPT levels and the degree of steatosis. Therefore, it has been reported that elaterium and CuB have both protective and curative effects (Agil et al., 1999).

1.3.8 Anti-inflammatory

In mice, the anti-inflammatory benefits of E. elaterium juice and CuB were tested against edema caused by serotonin and bradykinin. demonstrated by them that swelling was significantly inhibited proportionally to the dose (Yesilada, Tanaka, Tabata, & Sezik, 1989).

1.4 In-vivo Alzheimer's activity

Only 1% of these species have been evaluated as phytochemicals by scientists. This means that there is a lot of room for new bioactive chemicals to be discovered. Secondary metabolites, commonly known as phytochemicals, are substances whose full functions have not yet been revealed, as they are widely suspected to have an integral role in the defense systems of plants. These plant substances have been discovered to be effective anti-insect and anti-pathogenic microbe agents. They were

designed to achieve a self-defense process that could not be demonstrated through combinatorial synthesis.

Along with proteins, cucurbitacins (B, D, E, I, and L), glycosyl cucurbitacin, and lipids are also abundant in the plant (Greige-Gerges et al., 2007). In addition, fruit extracts continue to be used in many therapeutic systems in the Mediterranean region (Latté, 2009). *E. elaterium* contains several active ingredients, including compounds such as phenols, flavonoids, alkaloids, sterols, amino acids, vitamins, tocopherols, and fatty acids, which support their use in the food system, according to numerous recent studies (Abbassi et al., 2014; Touihri et al., 2015).

1.5 Animal models of AD

In terms of the challenges of resolving AD, interventional therapeutic options are limited due to the complex nature of the disease, therapeutic efficacy, and unknown targets. Finding the genetic factors that cause the production of AD (Reitz & Mayeux, 2014) may lead to the creation of effective tactics to combat this disease, opening up new treatment and prevention options (Kawas, 2003). In addition to pharmacological treatments, measures such as creating compounds that target proteins such as Tau and beta-amyloid-blocking enzymes involved in protein degradation and preventing protein aggregation can all help directly and effectively treat the condition. Techniques such as ApoE4 allele correction with viral vectors, CRISPR nanoparticles, and allelic deletion have been used with positive results in single-gene diseases. While the purposes and efficacy of viral vectors are hotly debated, target attainability (such as primer design and signal sequence sequencing) and good manual planning reduce the risk of default. Phytochemicals can be a great method as they can be controlled and their effects demonstrated by clear, quantitative measurements. Also, the biological resource to be investigated is very important. While cell culture studies produce reliable and realistic results, when the findings are applied to experimental animals, it may be even more spectacular to cycle the physiological response and make the treatment available as a human model. Herbal medicines, which have been used for thousands of years in countries that value traditional medicine such as China, Asia and Japan, aroused great interest in the scientific community. CuI, a triterpenoid found in *E. elaterium* (Bitter Melon, Donkey Cucumber, Cırtatan) plant grown in Turkey, has antioxidant properties and is generally used in the treatment of sinusitis by the public,

is formed as a result of Tau protein accumulation. This is the main cause of AD pathogenesis. We're just assuming it will help with deterrence. The anti-inflammatory and antioxidant properties of the plant are well known, and its leaf, flower, and fruit extracts are used in a wide variety of treatments (Bourebaba, Gilbert-López, Oukil, & Bedjou, 2020). Cucurbitacins, a class of triterpenoids found in all pumpkin species, are found in the plant. Cucurbitacins is a recognized tetracyclic triterpene in the Cucurbitaceae plant family. Bitter-tasting triterpenoids are thought to be designed as a countervailing mechanism against pests (Ríos, Escandell, & Recio, 2005) cancer (Jacquot et al., 2014), inflammation (Attard & Attard, 2008) with anti-oxidant (Pizzino et al., 2017) and antibacterial effects are among the pharmacological activities of these compounds. CuB is a putative target protein in PC12 (pheochromocytoma) cells in AD amyloid-beta pathology, and the GR / PLC / PKC and TrkA / Ras / Raf / ERK signaling pathways play critical roles in the neuroprotective effect of CuB (Jia et al., 2009). Based on this evidence, we speculate that the activity of this protein may be controlled by the anti-inflammatory, apoptotic, and RNS (reactive nitrogen species) aspects of AD, which aids in the tau protein pathogenesis. The mouse, which is the closest animal modeled to humans, has a large share in the research of diseases and has been preferred in physiological research for a very long time. While the rapid learning abilities of rats cause them to be preferred in behavioral studies, the similarity of physiology with humans and dimensions suitable for measurements are used as an advantage (Lindblad-Toh, 2004). However, the difficulty of understanding the physiological repercussions of genetic changes in mice compels researchers to conclude rat data. The ability to evaluate the animal's memory, cognition, and analytical ability in behavioral tests of models applied for AD is a sought-after feature. They can perform similar tasks as well as the large behavioral gap between rat and mouse (Deacon, 2006). Mice have a less complex background than rats and have less flexibility in adapting to new situations. This indicates that the mouse is less complex than the rat and presents difficulties in monitoring neurological behavior (Whishaw et al., 2001). According to the investigations mice make adaptive decisions in the distant future based on the information they have. While the ability to reflect on their own mental abilities (metacognition) unique to humans is observed in primates, the fact that mice have this capacity may put these animals in an advantageous position for some research (Kepecs et al., 2008). The advantage of the rat in neurological research is due to methods such as easily applicable stereotaxy or neurosurgery, histological and pathological

sampling, electrophysiological recording, and cerebrospinal fluid intake. Especially when Huntington, HIV, and immune system modeling is required, rats are organisms that can accurately represent the model, and more effective and reliable data can be obtained (Loeffler, 2004). Based on previous data, Sprague Dawley female rats, in a neuronal behavior model to keep an eye on tau neurofibrillary formation were selected in our study to the related mechanism of Alzheimer's in humans.

1.6 Rotarod test for motor functions

The act of coordination is linked to motor skills. The experiment on the behavior of animals was initially led by Colin Stewert (Baker, Lindsey, & Wesibroth, 2013). The events and the total turn are determined in the format starting from the simplest. In this setup, without focusing on the force, the animal's muscle tone and balance response to the rotarod at later times were measured (Jones & Roberts, 1968). The device consists of a rotating rod with 30-35 cm height, a tray with sensors to finish the assessment as the animals fall, and a screen. The driving time (seconds) and endurance parameters in the test give information about the level of motor activity. The test also provides an assessment of balance, grip and partial strength. It is especially preferred for testing the effects of traumatic brain injury, drugs or surgery

1.7 MWM test for cognitive functions

The most fundamental MWM technique is the place or spatial learning. The apparatus, which analyzes learning, including spatial and reference memory, showed that the animals could sequentially resolve the tank wall and analyze the platform distance. The inner surface of the tank has special colors and shapes, which play an important role in improving the spatial learning and memory evaluation of the animal. For this experiment, animals are allocated to a circular pool with a hidden platform. The platform helps the animal escape using various clues from the water. Different strategies were used to analyze factors that could affect the performance of rats, notably the ability to remember the location of the platform, the use of visible and preferential swimming (cm) cues to identify the platform, and swimming speed (cm/sec) all will be documented by an autonomous monitoring system (Vorhees & Williams, 2006).

1.8 EPM test for anxiety status

EPM testing plays an important role in assessing anxiety-related behavior in mice, which includes an innate fear of heights and a spontaneous natural curiosity about open spaces and new environments. The module includes vertically crossed open and closed arms in the middle and a central area. Mice have the option to move freely in all of their arms. The movements of the animals are recorded with a video camera. Indicators of confined space-induced anxiety in mice include frequency and latching time in open arms (Komada et al., 2008).

1.9 Aims and objective of the study

Five drugs have been approved by the U.S. Food and Drug Administration to temporarily relieve symptoms associated with mixed AD. The usefulness of these drugs varies by ethnicity. None of the current treatments affect the underlying etiology of this incurable disease (Alzheimer's & Dementia, 2012). There are no clear strategies for evaluating and treating AD as it is a brain disease. Because the problem is complex, no single treatment is possible. It is becoming more common in society and its gradual onset results in memory loss that remains hidden (Rogan, Lippa, & Dementias®, 2002). While gene therapies can help with a single gene, mutation, or polymorphism, their use in humans remains an open question. When chemicals enter the cell and cross the blood-brain barrier in terms of molecular structure, they can have cell growth, detrimental effects or the potential to cause problems. However, these are still viable treatment options. Phytotherapy, which has been used for centuries, is based on plant-derived phytochemicals. Humanity has accidentally cured many diseases by using it. There are many biomaterials. On the other hand, obtaining specific ligands is incredibly difficult. Using the molecular docking method to restrict these alternatives will save time and money.

Cucurbita may contribute to the suppression or slowing of tau protein development in an AD disease animal model, according to our concept. According to our concept, cucurbits may contribute to the suppression or slowing of tau protein development in an animal model of AD. CuI can be achieved through modulation of the target activity of TDO2 and NF-kB.

The goal of the study is given below;

i. Measuring whether the hippocampal tau protein is inhibited by evaluating the efficacy of CuI with different methods.

ii. Demonstration that CuI can control the expression change of *TDO2* and *NF-κB* in the inflammatory pathology of AD.

iii. Determining the effect of CuI on regaining lost memory in AD.

iv. To reveal the contribution of CuI in the correction of anxiety, one of the symptoms of AD.

CHAPTER II

LITERATURE REVIEW

Over time, man realized that nature has significant healing potential. People have figured out how to treat diseases with various herbs. They identified molecules found in naturally occurring compounds originating from plants that play important roles in human health and development. Along with their metabolites, these herbs are in the mainstream as a treatment option for various infections. About 80% of the population in poor countries prefers to use traditional health systems for their treatment (Grover & Yadav, 2004).

Parts of the plant such as seeds, roots, flowers, fruits, stems, and sometimes the whole plant are used for the isolation and separation of their active components. These isolated components exhibit a variety of activities such as anti-inflammatory, antioxidant, antibacterial, anti-Alzheimer's, anti-diabetic, analgesic, and anticancer agents. The first written record of the formulation was uncovered on a 5000-year-old Nagpur Sumerian clay slab of the use of medicinal plants. It had 12 crop regimens that repeatedly referred to over 250 selected species registered as alkaloids, including hashish, henbane, and mandrake (Kelly, 2009). The Chinese and Indian books are prominent representations of plant species (Petrovska, 2012).

Medicines are derived from natural sources. For example, aspirin is derived, an anti-inflammatory chemical (Kunwar, Bussmann, & ethnomedicine, 2008), curcumin (Lal et al., 1999; Satoskar, Shah, Shenoy, & toxicology, 1986; Srivastava, Dikshit, Srimal, & Dhawan, 1985) from *Curcuma longa*, termination (Lal et al., 2000), a tetra methoxy flavone from *Egletes viscose*, and anthocyanin flavonoids (Borissova, Valcheva, & Belcheva, 1994) from *Aronia melanocarpa* natural juice have been used as anti-inflammatory agents. It is traditionally used as a food infusion, fruit matrix, and even as an aerosol in cases of fever or illness. Although the drugs used in AD are not very

effective in the complete recovery of the disease, their symptomatic effects are tabulated. *Ecballium elaterium* plant has medicinal importance. Depending on the cultivation area, its components are cucurbitacins, flavonoids, polyphenols and coumarins. The presence of cucurbitacin in the plant draws attention and has a variety of uses. For this reason, research on plants are carried out to break new ground in the field of medicine.

Although interest in the potential anticancer therapy, cucurbitacin, has waned for decades, cucurbitacin's important role in STAT3 (Signal Transducers and Activators of Transducers and Activators of Transcription-3 inhibitory activity) and other signaling pathways, including the JAK pathway, as well as the MAPK pathway. In addition to the current findings showing that it regulates the STAT system, it is also known to be critical for the survival and spread of cancer cells. Due to these effects, it has attracted the attention of the pharmaceutical industry. Furthermore, findings from several studies suggest that cucurbitacins work synergistically with proven chemotherapy drugs such as doxorubicin and gemcitabine (Abbassi, Ayari, Mhamdi, & Toumi, 2014). CuI targets STAT3 signaling with reduced CSC-like properties and accelerated chemoradiotherapy sensitivity in CD133-positive NSCLC cells (Hsu et al., 2011). It is justified to recommend CuB as core chemotherapy before cancer onset and after a cancer diagnosis because of its intriguing effect against different tumor types through a wide range of molecular signaling pathways (S. Garg, S. C. Kaul, & R. J. I. j. o. o. Wadhwa, 2018). CucI affects aligned glioma tumor growth and survival by inhibiting targeting disruption of STAT3. Along the AMPK/mTOR/p70S6K pathway, cucurbitacin I induce protective autophagy and downregulates HIF-1. Glioblastoma is more amenable to CuI treatments when autophagy is limited. The pharmacological and anti-proliferative effects of CuI against glioblastoma are now well understood (Yuan et al., 2014). The free radical scavenger and antioxidant (cucurbitacin glucoside combination; CGC) abilities of cucurbitacin B + E glucoside were investigated using ESR spectroscopy. The capacity of CGC to convert already formed ABTS•+ to its original nature and inhibit MDA formation during linoleic acid oxidation, which was used to analyze its antioxidant activity, revealed the dose-dependent antioxidant effect in both ways, as would be expected from the antioxidants (Tannin-Spitz, Bergman, Grossman, & Communications, 2007). Structure-activity relationship (SAR) experiments with five analogues of cucurbitacin (Pizzino et al.) including A, B, E, I,

and Q—cause the identification of Cu Q, which prevents the activation of STAT3 rather than JAK2 activation while Cu A prevents the activation of JAK2 rather than STAT3 activation; and Cu B, E, and I prevent the activation of both. Moreover, these SAR experiments showed that the cucurbitacins' anti-JAK2 activity is lost when their C3 carbonyl is converted to a hydroxyl, while their anti-STAT3 activity is lost when their C11 carbonyl is added with a hydroxyl group (Sun et al., 2005). CuB, D, E, I, F, and R played an important role in the modulation of BACE.

With the help of the microarray, more than 140 genes are the main cause of migration and invasion of cells in a disease that is problematic to treat, such as malignant melanoma (Park et al., 2014). The disease-related up-regulation of LIMK1/2 was repressed with BMS-5 and CuI. Glioma cell viability diminished, cell adhesion improved, and migration and invasion declined after this treatment.

Lymph nodes, peripheral blood, and skin tumor cells are enriched in the hyperactive cutaneous CD4+ T-cell lymphoma Sézary illness (Sz). They applied western blotting to determine that both newly isolated tumor cells from Sz patients (n = 6) and the Sz-derived cell line Seax comprise the phosphorylated (P)-Stat3 protein. After an overnight in vitro culture was obtained without any exogenous cytokines (n = 3). P-Stat3 expression is reduced, confirming that Stat3 is not always activated. When the Jak/Stat3 inhibitor CuI was injected into the Seax cell line, P-Stat3 and Stat3 declined significantly in a time and concentration-dependent manner. Despite the absence of P-Stat3, cucurbitacin I reduced the expression of Stat3 in recently separated Sz cells (n=3) in a dosage method (Van Kester et al., 2008). However, the treatment included a combination of mouse and human fibroblast between CuC and glioma cells, it was not toxic to cells. Anti-stress effects and glial differentiation tests give positive results (S. Garg, S. C. Kaul, & R. Wadhwa, 2018).

In a scientific assessment, the relationships of CuB, E, and I with microtubules and actin filaments were explored for any negative consequences of cucurbitacins (Wang, Tanaka, Peixoto, & Wink, 2017). Hela and U20S cells were used to evaluate CuB, E, and I. Immunofluorescence staining, in vitro tubulin staining, and a polymerization test were used to examine green fluorescence protein (GFP). These three have an impact on cellular microtubule assembly and polymerization. Additionally, these three compounds reformed the mitotic spindle's shape and exhibited an arresting impact on

the G2/M phase during the cell cycle. By depolymerizing and clustering actin filaments, all three had an impact on the actin cytoskeleton.

CHAPTER III

MATERIAL AND METHOD

3.1 Materials

3.1.1 Chemicals and reagents

The chemicals and reagents used in the experimental process are given in Figure 3.1.1

Chemicals and Reagents	
Sodium Chloride (0.9%)	Bioflex, Turkey
Polymycin (10.000 I.U./30 mg)	Koçak Farma
Desefin (1g I.V.)	Deva
Batiqon	Biyosidal, Turkey
Polycarboxylate Cement	DEN.01.10304, Dentonics
Alfazyne	Alfason
Ketax	Vem
Okadaic acid	BioVision, 7M20L15430
aCSF	Tocris, 3525
Cucurbitacin I	Cayman, 14747
Formaldehyde	Merck, BCR546
Trizol	Tripure, ELK Biotechnology
Choloroform	Sigma, 24216-2-5L-R
Isoamyalcohol	Merck, 123-51-3
Ethyl alcohol	Merck, 493511
Methanol	Merck, 67-56-1
Bradford Assay	BioBasic, SK3031-1000, Canada
10XRIPA buffer	Abcam, ab156034
PMSF	Cell Signaling Technology, 8553
Phosphatase Inhibitor Coctail	Simple Stop™ 1, Goldbio, GB-450-1
5X Laemmli Sampling buffer	BioLegend, 426311
Western blot gel	TruPAGE™ Precast Gels 10%, Sigma, PCG2009-10EA
1X Tris Buffered Saline	Advansta, R-01039-020
1X Tris-Glycine-SDS buffer	Advansta, R-01036-020
1X Tris-Glycine	Advansta, R-01037-020
EZ-ECL kit	Sartorius, Biopharma, 20-500-500A, 20-500-500B
PVDF membrane	BIO-RAD, 1620177
Tween 20	AFG Bioscience, 224594
Western Blotting Filter Paper (0.83 mm thick, 7x8.4 cm)	ThermoFisher, 84783
TDO2 antibody	St.Johns Laboratories, STJ115148-100
NF-κB antibody	Thermo Fisher Scientific, 51-0500
HRP linked secondary antibody	abcam, ab6721
TDO2 gene expression assay	Qiagen, QT00181405
NF-κB gene expression assay	Qiagen, QT00370545
ACTB gene expression assay	Qiagen, QT00193473
QuantiTect Reverse Transcription Kit	Qiagen, 205311
SYBR Green Master Mix	QuantiFast SYBR Green PCR Kit, 204056
Protein ladder	BioLegend, 773302
Non-Fat Dry Milk	Santa Cruz Biotechnology, sc-2324
Surgery suture	Damalon, DN4K17
Sodium chloride	Biofleks
RNA stabilization solution	RNAlater™, Thermo Fisher
Hematoxylin	Roche Diagnostic, 05266726001
Blueing Reagent	Roche Diagnostics, 05266769001
Paraffin	Leica, Paraplast, 39603002
DAB kit	Thermo Scientific, TA-125-HD
EDTA	Sigma-Aldrich, E0270, Germany
Antigen Retrieval solution	ab208572, abcam, UK

Figure 3.1.1 Chemicals and reagent

3.1.2 Apparatus

The apparatus used in the experimental process are given in Figure 3.1.2.

Apparatus	
Surgical set	Fine Science Tools, Germany stainless
Syringe (1 ml, 5 ml, 10 ml)	Ayset, Turkey
Eppendorf tubes (0,5 ml)	Eppendorf, Germany
Eppendorf tubes (0,2 ml)	Eppendorf, Germany
Strips (0.1 ml)	RotoCycler™, Qiagen
Hamilton syrnge (25 µl)	Hamilton, United States
Drill	$OmniDrill_{35}$, World Precision Instrument
Stereotaxic chamber	Reset, World Precision Instrument
Rectal termometer	ATC-2000, World Precision Instrument
Rat Rotarod	Ugobasile, 47750, Italy
Morris Water Maze	Hand made
Elevated plus maze test	Hand made
Rotor-Gene™	Qiagen, Hilden, Germany
TissueLyser LT	Qiagen, 85600, Hilden, Germany
Western blotting equipment	Biorad, Trans-Blot® Cell
Western blot imaging	Fusion FX, Vilber Lourmat, France
Behavioral analysis program	Ethovision
Spectrophotometer	MultiSkan™ Go, Thermo Scientific™, Porto Salvo
Rotorgene Q	Qiagen, Hilden, Germany
Thermal Cycler	Veriti™ 96-Well Fast, 4375305
-80 °C freezer	Hettich, Almanya
Microcentrifuge	Eppendorf, Germany
Cooled centrifuge	Eppendorf, Germany
Power Supply	Bio-Rad, ABD
Shaker	ISOLAB, Germany
Shaker	Stuart, Staffordshire, England
Tissue processor	Leica, ASP300, US
Heated Paraffin Embedding Station	Leica, Arcadia H, US
Microtome	Leica, HistoCore AUTOCUT, US
Microscope	Olympus, USA

Figure 3.1.2 Apparatus for experimental analysis.

3.2 Methods

3.2.1 Ethical standards

The study was started with the ethical permission, procedure dated 20.10.2022, and reference number 2022/53 of the ethics committee for the animals used from Gaziantep

University Animal Experiments Local Ethics Committee (HADYEK). The entire study was carried out at Gaziantep University Experimental Animals Research Center (GAUNDAM) (Turkey).

3.2.2 Experimental layout for laboratory animals

The application of the Ministry of Agriculture and Forestry titled "About the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes" was monitored for animals. 36 *Sprague Dawley* female rats are individually housed in standard clear cages (39 cm x 28 cm x 18 cm) with a 12-hour day and dark cycle, constant temperature (22-25 °C) and humidity (60-65%), unrestricted feed and water access. hosted separately.

3.2.3 Analysis of herbs and phytochemicals

The *E. elaterium* plant was searched online by using MESH terms and keyword like "Medicinal Herbal". The multiple databases and search engines were searched and examined (https://researchguides.uic.edu/c.php?g=252194&p=1682724). Afterwards, a database named Dr. Duke's Phytochemical and Ethnobotanical were analyzed for the Alzheimer's disease and the mechanisms by using (https://phytochem.nal.usda.gov/phytochem/search/list). The basic reason of this database was as it provides the knowledge with advance filtering technique including plant, phytochemistry, metabolites, and disease options. The literature review of plants was evaluated. After extensive studied, the apoptosis, oxidative metabolism, inflammation and cancer effect of plants were assessed. In experimental study, it was concluded the chosen plant play efficient role in reducing the infection and neuronal damage. Afterwards, the active ingredients of desired plant were searched on multiple databases including openchem.org, stitch.embl.de/cgi/network, Targetmol-www.targetmol.com. 19 types of Cucurbitacins were determined having similar actions however only CuI was associated with AD according to Targetmol database.

3.2.4 Experimental design, groups and chemical doses

The experimental design of rats for CuI is shown in Figure 3.2.1.

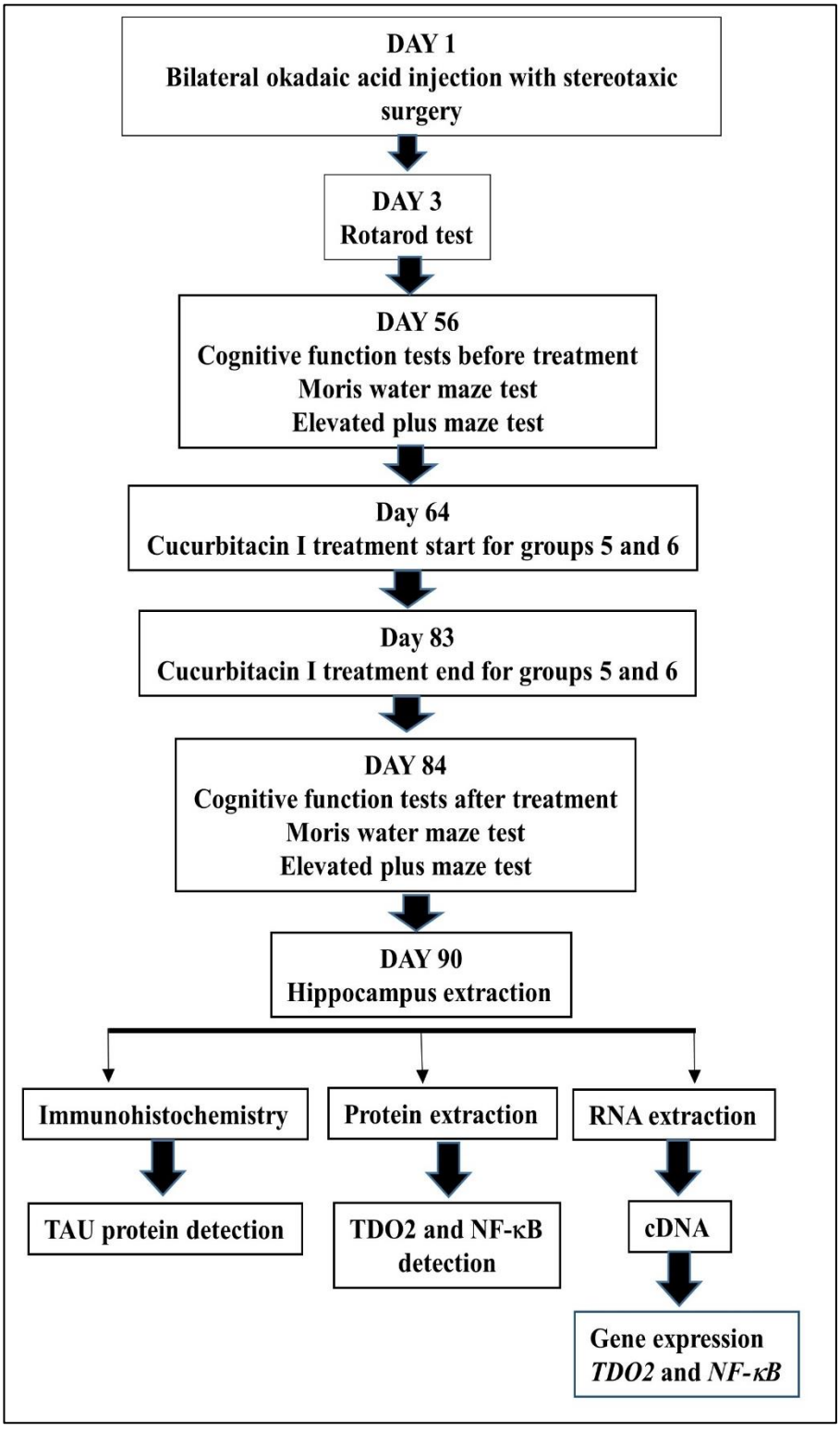


Figure 3.2.1 Experimental design of study.

Description about all groups and plant dose are provided in Table 3.1. One animal died under anesthesia at the start of the study.

Table 3.1 Animal numbers of chemical doses for experimental groups**.**

Experimental Groups	N	Chemicals	Doses	Administration/dose
Control	6	-------	---------	---------
Stereotaxic	5	-------	---------	---------
Stereotaxic+aCSF	6	aCSF	10 µl	ICV/1
Stereotaxic+OKA	6	OKA	200 ng/kg	ICV/1
Stereotaxic+OKA+CuI (LD)	6	CuI	2 mg/kg	i.p/1 in day for 20 days
Stereotaxic+OKA+CuI (HD)	6	CuI	4 mg/kg	i.p/1 in day for 20 days

N; Numbers of animals, aCSF; artificial cerebrospinal fluid, OKA; okadaic acid, CuI; cucurbitacin I, i.p; intraperitoneal, ICV; Intracerebroventricular.

3.2.5 Stereotaxic surgery and ICV injection of okadaic acid

The procedures were started with stereotaxic surgery. Firstly, Alfazyne+ketax (5-10 mg/kg + 30-50 mg/kg, i.p.) was used at one time to induce anesthesia in rats. Then, a midline sagittal cut was made on the scalp of the animal in the stereotaxic chamber. Finally, bregma coordinates were used to drill holes in the skull on both sides (0.8 mm posterior, 1.5 mm lateral above the sagittal suture, and approximately 3.6 mm below the brain surface), and then a 200 ng/kg dose of OKA was bilaterally injected into the holes to initiate AD (Figure 3.4). Intra-hippocampal injection of OKA causes memory impairment as well as striking neuropathological changes such as hippocampal neurodegeneration, tau protein phosphorylation that resembles paired helical filaments, and the development of plaque-like structures containing amyloid (Kamat, Rai, & Nath, 2013). For the precision of the model at this stage, TAU fibrils were confirmed by immunohistochemistry in 3 animals whose AD model was generated independently of the group.

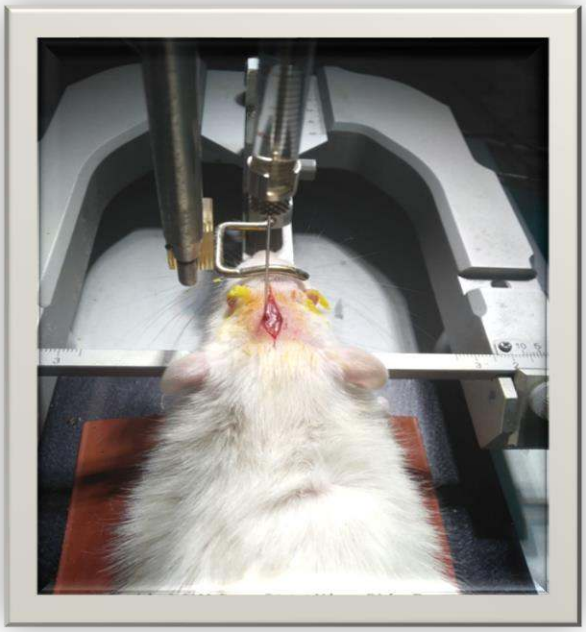


Figure 3.2.5.1 Stereotaxic surgery and bilateral OKA injection.

3.2.6 Rotarod performance test

The method of trial was adopted from (Rustay, Wahlsten, & Crabbe, 2003) with modification. The test was performed to measure balance and motor activity after stereotaxic. The rats were clematises for half an hour in the testing room. The experiment was performed on disinfectant (70% alcohol) surface, without sound, odor, and light. The equipment has a 63 cm height and a 6.3 cm diameter dowel. To produce a uniform surface and avoid slippage, dowel surfaces were buffed with dry sandpaper/320 grit wet. Because mice can use any seam to hold the rod and greatly increase their latency to fall, sandpaper was carefully attached to the dowel to minimize the seam (Wahlsten, Rustay, Metten, & Crabbe, 2003). Four animals were placed on the fixed rod for the ARR, one in each of the four lanes formed by Plexiglas rounds for two sessions of three trials. The motor was turned on once all of the animals were on the rod, and acceleration at a rate of 4–40 rpm/min for 5 minutes was fixed for rotation of rod. The documentation of rate and time of falling from instrument was made. The trial interval for each animal was set at 10 minutes. (Deacon, 2013) (Figure 3.3).

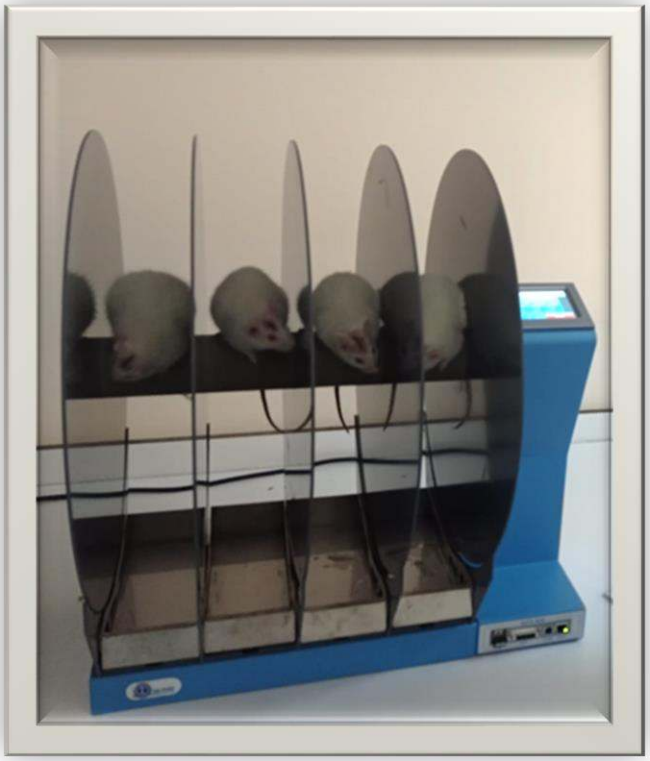


Figure 3.2.6.1 Motor activities assessment by Rotarod Performance Test.

3.2.7 Assessing memory and spatial learning

The MWM test were made according to (Nunez-Iglesias, Liu, Morgan, Finch, & Zhou, 2010) with little adaptation. Markers of different colors and shapes were placed on the four main poles of the tank (Figure 3.6).

Figure 3.2.7.1 MWM assembly and polar shapes.

The test will be carried out at controlled atmosphere at 21 °C in a 180-centimeter-diameter white color pool filled with harmless white paint. During training, a platform of a 14-cm-diameter will be kept 1 cm below the surface. Each rat will be subjected to four trials over the course of six days (Table 3.2.4). The starting position will be assigned at random from the four quadrants of the pool. In each test trial, the rat will have 90 seconds to search the hidden platform. If rats failed in finding the platform within 80 seconds, it will be directed gently towards the target. The rat will be kept on the platform for 20 seconds, at the termination of experiment. After, being saved until the next attempt, it will be returned to its own cage. 24 hours after their previous training attempt, animals will be allowed to swim for 60 seconds, without an escaping the platform, within the pool. The time (sec), distance (cm) and the speed of swimming(cm/sec) taken by the rat to reach the target, will all be write down by an autonomous tracking system.

Table 2.2.7 Directions of rats drop position.

Before CuI treatment						
Trials	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6 (Probe)**
Trial 1	NE	SW	SE	NW	NE	NW
Trial 2	SW	NW	NE	SE	SW	
Trial 3	NW	SE	NW	NE	NW	
Trial 4	SE	NE	SW	SW	SE	
After CuI treatment						
Trials	**Day 1**	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6 (Probe)**
Trial 1	NE	SW	SE	NW	NE	SE
Trial 2	SE	NW	SW	NE	NW	
Trial 3	NW	SE	NE	SW	SE	
Trial 4	SW	NE	NW	SE	SW	

SW; southwest, NW; northwest, NE; northeast, SE; southeast.

3.2.8 Elevated plus maze test for anxiety interaction and testing learning

This test was adopted from (Yang & Mailman, 2018). This was designed to see how worry affects learning tests. The letter T is formed by its four arms (45 cm x 50 cm x 10 cm) two of which are enclosed (the "closed arms") and two of which are completely exposed to the environment (the "open arms") (Figure 3.5). The rat is then set free to freely discover the maze, and its behavior is recorded. Typically, animals that are more anxious will spend less time in the open arms as compared to their time spend in closed arms, as they are more fearful to the exposed areas. On the other hand, animals that are less anxious will be more willing to explore the open arms. By measuring the amount of time that the rats spend in the open arms, researchers can get an idea of their level of anxiety. The experiment will continue until the rats successfully place all four paws on the open arms for a minimum of 300 seconds. This requirement will be met through multiple attempts. The length of time and distance traveled by the rats in both arms will be recorded and analyzed. This is done to prevent any inhibiting behavior from occurring (Walf & Frye, 2007).

Figure 3.2.8.1 Placement of the animal in the EPM assembly.

3.2.9 Dissection of hippocampus

At the conclusion of the behavioral trials, which had taken 14 weeks, rats underwent the anesthetic decapitation. After the anesthesia, complete brain of each rat was

remove and sagittal cut into two halves. The hippocampus was dissected using Glowinski and Iverson's method (Snyder, Jarrott, Turner, & Beart, 2020). To make a homogenate with a concentration of 10% (w/v), the first component was weighed and stored in an RNA after solution. Expression tests will be performed and kept at -80°C until the proteins are identified. The remaining half of the brain were in 10 % formaldehyde at +4 °C for immunohistochemistry. Sagittal pieces were cut using a cold microtome (40 mm thick).

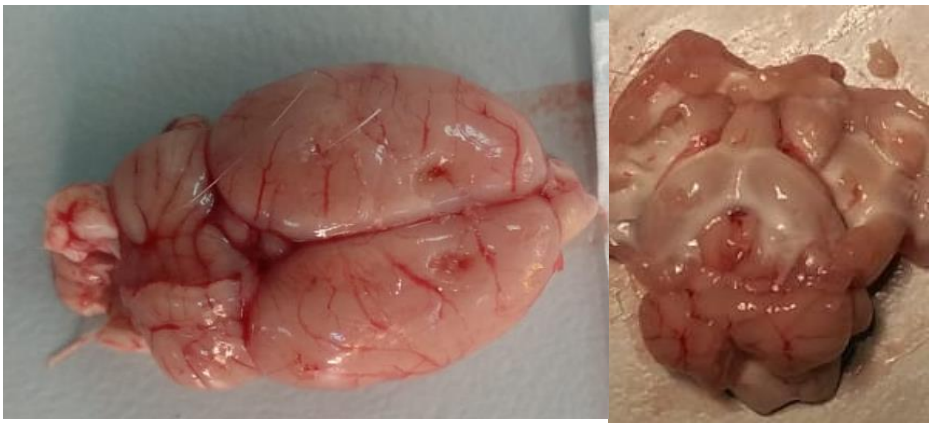


Figure 3.2.9.1 Whole brain and hippocampus of rat.

3.2.10 Protein analysis by western blotting

3.2.10.1 Extraction of protein from homogenized hippocampal tissue

The method was adapted with little modification (Kurien & Scofield, 2006). Temperature for all steps of extraction was sustained at 2-8 °C. Dissection of the hippocampus was performed on ice. To remove proteins from the tissue, 300 µL of cold lysis buffer was added to a 20 mg hippocampus tissue and the mixture was homogenized using Qiagene's Tissue Lyser LT homogenizer at 50 osc for approximately 5 minutes. The samples were then centrifuged at 16,000 rpm for 20 minutes at 4°C, the pellet was discarded and the supernatant collected and stored at -80°C.

3.2.10.2 Detection of proteins

Proteins were quantified using the Bradford assay method, which involves comparing the protein concentration of an unknown sample with a set of standards. This was done using a Bradford assay kit and a 96-well plate. Protein standard dilution called bovine

serum albumin (BSA), were easily developed within the test tubes, and the unknown sample of protein was also diluted to a specific quantity. The absorbance of the protein mixture was measured through the spectrophotometer having specific wavelength 595 nm, and the outcomes were compared to a standard chart to calculate the protein amounts.

3.2.10.3 Loading solution for proteins

The loading solution consists of 20 µg of protein sample, 25% of 5X Laemmli Sampling buffer, and ddH2O to get a final volume of 30 µl. Mercaptoethanol at a concentration of 2.5% (v/v) is also added to the loading solution.

3.2.10.4 Loading of protein solution into the gel

A 10% prepared polyacrylamide gel (TruPAGE™ Precast Gels, Sigma, PCG2009-10EA) was used. The gel was placed in a blotting tool and secured with clasps (Mini PROTEAN™ Tetra Cell, Bio-Rad, Cat. No. 1658004). A500 ml running buffer of 1X Tris-Glycine SDS (Advansta, R-01036-020) was used. Furthermore, 250 ml running buffer was used for the filling of the inner pool along with antioxidant (NuPAGE™, NP0005) of 600 µl quantity. However, the left over 250 ml was divided between the inner and outer pools, filling each to the designated line. A micropipette was used for the washing of the wells effectively. The PRERUN step was carried out for 15 minutes at 120 V, after which for 5 minutes at 95 °C the denaturation step of sample was conducted. The denatured sample then placed on ice. Finally, the wells are filled with the denatured sample and protein marker along with the gel that was operated for 1.5 hours at 120 V until the dye completely cover the base of the gel.

3.2.10.5 Blotting of proteins and PVDF membrane

Before the end of the gel run, transfer buffer (20X Tris-Glycine, Advansta, R-01037-020) was prepared. Once the gel run was finished, mold edges were completely broken off while one of the edge was marked and cut. Furthermore, the PVDF membrane was allocated through the forceps between the layers of two filter papers. PVDF membrane was dipped for approximately half minute in the methanol and then distilled water was used for rinsing purpose. After rinsing, the membrane was shift into the 1Xtransfer buffer and shaken through shaker for 15 minutes at the set 60 rpm. The blot assembly

was then prepared by placing a sponge in a transfer cassette and layering two filter papers and the membrane on top, making sure to eliminate any bubbles. The top of membrane was completely merged through the gel along with the placement of two botting papers and addition of transfer buffer. The transfer machine was connected to a power supply and the blot was transferred overnight for 40mA and 4°C. After the completion of the transfer, PDVF membrane was separated from the filter papers layer and gel.

3.2.10.6 Blocking of membranes

The membrane was incubated for approximately 1 hour within the 5% non-fat dry milk (Santa Cruz Biotechnology, sc-2324) after shaking at the room temperature. The blocking solution was prepared by mixing 100mL of 1X TBS (Tris Buffer saline, Advansta, R-01039-020) with 1 mL Tween 20, to this, 5 g of non-fat dry milk (TBST) was added in the solution for the preparation of the final solution. Once the blocking was finished, TBST solution was used for the washing of membrane thrice for a total of 10 minutes.

3.2.10.7 Primer antibody staining

The TDO2 and NF-κB on the membrane were incubated with a primary antibody solution (1:500 dilution) in a shaker set to 60 rpm and stored at 4°C in a refrigerator overnight. After the incubation period, the solution of primary antibody was collected for recycle and these antibodies on the membrane was removed by using TBST solution for removed by washing the membrane with TBS for a total of 10 minutes for each time.

3.2.10.8 Secondary antibody staining

A blocking buffer having 1:500 dilution of anti-rabbit IgG, HRP-linked secondary antibody applied to the PDVF and incubated at room temperature with shaking for 1 hour. After incubation, 20 mL TBST was used for three times washing of membrane for a total of 10 minutes in a shaker, followed by two 2-minute washes with distilled water.

3.2.10.9 Imaging and analysis of protein bands

The EZ-ECL chemiluminescence kit (Sartorius, Biopharma, 20-500-500A, 20-500-500B) was the tool that used for the visualization of protein bands in this experiment. Equivalent volumes of Solution A and Solution B were mixed in a 1:1 ratio and incubated in the dark for about 5 minutes. After incubation, Fusion FX chemiluminescence imaging device (Vilber Lourmat) was sued for the visualization of protein bands. The recorded images of the protein bands were analyzed using the ImageJ program (Version 1.53f) to measure densitometry and normalization to GSK-3β protein levels.

3.2.11 Immunohistochemical staining

Sections will be treated with a solution of 0.3% H2O2 and 0.3% Triton X-100 for 15 minutes at normal temperature, cleaned with PS, and solution of 5% normal goat serum with 0.1% Triton X-100was used for blocking for 30 minutes. The units will then be incubated with tau protein-specific primary antibodies overnight at 4°C, followed by secondary antibodies avidin/biotinylated horseradish peroxidase. The peroxidase substrate will be used for staining of unites that will be ultimately dried, and covered with coverslips before being photographed using a fluorescence microscope. The thtriazoleol (Invitrogen, Carlsbad, CA) technique will be used to extract RNA (Chomczynski and Sacchi, 1987). The Ventana Benchmark (Roche Diagnostics Turkey) automatic immunostaining equipment was executed for immunohistochemical staining after a 120-minute processing time at room temperature. A trained pathologist used an Olympus BX51 light microscope to examine glass slides that had acquired immunohistochemistry staining. To capture images of microscopic objects, a digital camera that was attached to another light microscope was deployed. On the basis of the presence of cytoplasmic staining, tau staining was examined. On the same day that fresh specimens were stained in 10% formalin, tissue review was achieved. With a tissue processor (Leica, ASP300), the samples were processed through a series of ethanol concentrations to dehydrate the water: 70% ethanol, 90% ethanol and 100% ethanol for 15 minutes whereas 100% ethanol for 30 minutes, and 100% ethanol for 45 minutes. The ethanol in the tissue was neutralized by cleaning with xylene: 20 seconds, 20 minutes, and 20 minutes. Wax infiltration of the tissue occurred during three intervals of one, two, and three hours.

With the support of a tissue embedding tool, tissues were paraffin-blocked (Arcadia H, Leica). On the vertana ULTRA equipment, tau protein immunohistochemical staining (Invitrogen, 13-6400) was accomplished through the ultraview DAB kit. The methodology for tissue staining was as follows: 1- For 30 minutes, the preparations were heated at 68°C in an oven. 2. Used antigen removal to treat with EDTA for 20 minutes at 95 °C. 3- Tau was treated to the main antibody for four minutes. 4- For background staining, it was left in hematoxylin for 28 minutes. 5- To fix the hematoxylin, it was left in the bluing reagent solution for 4 minutes. 6 - Microscopic analysis was carried out again after Entellan was enclosed.

3.2.12 Gene expression analysis of hippocampal tissues

3.2.12.1 Total RNA extraction from hippocampus

A new modified and alternative method was used for the conduction of Total RNA extraction (Avison, 2007) with all steps carried out on ice. Tissue samples stored at -80°C were slowly brought to -20°C and then defrosted at normal temperature. A 50 mg sample of tissue was weighed and mixed with 1 ml of Tripure (750 µl Tripure + 250 µl dH2O, ELK Biotechnology). The mixture was incubated for 5 minutes at normal room temperature, and then addition of chloroform per 1 ml of trizole of 0.2 ml was done that vortexed for around 15 seconds. The whole mixture was centrifuged for 10 minutes at 4°C at the set 13,000 rpm, and the sterile tube was used for the transfer of supernatant. Isopropanol of 0.5 ml per 1 ml of trizol was added in the liquid solution, and the mixture was incubated at room temperature for 10 minutes. The whole mixture was centrifuged at 4°C for 10 minutes at the set 13,000 rpm, and the supernatant was thrown out. Furthermore, 1 ml of 75% EtOH per 1 ml of trizol was used for the washing of the pellet that was centrifuged for 10 minutes at 4°C; at 13,000 rpm. The pellet was immersed in the in 50 µl of RNase-free water that finally stored at -80°C.

3.2.12.2 RNA Quantitation within samples

Plate spectrophotometer (MultiSkan™ Go, Thermo Scientific™) was used for the quantification of concentration of RNA by assessing the absorbance at 260 nm. After assessment; the sample of RNA was immersed in the 10 ng/µL in the complementary DNA (cDNA) reaction.

3.2.12.3 cDNA obtained from total RNA samples

Quantitect RT kit (Qiagen, 205311) was used for the conduction of the cDNA analysis in this study. A cDNA library was generated through total RNA samples that will ultimately helped in the expression of TDO2, NF-κB genes and the endogenous control ACTB (β-Actin) (Qiagen, 249900) using reverse RT-PCR with gene-specific primers. A 10 µl RT-PCR reaction mixture was prepared by combining RNA sample of 10 ng, 10X Nucleic Mix, 5X RT buffer, 0.25 mM MultiScribe Reverse Transcriptase, and ddH2O. The thermal cycler (Veriti, Germany) was used for the incubation of RNA samples around 30 minutes at 16°C. The first cycle for 30 minutes at 42°C, the other cycle around 5 minutes at 85°C, 1 cycle. The resulting cDNA was kept around -80°C.

3.2.12.4 Analysis of specific gene expression through cDNA samples

The expression profiles of *TDO2*, *NF-κB* and normalization gene *ACTB* (endogenous control) was performed through the resulting cDNA library by using Real-Time PCR system especially Rotor Gene Q. The reaction mixture was prepared by combining 20 µl product of PCR, 5 µl of cDNA, 2X SYBR Green Master Mix, Universal primer, 10X Assay, and distilled water to develop the desired 20 µl volume. The RT-PCR conditions were described as: 1 cycle at 95°C for 10 minutes, for 40 cycles 95°C for 15 seconds at, 60°C for 30 seconds at, and 70°C for 30 seconds.

3.3 Statistical analysis

SPSS 22.0 (IBM, 2013) was used for the detailed analysis of obtained data. The minimum sample size for the study was calculated to be 6 animals in each group based on historical data, 5% Type I error, and a power of 80% (0.20 Type II error) in order to determine 0.50 across the groups difference. Across the groups, difference was determined by using one-way ANOVA and Tukey post-hoc tests. Shapiro-Wilk test was used for the determination of the normality of the data using the, and after normality of the test, Mann-Whitney U or Kruskal-Wallis tests were applied to the data for the comparison the groups. Categorical data were presented in terms of frequency (%), while continuous data were described through as the Mean ± Standard Deviation or Median value (percentiles) according to data distribution. For analysis of gene expression, ΔCt values were obtained by comparing the investigated gene

expressions to the *ACTB* housekeeping gene, and $2^{-\Delta\Delta Ct}$ was used for the determination of the values of fold change. Protein expression was examined using the Image J program and quantified based on western blot band densities.

CHAPTER IV

RESULTS

4.1 Result of rotarod test

The rotarod test was conducted to compare the motor activity in surgical and control groups. The figure 4.1 includes major points the fall of speed and latency to fall. The results showed that stereotaxic surgery and administration of drug is strongly comparable to control group.

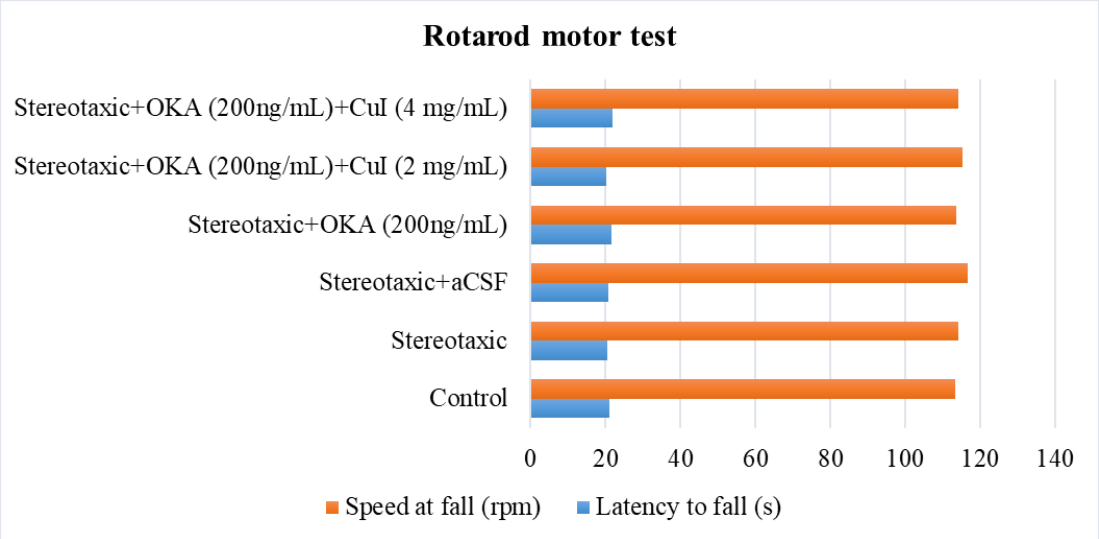


Figure 4.1 Speed at fall and latency to fall for experimental group.

4.2 Result of MWM test

The MWM test was analyzed, starting when the rats were 15 weeks old, discontinued at 30-dayfor the AD progression with OKA. The learning process in all six groups is shown (Figure 4.2.1).

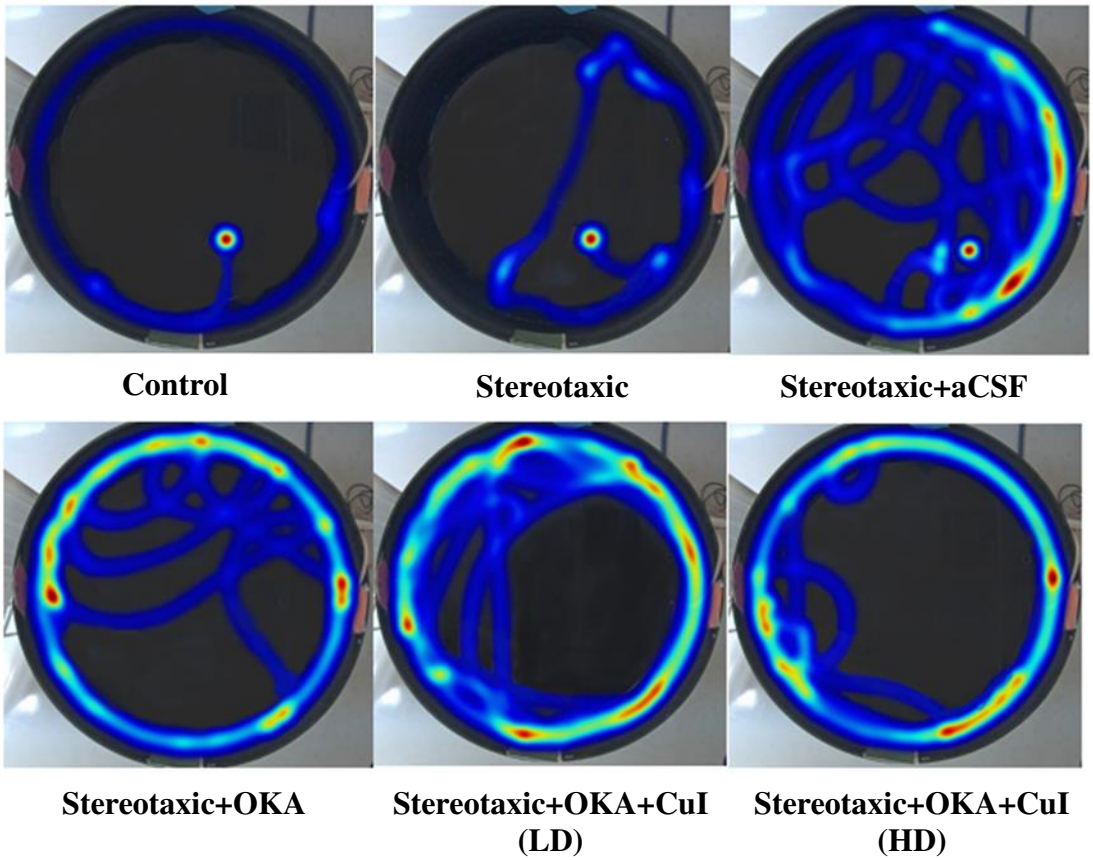
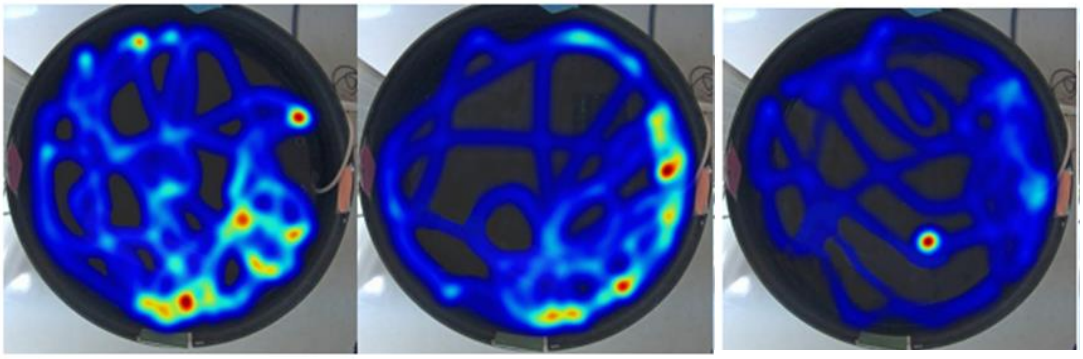


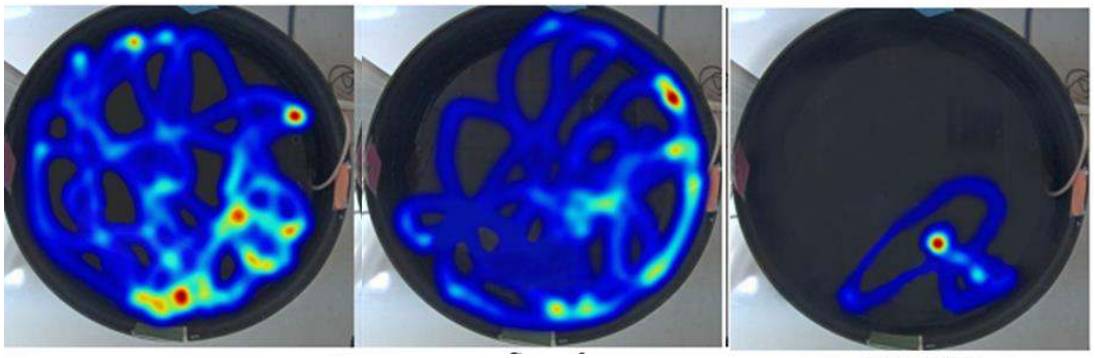
Figure 4.2.1 MWM test of all AD experimental groups before CuI treatment.

While the animals in the groups that could not be intervened before the treatment found the platform, the animals in the 3 groups (4, 5, and 6) treated with OCA could not learn the platform-finding behavior. During the five-day learning phase, rats in the AD group positioned the platform more slowly and did not perform platform-seeking behavior by making circular movements in the probing trial. This result showed that OCA application started the AD process cognitively and learning was delayed. The time to find the platform and the circulation distance were shortened in the post-treatment groups (Figure 4.2.2) (Figure 4.2.3).



Stereotaxic+OKA | **Stereotaxic+OKA+CuI (LD) before treatment** | **Stereotaxic+OKA+CuI (LD) after treatment**

Figure 4.2.2 MWM test of before and after CuI treatment for LD according to AD.

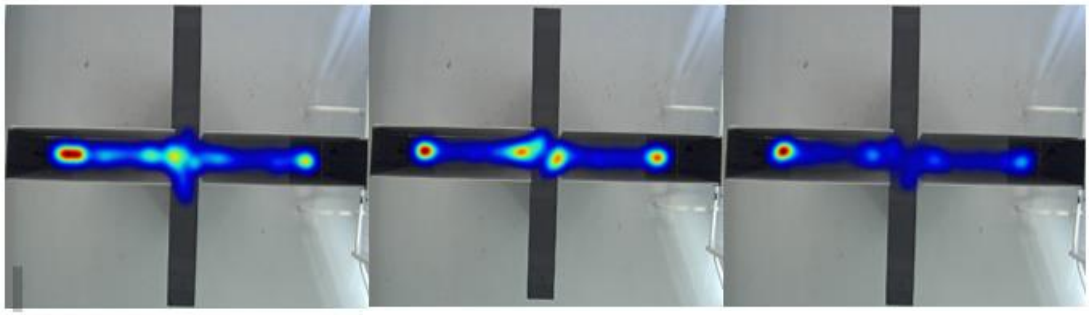


Stereotaxic+OKA | **Stereotaxic+OKA+CuI (HD) before treatment** | **Stereotaxic+OKA+CuI (HD) after treatment**

Figure 4.2.3 MWM test of before and after CuI treatment for HD according to AD.

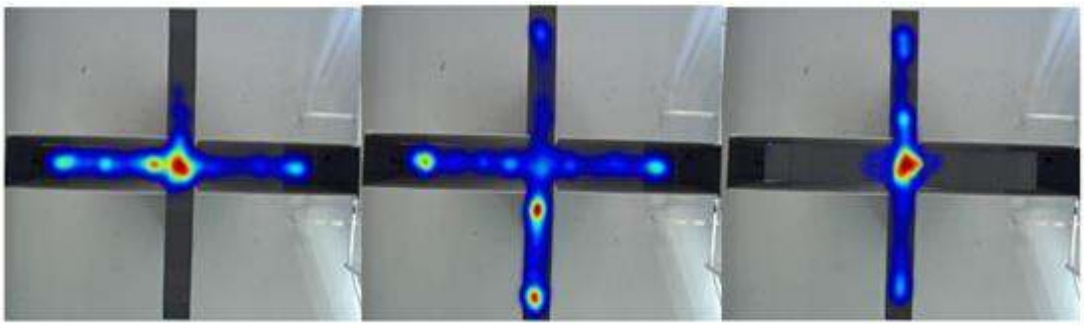
4.3 Result EPM test

Anxiety was measured by distance traveled in the closed arm, altered voiding pattern, and increased urination in groups 4.5 and 6 OCA, while the short distance in the open arm was examined before treatment in EPM. It was observed that the distance covered in the closed arm decreased and the distance covered in the open arm increased after drug administration. No defecation or urination was detected (Figure 4.3.1). The findings showed that CuI reduced symptoms of the AD model, reduced anxiety, and complete cessation of urination and defecation indicated advanced recovery.



Stereotaxic+OKA **Stereotaxic+OKA+CuI (LD)** **Stereotaxic+OKA+CuI (HD)**

Figure 4.3.1 EPM test of AD experimental groups before CuI treatment.



Stereotaxic+OKA **Stereotaxic+OKA+CuI (LD)** **Stereotaxic+OKA+CuI (HD)**

Figure 4.3.2 EPM test of AD experimental groups after CuI treatment.

4.4 Expression of neuronal proteins at hippocampal level

Expression of TDO2 and NF-κB containing proteins in hippocampal tissue is shown in Figure 4.4.1. NF-κB is an important neurodegenerative fuel in the brain and a prominent marker in AD, so its activity would be expected to be high in the patient group and lower in the treatment group. TDO2 is important for protein homeostasis and helps reduce the toxicity of other misfolded proteins. Expression of TDO2 (Figure 4.4.2) and NF-κB (Figure 4.4.3) was high in OCA group and low in CuI group. It was observed that TDO2 and NF-κB protein levels increased in the AD group and decreased in the treatment group (Table 4.4.1). These results suggest that CuI treatment may reduce TDO2 and NF-κB levels and modulate their activities therapeutically.

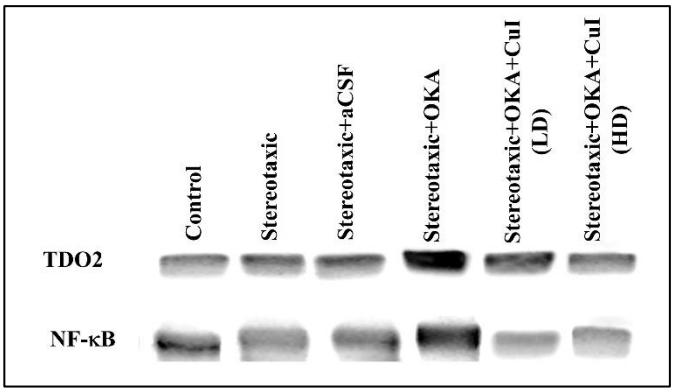


Figure 4.4.1 TDO2 and NF-κB protein expression in rat hippocampus tissue.

The relative band intensities of the proteins compared to the control group indicate the reduction of protein expression in the treatment group. Consistent with gene expressions, it was determined that CuI caused a decrease in the expression of textural proteins as a result of expression modulation.

Table 4.4.1 Protein density results of TDO2 and NF-κB in rat hippocampus.

	TDO2		NF-κB	
Experimental Groups	**Mean**	**SD**	**Mean**	**SD**
Control	116,616	20,242	70,722	33,300
Stereotaxic	91,197	16,557	92,839	24,443
Stereotaxic+aCSF	88,911	23,602	63,592	38,218
Stereotaxic+OKA	126,448	45,937	131,704	21,561
Stereotaxic+OKA+CuI(LD)	85,67	81,205	124,155	20,358
Stereotaxic+OKA+CuI(HD)	81,594	30,819	106,017	11,987

SD; standard deviation.

4.5 Immunohistochemically result hippocampal sections

When the histopathological morphology of the OKA group was examined after H&E and TAU antibody staining, TAU protein accumulation was observed to be quite high. Almost no TAU pathology was observed in the CuI-treated group (Fig. 4.5.1). The figure shows that there was staining in the untreated group, weak staining at low CuI dose and no staining at high CuI dose.

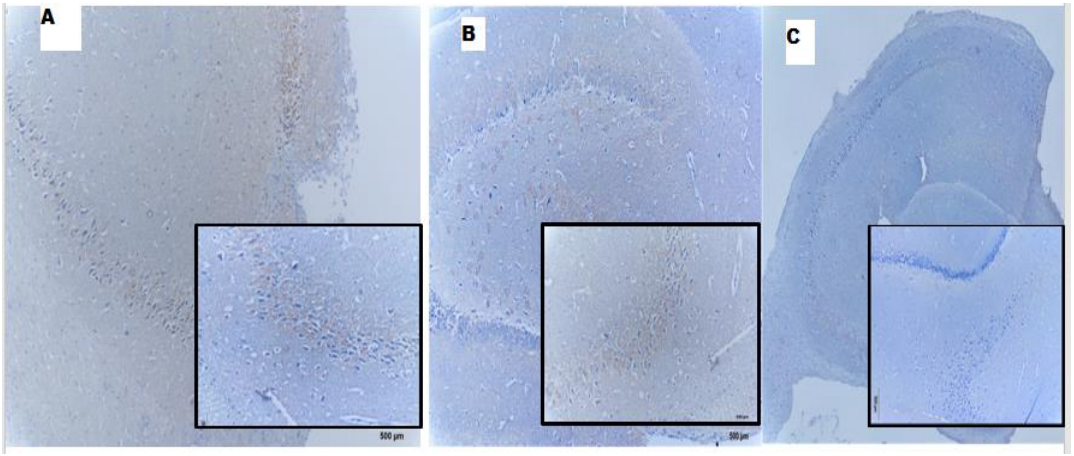


Figure 4.5.1 Cytoplasmic staining in the hippocampus sections. A: Untreated group (Maginification: x 100), B: Treated group with low dose (Maginification: x 100), C: Treated group with high dose (Maginification: x 40)

4.6 Gene expression results of hippocampal tissues

Analysis of gene expression showed increased *TDO2* and *NF-κB* expression levels in the hippocampus of the AD sample ($P \leq 0.05$). Expression changes of *TDO2* and *NF-κB* genes between groups in rat hippocampus tissue are shown in Figure 4.6.1 and Figure 4.6.2. It was determined that such genes were increased and overexpressed in brains with aging, while the expression was decreased in the CuI treatment groups in a dose-dependent manner and effectively increased in the OKA group.

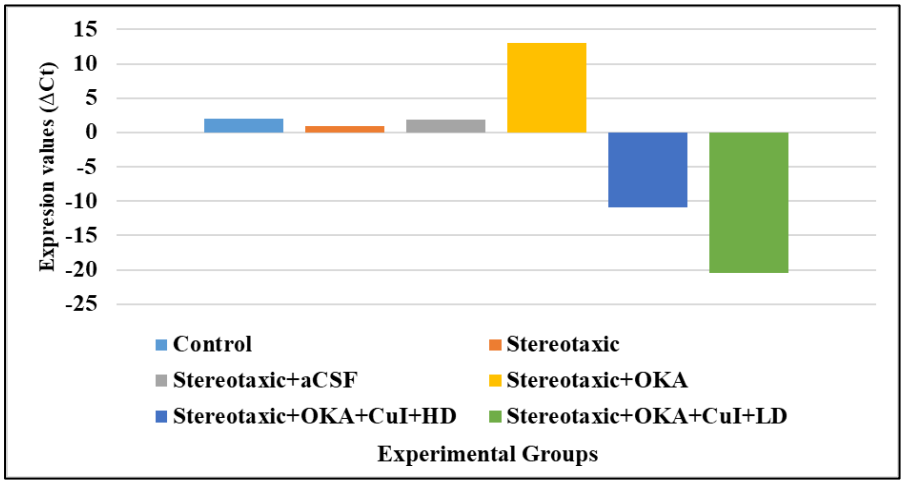


Figure 4.6.1 Hippocampal *TDO2* gene expression differences.

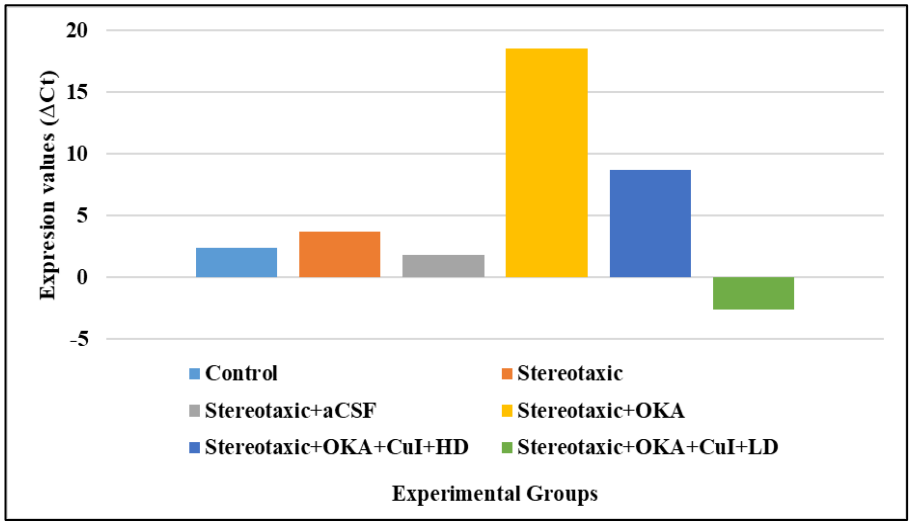


Figure 4.6.2 Hippocampal *NF-κB* gene expression differences.

CHAPTER V

DISCUSSION

Since the dawn of time, plant-based treatments for curing illnesses are being utilized by humans, recently attracted a lot of attention, use of therapeutic herbs due to efficacy and safety in many distinct animals. Because medicinal plants have a higher chemical diversity due to secondary metabolites, they offer an endless wide range of chemicals. The chemical used may show different type of response; cure, delay or alleviating the disease as well as have some toxic effects. These should be thoroughly investigated prior to administration in any individual (Gupta, 1998). The development of AD drug is the main focus by researchers, it can be traditionally used medicinal plants, their compounds, even their mixtures, already synthesized drug and manufacturing the new ones. The newly newer ones need refinement, especially with the use of animal models to estimate toxicity and efficacy in preclinical models. Animal models unquestionably offer valuable information for study, but only 70% of these studies can accurately predict human toxicity (Cummings, 2018). These early-stage animal toxicity studies may enable for a huge number of medications with potential human risk to be unduly kept in the trial process. CuI has potential to cure many diseases mainly cancer (Delgado-Tiburcio, 2022). The antitoxic neuroprotective property of CuI with high and low dose is used in this study of AD (with the dose determined according to the literature data).

The most common form of dementia, Alzheimer's disease (AD), is primarily described as the presence of neuritic plaques and tangles of neurofibrillary tissues. In elderly patients, these two mechanisms may be interdependent. The reduction or elimination of Tau fibrils could potentially protect against neuronal death, as they can contribute to the maintenance of neuronal activity. It is also not clear whether Aβ deposition leads to the formation of tau or vice versa. The pathology and histology of memory loss are associated with Aβ and tau, which are both soluble building blocks. Aβ is generally a fragment, while tau brain-specific protein that highly associated with the microtubule

in structures and alignment. These pathologies lead to damage at the synaptic level and loss of neurons and surrounding tissues. Previous literature described that both Aβ and tau played significant role in maintaining the health of neuron and enhance the recovery of neurons regardless of their specific pathological condition (Bloom, 2014).

Synaptic dysfunction and neuronal death are attributed to the interaction between tau and extracellular Aβ. This suggests that Aβ is involved in the process that tau goes through before becoming toxically hyperphosphorylated and mediates this process. The feedback loop, which raises the Aβ toxicity, is also demonstrated to be a result of tau. Although it is proposed that the pathology begins in this manner, it is known that both of them are capable of self-replication. As a result, the advantage of our research will contribute to the improvement or alleviation of the disease's pathophysiology.

Phytochemicals from plants may be a promising solution for the complex pathology of AD. These plant's molecules are potentially more effective for the treatment purposes having less toxic level as compared to any synthetic drug. However, it is important to standardize these compounds to recognize the active ingredients and determine appropriate doses for use in experimental studies. Molecular docking can also be used to identify the target proteins of these compounds, which can help to streamline the experimental design and save time and resources. Some of the effectively used phytochemicals in AD include berberine, caffeine, curcumin, galantamine, huperzine A, nicotine, nobiletin, physostigmine, quercetin, resveratrol, sinecatechins, and terpenoids, which can be found in plants such as *Coptis chinensis*, *Erigeron annuus*, *Curcuma longa*, *Galanthus caucasicus*, *Galanthus woronowii*, Narcissus, *Leucojum aestivum*, *Lycoris radiata*, *Huperzia serrata*, Nicotiana sp, *Citrus peels*, *Physostigellius alvinozansis*, *Camellius alvinosinenbasis*, Camellius, and *Ginkgo biloba* (D'Onofrio et al., 2017). These compounds may improve AD symptoms by acting through various mechanisms, including apoptosis, oxidative stress, synaptic plasticity, neuroprotection, and neuroinflammation.

The TDO2 and NF-κB protein are activated in different ways in AD. The gene TDO2 encodes tryptophan 2, 3-dioxygenase (TDO2), was first isolated in 2009. In our study, the expression of TDO2 is high in hippocampal neurons. It holds systemic tryptophan levels fixed by serving as the first and rate-limiting step of tryptophan (Trp) metabolism along the kynurenine (Kyn) pathway. Furthermore, it is suggesting that

TDO2 regulates protein homeostasis generally, arguing that TDO2 controls the metabolism that affects age related protein. Van der Goot explains that tryptophan is a necessary amino acid that must be absorbed through meals. Tryptophan levels are primarily regulated by the enzyme TDO2, hence turning this enzyme on and off can make it function as a switch by controlling tryptophan levels. As a result, this amino acid is an excellent choice for alerting pathways that control metabolism and animal health to environmental changes (Van Der Goot, 2012).

Van der Goot continues, "We then evaluated whether reduction of TDO2 would also be able to inhibit the toxicity of these endogenous metastable proteins and, significantly, TDO2 knockdown repaired a developmental abnormality."

Nuclear factor kappa B (NF-κB), a protein that regulates transcription that includes NF-B1, NF-B2, RelA, RelB, and c-Rel. This family regulates the expression of numerous genes involved in inflammation and immunity. It binds to the NF-κB enhancer, a critical DNA region, either in hetero- or homodimeric form, to facilitate the transcription of the target gene (Oeckinghaus, 2009). These genes bind to the molecular pathways involved in NF- κB and translocation from the nucleus are frequently tightly regulated, yet they work in concert with the activation of NF-κB-dependent gene expression (Makarov, 2001).

Recent studies have shown that nuclear factor kappa B (NF-κB) plays a significant impact in conditions like autoimmune disorders, inflammatory, cardiovascular, and neurodegenerative diseases. NF-κB protein is thus a new target for therapeutic intervention. Different pathological situations in humans, including cancer, autoimmune illnesses, inflammatory disorders, and neurodegenerative diseases, are brought on by activation of the IB kinase/NF-κB signaling pathway. As a result, different cascade pathways play a significant role in controlling IB kinase/NF-κB's activity. This may lead to expression of pro-inflammatory genes, such as those encoding cytokines, chemokines, and adhesion molecules. An inhibitor molecule protein (IkB) binds to the cytosolic sequestered NF-κB, which is then phosphorylated and translocated into the nucleus where it further activates a number of genes required for altering a number of cellular activities. Numerous studies have supported the

involvement of several NF-κB family member proteins in mediating and regulating cellular cascades and the expression of a variety of genes' products.

After 20 days of CuI treatment, the rate of recovery of cognitive and intellectual functions to a certain level confirmed that Cui will be effective in producing same results in humans but the duration will be short like in rats. This is actually due to the phytochemicals multi-pathway effect including protection of neurons and structure, hemostasis of mitochondria, antioxidant and anti-inflammatory properties (Limanaqi et al., 2020). The analysis of the current data confirmed that tau pathology can easily be managed and controlled through the oxidative metabolism and inflammation. Therefore, CuI is highly multi-target therapeutic effective agent in reducing the chances of neurodegeneration due to potential phytochemicals.

There are number of previous literature that focuses on the effectiveness of phytochemicals on mental and cognitive ability of brain function. Among all the most common is the flavonoid effect on behavioral and cognitive function. Learning and memory recovery is achieve through kynurenine (Kyn) and NF-κB signaling pathways mediated by flavonoids. The pathway involve in proliferation and survival of neuron, decrease the level of oxidative stress and AD treatment (Bakoyiannis, Daskalopoulou, Pergialiotis, & Perrea, 2019). Therefore, flavonoids showed advance properties in improving the decline in the cognitive and mental function. The other therapeutic group, including CuI, is terpenoids. There is a need for new therapeutics in addition to the acetylcholinesterase inhibitors involved in the treatment process of AD. Past studies conduct in vitro and in vivo animal researches on the effectiveness of terpenoids on cognitive function such as cornel iridoid glycoside, oleanolic acid, tenuifolin, cryptotanshinon and ursolic acid (Yoo & Park, 2012). CuI is one of the potential treatment agent for the acquisition and maintenance of cognition in the treatment of AD.

Anxiety as psychopathological condition effects individual at multiple sectors of their life. The recent medications prescribed by the physicians had serious sedative effect. However, plant's phytochemicals have therapeutic effects with minimum to no side effects. The medically authentic anti-anxiety herbs produce significant improvement and treatment option in management of such diseases. It is the most important point of phytochemicals as the current study showed the effect of CuI on anxiety. Before the

treatment, the multiple observation of defecation and urination of rats were recorded however, it was not assessed after the treatment and in the extended open arm time. The observation was not taking after the treatment due to the serious anti-anxiety effect of CuI. A study observing the diet-related anxiety concluded that there are two pathways in which first pathway generate due to the deficiency of multiple precursors including neurotransmitters, amino acids, vitamins and some cofactors, while the second pathway completely raised from the brain including amygdala, hypothalamus, pituitary and adrenal axis (Weeks, Weeks, Kim, Kessler, & Perez, 2021). Among all the structures, amygdala is responsible for our perception of threat. However, it also works in interacting with hippocampus that is learning and memory center. Both structures, work in order to detect the threat and managing the response according to situation.

According to the results of the current study, application of anxiolytic nutraceuticals played an important role in reducing and managing the anxiety and it should have considered as an alternative solution for such diseases. The EPM model of our study confirmed that innate fear among the enclosed rats caused anxiety. This is identifying due to the release of the stress factors that described their perception and processing of any threat condition. For analyzing the anxiety level among the rats, test highly focused on the animal's ability of experiencing the natural fear and their choices to escape form the closed arm into the exposed arm. Due to reduce communication in AD, the level of fear and threat raised among the rats as they were not being able to use their memory that forced them to stay in the closed arm of EPM model. CuI played significant role in enhancing neuroplasticity and TDO2 and NF-κB reduce the level of threat among the rats. CuI, TDO2 and NF-κB helps in improving the level of anxiety that was assessed by reduce and absence of urination and excretory behavior of sampled animals.

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

In summary, the purpose of the current research was to investigate the ability of a innovative phytochemical, CuI, to decrease or eliminate the accumulation of tau protein in Alzheimer's disease, a condition with limited therapeutic options. The mechanisms involved in cognitive processes were evaluated through gene and protein expression analysis. The results showed that CuI had a positive impact on hyperphosphorylated tau in AD through its effects on TDO2 and NF-κB. TDO2 helps to maintain protein levels and reduces the amount of tau protein, leading to an improvement in cognitive decline and anxiety in rats. These findings suggest that CuI may have therapeutic potential in the treatment of AD that is needed to confirm these results by advanced study on human.

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