

Investigation of Potential Anti-Alzheimer's Compounds through *In Silico* and *In Vitro* Studies

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Pharmacy

Submitted by

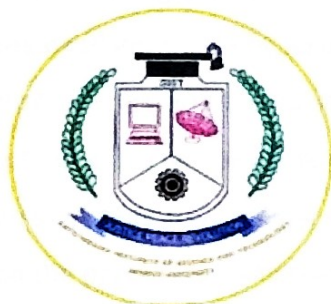
ANANDRAJ R (39050004)

DEEPAK M (39050011)

DILLIBABU S (39050014)

SOUNDRAPANDIAN T (39050051)

THANIKACHALAM C (39050056)



SCHOOL OF PHARMACY

SATHYABAMA INSTITUTE OF SCIENCE & TECHNOLOGY

(Institution Deemed to be University-U/s 3 of the UGC Act, 1956)

Rajiv Gandhi Salai, CHENNAI – 600119

May, 2023



Dr.M.ArunSundar, M.Pharm., Ph.D.

Associate Professor

BONAFIDE CERTIFICATE

This is to certify that thesis entitled "**Investigation of Potential Anti-Alzheimer's Compounds through *In Silico* and *In Vitro* Studies**" submitted by

ANANDRAJ R (39050004)

DEEPAK M (39050011)

DILLIBABU S (39050014)

SOUNDRAPANDIAN T (39050051)

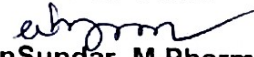
THANIKACHALAM C (39050056)

to The SATHYABAMA INSTITUTE OF SCENCE AND TECHNOLOGY Chennai – 600119 for the award of the degree of **Bachelor of Pharmacy** by Research is a bonafide record of research work carried out by him/her under my supervision. The contents of this thesis, in full or in parts, have not been submitted to any other Institute or University for the award of any degree, diploma, associateship, fellowship or any similar title and that it represents entirely an independent work on the part of the candidate.

Chennai-600119

Date: 15.04.23

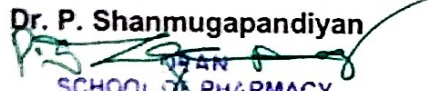
Research Guide


Dr.M.ArunSundar, M.Pharm., Ph.D.



Approved by Dean

Dr. P. Shanmugapandiyan


SCHOOL OF PHARMACY
SATHYABAMA
INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)
Jeppiaar Nagar, Rajiv Gandhi Salai
Chennai 600 119.

Authenticated by Examiners

1. 
- 2.



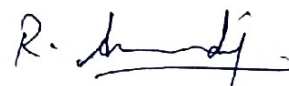
Date: 15.04.23

DECLARATION

I do hereby declare that the work presented in the thesis entitled "**Investigation of Potential Anti-Alzheimer's Compounds through *In Silico* and *In Vitro* Studies**", submitted by me to the **SATHYABAMA INSTITUTE OF SCIENCE & TECHNOLOGY**, Chennai-600119 for the award of the degree of **Bachelor of Pharmacy (2019-2023)** by Research is a original record of research work carried out by me under the supervision of "**Dr.M.ArunSundar, M.Pharm., Ph.D., Associate Professor, Department of Pharmacy**". The contents of this dissertation, in full or in parts, have not been submitted to any other Institute or University for the award of any degree or diploma, membership, fellowship, associateship etc... In keeping with the general practice in reporting scientific observation, due acknowledgement has been made whenever the work described is based on the findings of other investigators.

Place: CHENNAI

Date: 15.04.23



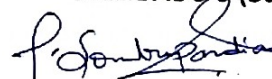
ANANDRAJ R (39050004)



DEEPAK M (39050011)



DILLIBABU S (39050014)



SOUNDRAPANDIAN T (39050051)



THANIKACHALAM C (39050056)

INTRODUCTION

1.1 ALZHEIMER'S DISEASE:

Alzheimer's disease (AD) is the most common neurodegenerative dementia and affects millions of individuals, with many millions more affected as caregivers and family (1). AD's discovery as a neurodegenerative disease is attributed to Alois Alzheimer, a German neurologist who examined a 51-year-old woman named Auguste Deter, who was suffering with loss of memory, language, disorientation, and hallucinations. Her autopsy revealed plaques and tangles in the cerebral cortex (2). In 2018, the number of people living with dementia reached approximately 50 million, which is expected to increase to 152 million by 2050. The global estimated cost of dementia in 2018 was US\$ 1 trillion, and this number is set to increase. The most common cause of dementia is Alzheimer's disease (AD), which is characterized by memory and cognitive dysfunction (3). AD is thought to result most probably from an imbalance in the production and clearance of amyloid beta (4).

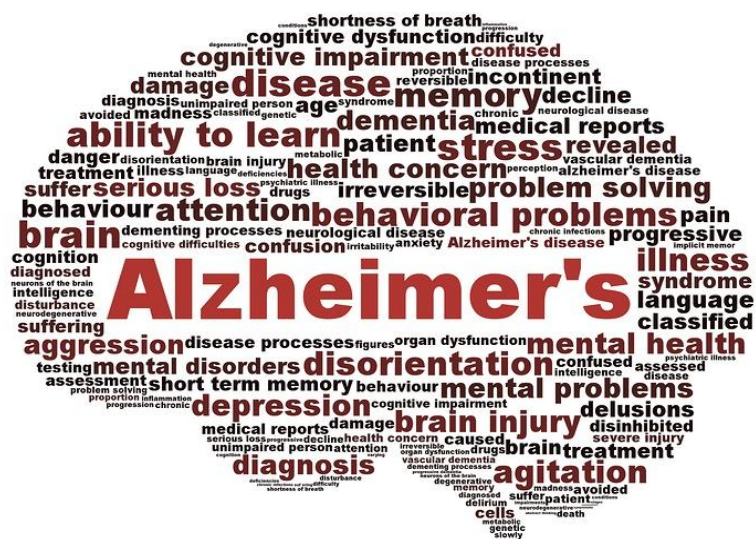


Fig 1.1. Alzheimer's disease - Word cloud

White matter abnormalities in AD with a focus on myelin damage and oligodendrocyte lineage cells and to review the relationship between white matter changes and the pathological hallmarks of AD (5). In recent years, the number of

studies on the role of nutrition in AD have been increased. Several dietary supplements such as antioxidants, vitamins, polyphenols, and fish were reported to decrease the risk of AD, whereas saturated fatty acids and high-calorie intake were associated with increasing the risk of AD (6). Several risk factors are related to the development of Alzheimer's disease. Adding to this list, older people with AD usually have medical conditions such as cardiovascular disease (CVD), obesity, diabetes, and others. All of these conditions are associated with increased risk of AD (7).

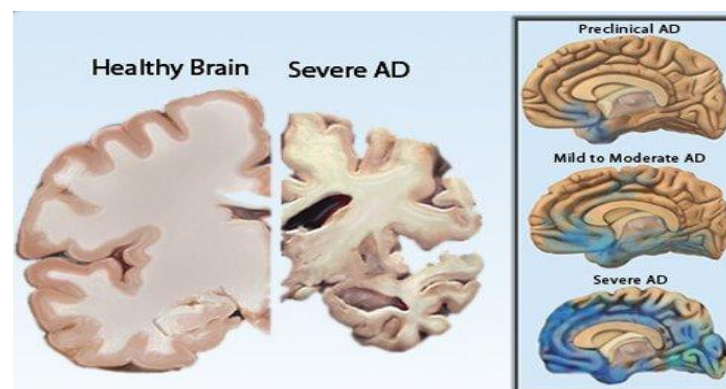


Fig 1.2 Alzheimer's affected brain

Alzheimer's disease has been increasing steadily, and with today's aging population. WHO estimates that more than 55 million people (8.1 % of women and 5.4% of men over 65 years) are living with dementia. This number is estimated to rise to 78 million by 2030 and to 139 million by 2050. Aggregation of amyloid- β ($A\beta$) peptides into fibrils or other self-assembled states is central to Alzheimer's disease (AD) pathogenesis (8). Alois Alzheimer noticed a presence of amyloid plaques and a massive loss of neurons while examining the brain of his first patient that suffered from memory loss and change of personality before dying and described the condition as a serious disease of the cerebral cortex. Emil Kraepelin named this medical condition Alzheimer's disease for the first-time (9) cardinal symptoms of Alzheimer's disease (AD) in the early stages are memory loss and modest changes in behavior (10).

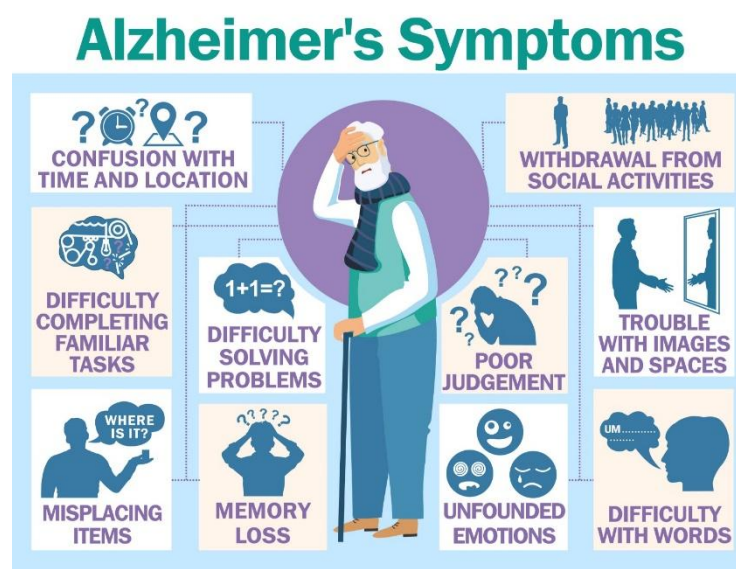


Fig 1.3 Alzheimer's symptoms

1.1.1 AMYLOID- β :

Amyloid- β ($A\beta$) protein was proposed as the trigger for a cascade of events in the brain that lead to Alzheimer's disease (9). Amyloid- β ($A\beta$) deposit in the parenchyma is a major characteristic in Alzheimer's disease (AD), and the impaired glymphatic clearance contributes to the $A\beta$ accumulation (10). Aggregation of amyloid- β ($A\beta$) peptides into fibrils or other self-assembled states is central to Alzheimer's disease (AD) pathogenesis (11). Beta-amyloid ($A\beta$) is present in the brain's interstitial fluid (ISF) and is considered a metabolic "waste product" (12). Essentially all neurodegenerative diseases are associated with misaccumulation of cellular waste products. Of these, misfolded or hyperphosphorylated proteins are among the most difficult for the brain to dispose. For example, tau and β -amyloid can accumulate as stable aggregates that are neurotoxic in conditions such as Alzheimer's disease (13).

A widely accepted assumption is that AD begins with abnormal processing of amyloid precursor protein (APP), which then leads to excess production or reduced clearance of β -amyloid ($A\beta$) in the cortex (14). There are several $A\beta$ species in the brain and plasma, such as $A\beta_{1-40}$ and $A\beta_{1-42}$, consisting of 40 and 42 amino acids, respectively. $A\beta_{1-42}$ is more toxic and aggregates more easily than $A\beta_{1-40}$ (15).

1.1.2 TAU PROTEIN:

The 2 proteins that, in their aggregated state, histopathologically define AD are β -amyloid ($A\beta$) and tau (16). tau is hyperphosphorylated, accumulating in the form of intracellular neurofibrillary tangles. This compromises its microtubule-binding ability and promotes neurodegeneration, and leads to accumulation of microtubule-transported APP, further contributing to neurodegeneration (17). A number of studies have shown increased concentrations of microtubule-associated tau protein in cerebrospinal fluid (CSF) of individuals clinically diagnosed as having probable.

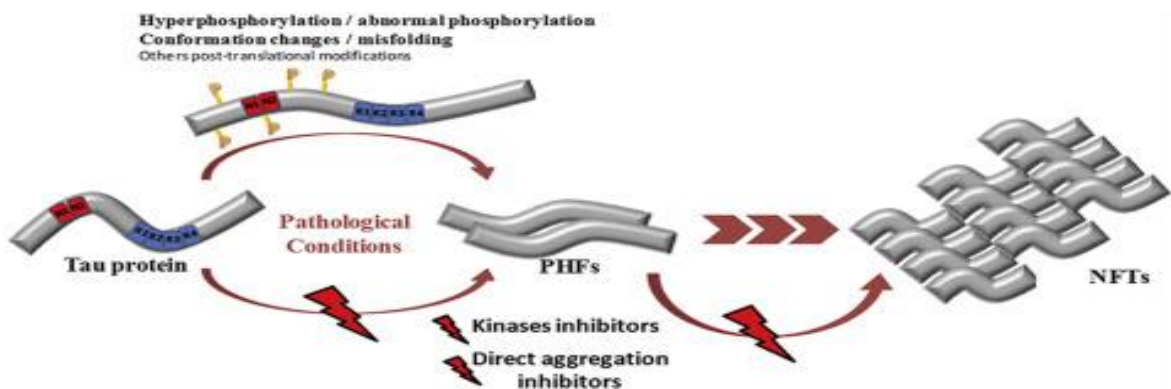


Fig 1.4 Tau protein and neurofibrillary tangles

Alzheimer's disease (AD), suggesting that CSF tau measurements might be useful in the diagnosis of AD and in the evaluation of treatment strategies (18). The normal tau protein is a human brain phosphoprotein with six isoforms ranging in size from 352-441 amino acids (19). Tau protein transference via the extracellular space, the compartment that is cleared by the glymphatic pathway. Neurofibrillary tangles (NFTs) are one of the key pathological hallmarks of Alzheimer's disease. NFTs primarily consist of phosphorylated tau (pTau). Clearance of extracellular tau from the brain is much slower than that of $A\beta$, and specific transporters for tau exit across the blood-brain barrier have not been identified (20).

1.1.3 NEUROINFLAMMATION:

Neuroinflammation associated with AD is another factor which could exacerbate glymphatic clearance impairment (21). Neuroinflammation might be a vital component in the initiation of AD pathology. Epidemiological studies have shown positive associations between dementia and previous reports of infection, smoking and diabetes (22). The release of pro-inflammatory molecules can lead to synaptic dysfunction, neuronal death and inhibition of neurogenesis (23). Amyloid- β can directly cause neuronal damage and death in differentiated neurons as in the AD brain. Indirectly, it can stimulate the production of reactive oxygen species (ROS); β -pleated aggregates, fibrillar Ab, and NFTs are also able to directly activate the classical complement pathway in vitro (24), understandings of the meningeal lymphatic and glymphatic system has led to the hypothesis that glymphatic dysfunction is an early side-effect of neuro-inflammation (25). Besides BBB disruption, inflammation can also contribute to glymphatic dysfunction. Inflammation slows down the convective flow, decreases CSF-to-ISF turnover and impairs glymphatic clearance (26).

Table 1.1. RISK FACTORS FOR AD

S.No	Risk Factors	Cause
1.	Age	Age is the strongest risk factor for AD. The risk of AD doubles every 5 years after the age of 65 and there is a 50% chance of having AD after the age of 85 (27).
2.	Gender	Age-adjusted rates for women were significantly higher than for men. This female excess is confirmed by 13 population surveys that report overall rates for AD by sex (28).
3.	Traumatic brain injury	the increased frequency of traumatic brain injury (TBI) in the previous history of AD patients. TBI could cause damage to the BBB resulting in leakage of plasma proteins and sensitization of the immune system to brain antigens normally isolated from them. Subsequently, a number of studies reported a connection between head injury and AD

		(29).
4.	Social class and education	Social class is a useful variable with which to begin an epidemiological examination of AD, because it could point to differential exposure or vulnerability. But the inquiry is hampered by the likelihood of biased case-finding, since many of the instruments used to detect dementia are highly sensitive to education, which is closely related to social class (30).
5.	Life style	• Alcohol drinking has been proposed as a possible risk factor for cognitive impairment and dementia, though understanding the association has proved difficult. Some studies have found no association between alcohol drinking and cognitive impairment or Alzheimer's disease, whereas others have found an association between heavy drinking and increased risk of dementia (31). • smoking was associated with an increased risk of AD (pooled relative risk, 1.79; 95 % CI, 1.43-2.23), Thus, in contrast to the initial hypothesis of a possible protective effect, cigarette smoking actually increases the risk of AD (32).

1.1.4 NEUROGENETICS:

As a polygenic disorder, the etiopathogenesis of AD is based on several genetic and epigenetic (genetic-environmental interaction) factors. A gamut of additional genes including apolipoprotein E (APOE) E4 allele has been found to be potential risk factors for AD. The genetic basis of early onset of Alzheimer's disease is found to be based on three main genes which include amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2). According to AlzForum, additionally, two genes are linked with AD by means of genetics or the neuropathology of the encoded proteins (TREM2 - Triggering

receptor expressed on myeloid cells 2; MAPT - microtubule-associated protein). A recent study showed that, of 53 mutation carriers identified in the cerebrospinal fluid (CSF) biomarkers, forty-six (87%) depicted aberrant changes in all the three CSF biomarkers including total tau protein (Tau), phospho-tau protein (P-Tau), and amyloid β (A β).

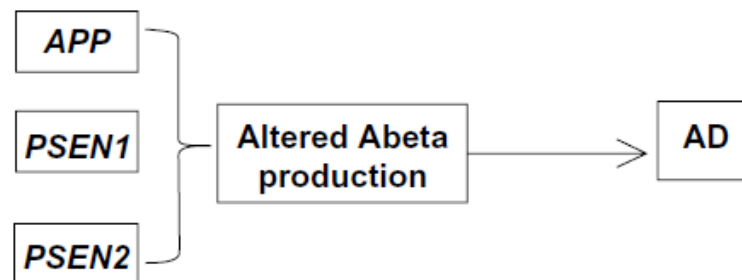


Fig 1.5 Neurogenetics

The amyloid precursor protein (APP) gene, presenilin (PSEN1 and PSEN2) genes involved in early onset Alzheimer's disease (AD); Abeta - Amyloid beta.

1.2 GLYMPHATIC SYSTEM:

The glymphatic system (or glymphatic clearance pathway, or paravascular system) was described and named in 2013 as a system for waste clearance in the central nervous system (CNS) of vertebrates. According to this model, cerebrospinal fluid (CSF) flows into the paravascular space around cerebral arteries, combining with interstitial fluid (ISF) and parenchymal solutes, and exiting down venous paravascular spaces. The name "glymphatic system" was coined by the Danish neuroscientist Maiken Nedergaard in recognition of its dependence upon glial cells and the similarity of its functions to those of the peripheral lymphatic system. Glymphatic flow was initially believed to be the complete answer to the long-standing question of how the sensitive neural tissue of the CNS functions in the perceived absence of a lymphatic drainage pathway for extracellular proteins, excess fluid, and metabolic waste products. The glymphatic pathway is connected to a classic lymphatic network, associated with dural meninges covering the brain, as well as sheaths of cranial nerves and blood vessels, or drains via the olfactory route, then exiting through cranial foramina. The glymphatic system is a recently discovered macroscopic waste clearance system that utilizes a unique system of perivascular channels, formed by astroglial

cells, to promote efficient elimination of soluble proteins and metabolites from the central nervous system (33). The glymphatic (a portmanteau of glial and lymphatic) system was first identified in the rodent brain in 2012 (34). Glymphatic clearance plays a critical role in metabolic homeostasis in the brain (35). The term is also coined considering that astrocytes carrying the aquaporin-4 (AQP-4) water channel support the CSF-ISF exchange and solute clearance (36). A β clearance impairment reduced the waste elimination from the brain and contributed to the A β deposits in brain parenchyma (37). Glymphatic clearance plays a critical role in metabolic homeostasis in the brain. In addition to solute clearance, other functions of the glymphatic system were further identified. CSF influx was observed to be a vessel for glucose and other nutrients, to be up taken by neurons and astrocytes (38). The glymphatic system is a glial-dependent waste clearance pathway in the brain, in place of lymphatic vessels, dedicated to drain away soluble waste proteins and metabolic products. Specifically, the glymphatic network serves as a “front end” for waste clearance, and is connected downstream to an authentic lymphatic network, associated with dura covering the brain as well as cranial nerves and large vessels at the skull exits. The anatomical and functional interconnections between these two networks are not completely understood (39). Waste removal from the central nervous system is essential for maintaining brain homeostasis across the lifespan. Two interconnected, dynamic networks were recently uncovered, which may provide new information concerning the conundrum of how the brain manages waste removal in the absence of authentic lymphatic vessels (LVs). The glymphatic system serves as the brain’s “front end” waste drainage pathway that includes a perivascular network for cerebrospinal fluid (CSF) transport (40). The first pioneering studies documented that soluble amyloid beta (A β) protein and tau oligomers – metabolic waste products whose buildup is associated with Alzheimer’s disease (AD) – were transported from the interstitial fluid (ISF) space and out of the brain via the glymphatic system (41). The waste clearance system is driven by the water coming through the perivascular space of the artery. It perfuses through the brain parenchyma moving the waste molecules and, finally, move out of the brain through the perivascular space of the vein. This process is somehow helped by the astrocytes, with aquaporin channels on their foot processes (42). The glymphatic system consists of three principal sequential anatomic segments: (i) CSF inflow along the

perivascular spaces surrounding penetrating arteries, (ii) dispersion of CSF through the wider interstitium, and (iii) efflux of ISF along the large-caliber draining veins to re-enter the CSF within the ventricular and cisternal compartments.

From the subarachnoid space, CSF is driven into the Virchow-Robin spaces by a combination of arterial pulsatility, respiration, slow vasomotion, and CSF pressure gradients. The loose fibrous matrix of the perivascular space can be viewed as a low resistance highway for CSF influx. The subsequent transport of CSF into the dense and complex brain parenchyma is facilitated by AQP4 water channels expressed in a highly polarized manor in astrocytic endfeet that end sheathe the brain vasculature (43).

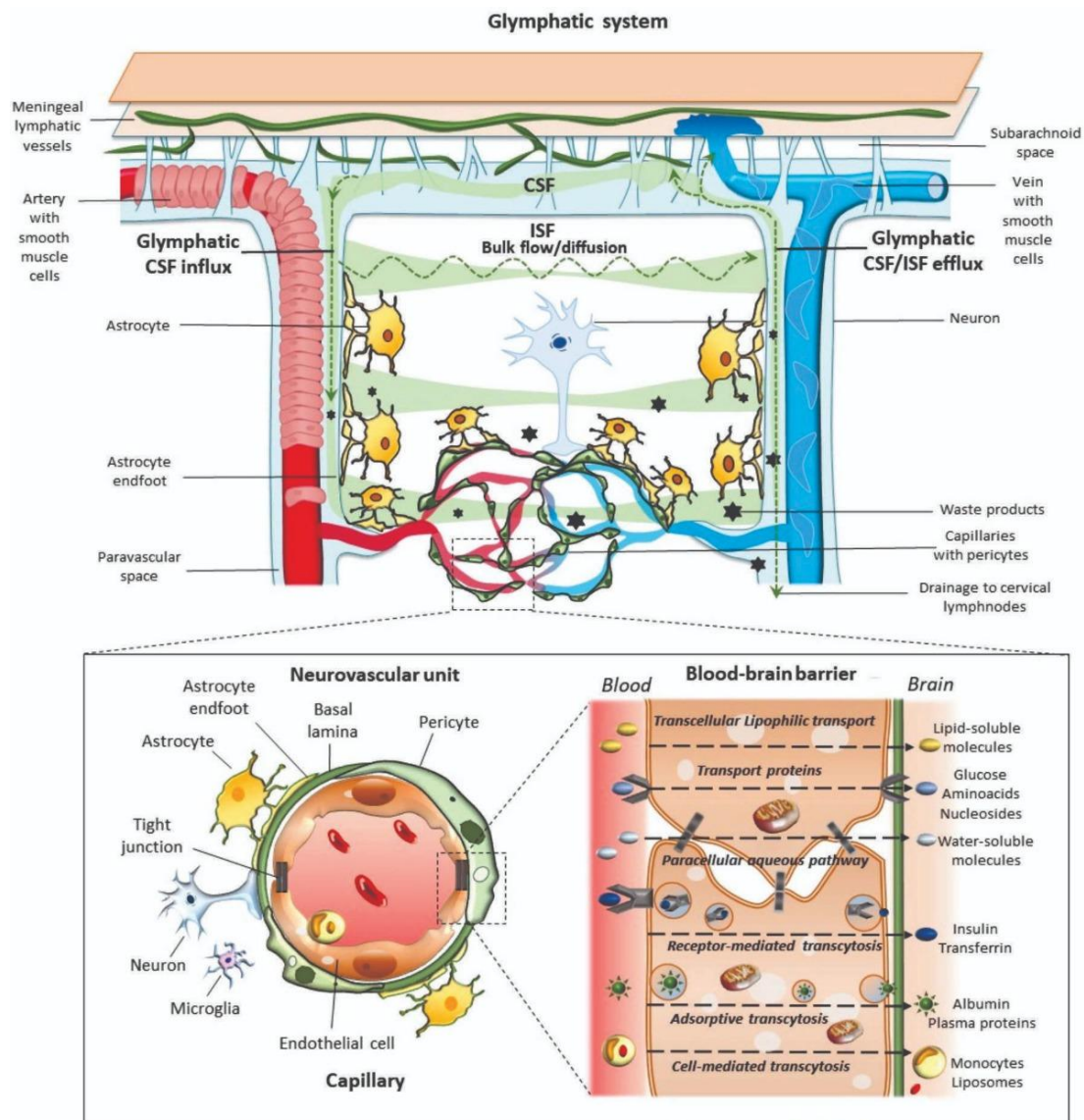


Fig 1.6 GLYMPHATIC SYSTEM

1.2.1 GLYMPHATIC SYSTEM IN ANIMAL MODELS:

The glial-lymphatic or glymphatic pathway is a fluid clearance pathway recently identified in the rodent brain. This pathway subserves the flow of cerebrospinal fluid (CSF) into the brain along arterial perivascular spaces and thence into the brain interstitium facilitated by aquaporin-4 (AQP4) water channels. The pathway then directs flows towards the venous perivascular and perineuronal spaces, ultimately clearing solutes from the neuropil into meningeal and cervical lymphatic drainage vessels. Glymphatic dysfunction has been demonstrated in animal models of traumatic brain injury (TBI), Alzheimer's disease (AD) and micro-infarct disease, most likely in relation to perturbed expression of AQP4. The recent characterizations of the glymphatic and meningeal lymphatic systems calls for reevaluation of the anatomical routes for CSF-ISF flow and the physiological role that these pathways play in CNS health. Furthermore, PET studies reveal that A β accumulates in the healthy brain after a single night of sleep deprivation, suggesting that the human glymphatic pathway might also be primarily active during sleep. The observed reduction in CSF clearance was associated with increasing grey-matter concentrations of A β in the human brain, consistent with findings in mice showing that decreased glymphatic function leads to A β accumulation. Research is needed to confirm whether specific factors driving glymphatic flow in rodents also apply to humans. Longitudinal imaging studies evaluating human CSF dynamics will determine whether a causal link exists between reduced brain solute clearance and the development of neurodegenerative diseases (44).

1.2.2 ROLE OF AQUAPORIN-4:

Aquaporin-4, also known as AQP-4, is a water channel protein encoded by the AQP4 gene in humans. AQP-4 belongs to the aquaporin family of integral membrane proteins that conduct water through the cell membrane. A limited number of aquaporins are found within the central nervous system. Aquaporin-4 was first identified in 1986. It was the first evidence of the existence of water transport channels. Aquaporin-4 (AQP4) is one of the most abundant molecules in the brain and is particularly prevalent in astrocytic membranes at the blood-brain

and brain-liquor interfaces. Only recently has evidence accumulated to suggest that AQP4 is involved in such diverse functions as regulation of extracellular space volume, potassium buffering, cerebrospinal fluid circulation, interstitial fluid resorption, waste clearance, neuroinflammation, osmosensation, cell migration, and Ca (2+) signaling (45). The method that was used to discover the existence of the transport channels was through knockout experiments. With this technique they were able to show the significant role of AQP4 in CNS injuries and brain water imbalances. Additionally, AQP4 can also be located in epithelial cells of many organs throughout the human body, such as the kidney, intestine, salivary glands, sensory organs, and skeletal muscles (46). AQP4 is also expressed in astrocytes and is upregulated by direct insult to the central nervous system (47). Based on work in animal models, aquaporin-4 may have a role in several other diseases including Alzheimer's disease, amyotrophic lateral sclerosis, Parkinson's disease, multiple sclerosis, and epilepsy, and appears to have a role in pathological response to traumatic brain injury and stroke. In most studies, AQP4 appears to be important in facilitating extravascular transport. However, the explanation of its effects is almost certainly not that it facilitates flow by providing a conduit for the flow of water between perivascular spaces and interstitial spaces of the parenchyma. The importance of AQP4 for entry and exit of solutes must lie in somehow facilitating movement of solutes including NaCl. Note that in the steady-state, the glymphatic hypothesis requires that periarterial inflow, net flow through the parenchyma and perivenous outflow must be the same -- there can be no accumulation or depletion of fluid in the perivascular spaces or interstitium, otherwise the volumes would be changing. This hypothesis takes no account of fluid derived from secretion across the blood– brain barrier.

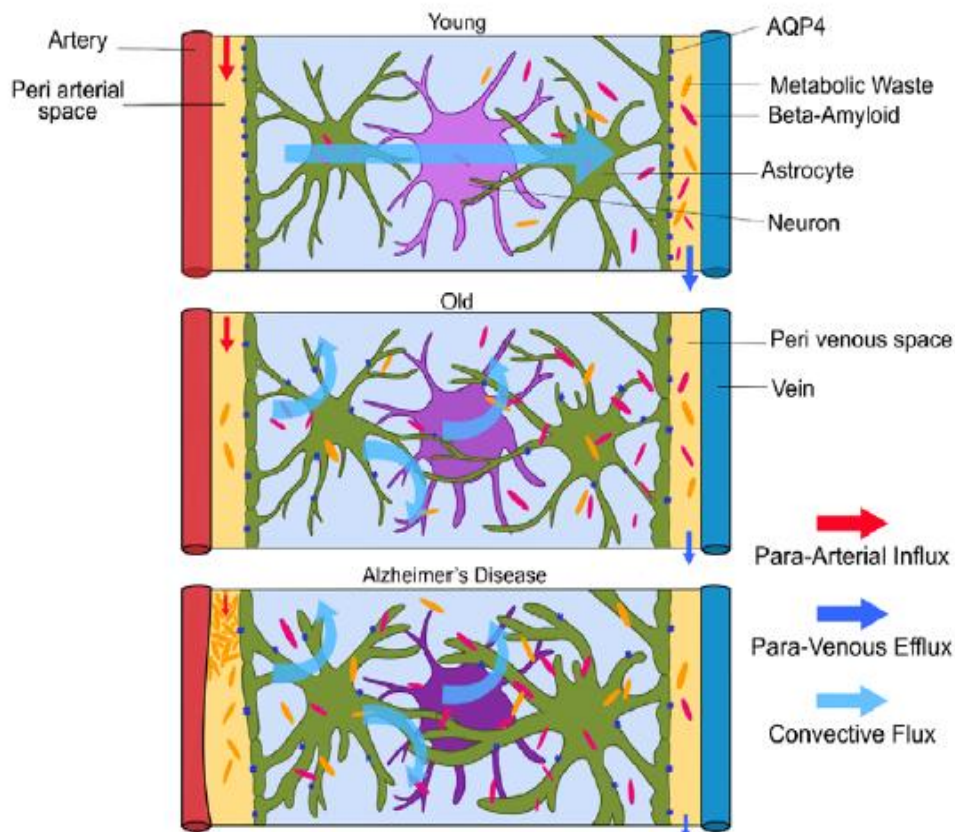


Fig 1.7 Model of glymphatic function in Young, Old and Alzheimer's disease.

In young people, CSF travels along periarterial routes, entering the brain parenchyma, and washes solutes and waste products into the veins. In older people, the loss of AQP4 water channels will result in reduced glymphatic clearance. In those with Alzheimer's disease, the accumulation of amyloid-beta impairs fluid movement within the interstitial space, decreasing glymphatic clearance.

1.2.3 MECHANISMS:

The glymphatic pathway is a highly-organized fluid transport system. In its initial segments, CSF from the subarachnoid space flows into brain through perivascular spaces of the large leptomeningeal arteries. The findings that interstitial bulk flow may be drained from the brain via lymphatic channels (ie, cervical lymph nodes) suggest that a conventional lymphatic system might exist in the mammalian brain. The glymphatic system, appropriately named based on its functional similarity to the lymphatic system in the periphery,¹¹ acts as a brain-wide convective flux of CSF and ISF that is strictly dependent on isoform water

channel aquaporin-4 (AQP4) expressed in astrocyte foot processes. The brain is well equipped for internal lipid transport via its own supply of lipid carrier particles of the high-density lipoprotein type secreted by astrocytes (48). Thus, a role of the glymphatic system is to assist in the clearance of unwanted and potentially noxious proteins, and seeking treatments that enhance drainage of amyloid- β and microtubule-associated tau is rational. Many other solutes have specific transporters at the blood-brain barrier and although they do not cross in such quantities, they can nevertheless move by the intravascular route in sufficient amount to satisfy the requirements of the brain. Transfer of the fluid from the periarterial space into the parenchymal interstitium with some of the water passing through AQP4 in the end foot membrane facing the endothelial cells. In some versions of the glymphatic hypothesis the perivenous outflow is thought to enter the subarachnoid spaces where it mixes with CSF. These versions can easily be modified and extended to include outflow via white matter tracts leading to the ventricles (49). How the rapid flow through the space was reconciled with the much slower entry into the parenchyma along the penetrating arteries. Presumably entry into the periarterial spaces of markers added to CSF occurs in the basal cisterns where CSF from the cisterna magna first encounters the arteries. This entry may occur via stomata found in the pial sheath of all arteries inspected in the subarachnoid space. I put forward a counter-view that no sheath is needed to explain the observations because the subarachnoid space on the dorsal surface of the brain is collapsed everywhere except in the periarterial regions. Their data and the firm evidence from electron microscopy for the presence of a sheath (50). Evidence that periarterial spaces surrounding subarachnoid arteries are used as conduits for fluid flow and solute movements also comes from experiments tracking the course of microspheres added to CSF in the cisterna magna. Pulsatile back and forth movements of the microspheres in time with the cardiac pulse were seen. Furthermore, when the trajectories of these were plotted on top of an image of the blood vessels, they were alongside the arteries. They found that effective perivascular pumping would require changes in the dimensions of the perivascular spaces to be near 50%.

In the glymphatic pathway, convective influx of CSF is balanced by perivenous efflux of ISF, which rids the neuropil of toxic proteinaceous

metabolites, including A β (51). CSF production contributes to parenchymal influx, and inhibiting CSF production in rodents by treatment with acetazolamide significantly impairs CSF clearance. drainage route for CSF-derived soluble molecules. Recent studies demonstrated that meningeal lymphatic vessels also regulated the fitness of glymphatic pathway it is influenced by the production and drainage of CSF fluid and it is involved in regulating cell volume and extracellular space dimensions.

1.3 TREATMENT:

1.3.1 APPROVED DRUGS FOR ALZHEIMER DISEASE:

Current treatments for patients with Alzheimer's disease aim to improve behavioral, cognitive, and non-cognitive symptoms. The acetylcholinesterase (AChE) inhibitors, rivastigmine, donepezil, and galantamine, and the N-methyl-D-aspartate (NMDA) receptor antagonist, memantine, have been the main pharmacological treatments for Alzheimer's disease (52).

1. **Donepezil's** cost-effectiveness was demonstrated regardless of whether outcomes were measured in terms of improvements in cognitive impairment, functional impairment or health-related quality of life, and whether costs were measured just for the health and social care system or for society as a whole (53).
2. **Rivastigmine** is a modern cholinesterase inhibitor with established therapeutic efficacy. Rivastigmine is particularly good in patients fulfilling the criteria of dementia with Lewy body (54).
3. **Galantamine** (Reminyl), an approved treatment for Alzheimer's disease (AD), is a potent allosteric potentiating ligand (APL) of human $\alpha 3\beta 4$, $\alpha 4\beta 2$, and $\alpha 6\beta 4$ nicotinic receptors (nAChRs). galantamine acts as an nAChR inhibitor. The other approved AD drugs, donepezil and rivastigmine, are devoid of the nicotinic APL action; at micromolar concentrations they also block nAChR activity (55).
4. N-methyl-D-aspartate (NMDA) antagonist **memantine** appears to be helpful in patients with moderate-to-severe Alzheimer's disease. There is evidence that an increased plasma homocysteine level is an independent risk factor for the development of Alzheimer's disease (56).

5. The **Donepezil and Memantine** in Moderate to Severe Alzheimer's Disease (DOMINO) study was a multicenter, double-blind, placebo-controlled, clinical trial with a two-by-two factorial design. The outcomes were assessed for 52 weeks (57).
6. **Aducanumab:** The FDA recently approved aducanumab (Aduhelm) for people with mild symptoms of Alzheimer disease, such as individuals who are still independent in basic daily functioning. It reduced brain amyloid plaque (protein deposits), a main feature of Alzheimer disease. Plaque reduction, however, has not been a reliable marker for cognitive function in past trials. Not everyone with plaques has or will get Alzheimer disease, and other plaque-reducing medications have not shown meaningful patient benefit. Adverse effects were common. In the trials, 41% of patients experienced brain swelling or bleeding. While most cases were mild and managed with dose reduction, 1% to 2% of patients required hospitalization or had long-lasting impairment. A recent patient death linked to aducanumab is under investigation.
7. **Lecanemab** reduced markers of amyloid in early Alzheimer's disease and resulted in moderately less decline on measures of cognition and function than placebo at 18 months but was associated with adverse events. Longer trials are warranted to determine the efficacy and safety of lecanemab in early Alzheimer's disease.

1.3.2 DRUGS UNDER CLINICAL TRIAL:

In addition to the anti-amyloid and anti-tau targeted drugs various other drugs are under clinical trials which include Nicotinic Receptors Agonist, Antioxidants, PPAR gamma agonist, Gamma secretase inhibitor, 5HT-6 Antagonist, Statins and Others.

Table 1.2 Drugs under clinical trials:

Class	Drugs
Nicotinic Receptors Agonist	4OH-GTS-21
Antioxidants	• Ginkgo biloba • Vitamin-E • Melatonin

PPAR gamma agonist	Pioglitazone
Gamma secretase inhibitor	Semagacestat
5HT-6 Antagonist	SB-271046
Statins	• Simvastatin • Pravastatin
Others	• heavy metal chelators • Estrogens • Antiinflammatory drugs

1.3.3 GLYMPHATIC SYSTEM TARGETED-TREATMENT:

1. L-3-n-butylphthalide (NBP) showed the potential neuroprotective effect in the rodent models of AD. The effects of NBP on the glymphatic system were explored in this study. In the wild-type mice, both CSF tracer influx and perivascular drainage increased after NBP treatment compared with vehicle treatment (58).

2. N-3 polyunsaturated fatty acids (PUFAs), by use of fat-1 transgenic mice and oral administration of fish oil, significantly promote interstitial A β clearance from the brain and resist A β injury. n-3 PUFAs are reported to have the ability to decrease amyloid- β (A β) production, modulate A β aggregation, or counteract A β downstream toxicity and it exert protective roles in reducing A β accumulation via mediating the glymphatic system function (59).

3. DSCR1 (Down syndrome critical region 1) is highly expressed in the brain and heart. DSCR1 affects the development of dorsal meningeal lymphatic vessels and facilitates A β clearance in a 5XFAD mouse model. DSCR1 as a new regulator of meningeal lymphatic vessel growth and suggested it as a potential therapeutic target for the treatment of AD and AD-related dementias (60).

1.3.4 PRO DRUGS/ MIMETICS:

Many prodrugs and biomimetics are also used in various animal models for the treatment of AD. Examples; ramiprosate, a glycosaminoglycan mimetic; 7,8-dihydroxyflavone (7,8-DHF), a BDNF mimetic; Huperzine A (Hup A), isolated from the Chinese herb *Huperzia serrata* (Thunb) selective AChE inhibitor; ALZ-801 is an oral, small-molecule inhibitor of beta amyloid (A β) oligomer formation; Memogain has been recently described, this pro-drug of galantamine, etc.

1.3.5 ALTERNATIVE THERAPIES:

a) Acupuncture:

Acupuncture and electroacupuncture (EA) have been commonly used as a complementary therapy by TCM practitioners in China for the treatment of various neurodegenerative disorders (61).

b) Physical Exercise:

Exercise causes changes in the brain at the anatomic, cellular, and molecular levels by inducing a cascade of cellular and molecular processes that promote different physiologic phenomena, including angiogenesis, neurogenesis, synaptogenesis, and stimulation of neurotrophic factors that enhance learning, memory, and brain plasticity (62).

c) Ethnobotanicals:

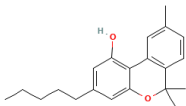
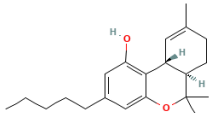
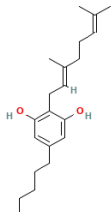
Many ethnobotanicals (plant extracts and plant derived active constituents) have been studied for the treatment of neurodegenerative diseases including AD. In the current study cannabis derived compounds (Cannabinoids) were assessed for their possible glymphatic clearance and neuroprotective effects.

1.3.6 CANNABIS SATIVA:

Cannabis is the botanical name of a genus within the Cannabaceae family. The genus includes three species, *C. sativa*, *C. ruderalis* and *C. indica*. The vernacular name of *Cannabis sativa*: Ganja-chedi in Tamil, Marijuana in English, jia in Bengali and Bhang ganja in Assamese. The extracts of *C. sativa* have been demonstrated to have analgesic, antiemetic, anxiolytic, anticonvulsant activity and neuro-protective effect (63). There are over 100 different Phyto cannabinoids, including cannabidiol (CBD), cannabigerol (CBG), cannabichromene (CBC), Δ 9-

tetrahydrocannabivarin (Δ^9 -THCV), cannabidivarin (CBDV), Δ^9 -tetrahydrocannabinol (Δ^9 -THC), Δ^9 -tetrahydrocannabiphorol (Δ^9 -THCP), Δ^9 -tetrahydro-cannabutol (Δ^9 -THCB), Δ^9 -tetrahydrocannabivarinic acid (Δ^9 -THCVA) and Δ^9 - tetrahydrocannabinolic acid (Δ^9 -THCA) etc., (64).

Table 1.3 Different types of phyto cannabinoids:

S.no	DRUG	CHEMICAL STRUCTURE	DESCRIPTION
1	Cannabinol		Cannabinol is a cannabinoid isolated from the plant <i>Cannabis</i> that is a metabolite of tetrahydrocannabinol (THC), with potential immunosuppressive and anti-inflammatory activities (65).
2	Δ^9 -tetrahydrocannabinol		Dronabinol is an isomer of tetrahydrocannabinol (THC) that is the main and most active isomer found in the <i>Cannabis sativa</i> L. plant, with potential anti-emetic, analgesic and appetite stimulating activities (66).
3	Cannabigerol		Cannabigerol is a member of the class of resorcinols that is resorcinol which is substituted by a (2E)-3,7-dimethylocta-2,6-dien-1-yl group at position 2 and by a pentyl group at position 5 (67).

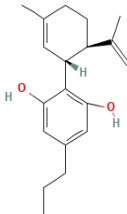
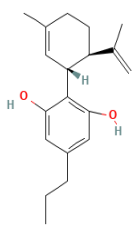
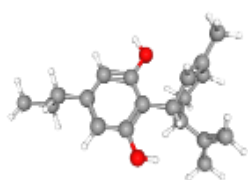
4	Cannabidivarin		Cannabidivarin, also known as cannabidivarol or CBDV, is a non-psychoactive cannabinoid and CBDV have demonstrated anticonvulsant activity in animal and human models (68).
---	----------------	---	---

Table 1.4 Cannabidivarin 2D and 3D structures:

CANNABIDIVARIN:

2D Structure	3D Structure
	

Cannabidivarin, also known as cannabidivarol or CBDV, is a non-psychoactive cannabinoid found within [DB14009]. It is one of over 100 cannabinoids identified from the *Cannabis* plant that can modulate the physiological activity of *Cannabis*, or marijuana. Compared to its homolog, [DB09061], CBDV is shortened by two methyl (CH₂) groups on its side chain. Notably, both [DB09061] and CBDV have demonstrated anticonvulsant activity in animal and human models and are demonstrating promising clinical trial results (68).

CHAPTER 2

LITERATURE SURVEY

Tiwari et al. (2019) was reviewed on the topic Alzheimer's disease: pathogenesis, diagnostics, and therapeutics (2). The present review discusses the current state of the art in AD therapeutics and diagnostics, including labeling and imaging techniques employed as contrast agents for better visualization and sensing of the plaques. The review points to an urgent need for nanotechnology as an efficient therapeutic strategy to increase the bioavailability of drugs in the central nervous system.

Ishida et al. (2022) Researched on the 5-Caffeoylquinic Acid Ameliorates Cognitive Decline and Reduces A β Deposition by Modulating A β Clearance Pathways in APP/PS2 Transgenic Mice (3). Histochemical analysis revealed that 5-CQA substantially reduced A β plaque formation and neuronal loss in the hippocampi. And They showed the result that 5-CQA reduces A β deposition in the brain by modulating the A β clearance pathways and ameliorating cognitive decline and neuronal loss in APP/PS2 mice. Thus, 5-CQA may be effective in preventing cognitive dysfunction in AD.

Yang et al. (2016) have done a review on the title Aquaporin-4 and Alzheimer's disease (4). Their recent studies have revealed that AQP4 is important to the clearance of A β in brain via lymphatic clearance, transcytotic delivery, and glial degradation, as well as to the synaptic function. Thus, AQP4 likely plays an important role in the pathogenesis of AD.

Hu et al. (2019) were made an article named Nutrition and the Risk of Alzheimer's Disease (6). Increasing epidemiological studies suggest that diet and nutrition might be important modifiable risk factors for AD. Adherence to a healthy diet, the Japanese diet, and the Mediterranean diet is associated with a lower risk of AD. So this paper will focus on the evidence linking many nutrients, foods, and dietary patterns to AD.

Qiang et al. (2017) Have worked on the Structural variation in amyloid- β fibrils from Alzheimer's disease clinical subtypes (8). Here they compared two atypical Alzheimer's disease clinical subtypes-the rapidly progressive form (r-AD) and the posterior cortical atrophy variant (PCA-AD)-with a typical prolonged-duration form (t-AD). These results demonstrate the existence of a specific predominant A β 40 fibril structure in t-AD and PCA-AD, suggest that r-AD may

relate to additional fibril structures and indicate that there is a qualitative difference between A β 40 and A β 42 aggregates in the brain tissue of patients with Alzheimer's disease.

Nedergaard (2013) In solo he worked the title "Neuroscience. Garbage truck of the brain". In his journal he shows that an intercellular "glymphatic" pathway clears cell waste from the brain (13). So, it may be a new target's for treating neurodegenerative diseases like Alzheimer's (AD), dementia, Parkinson's Disease (PD), and etc, . . .

Sery et al. (2013) was write an review on Molecular mechanisms of neuropathological changes in Alzheimer's disease: a review (17). The main aim of recent research is focused on the question what is primary mechanism leading to the molecular development of AD pathology. Here their review shows that the novel insight into a number of cellular signaling mechanisms, as well as mitochondrial function in Alzheimer disease could also bring explanations of initial processes leading to the development of this pathology.

Harrison et al. (2020) have made an research in Impaired glymphatic function and clearance of tau in an Alzheimer's disease model (51). They establish the central role of AQP4 in the glymphatic clearance of tau from the brain; and they show that this system presents as a novel druggable target for the treatment of Alzheimer's disease, and possibly other neurodegenerative diseases alike.

Lyman et al. (2014) were done a review on Neuroinflammation: the role and consequences (23). They gathered evidence here indicates that neuroinflammation causes and accelerates long-term neurodegenerative disease, playing a central role in the very early development of chronic conditions including dementia. They discuss wide scope and numerous complexities of neuroinflammation suggest that combinations of different preventative and therapeutic approaches may be efficacious.

Qiu et al. (2009) Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention (32). They brief about the long-term multidomain interventions toward the optimal control of multiple vascular risk factors and the maintenance of socially integrated lifestyles and mentally

stimulating activities are expected to reduce the risk or postpone the clinical onset of dementia, including Alzheimer's disease.

Iliff et al. (2012) have researched on A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β (40). On the basis of in vivo two-photon imaging of small fluorescent tracers, they showed that CSF enters the parenchyma along paravascular spaces that surround penetrating arteries and that brain interstitial fluid is cleared along paravenous drainage pathways. At the last they brief about the Clearance through paravenous flow may also regulate extracellular levels of proteins involved with neurodegenerative conditions, its impairment perhaps contributing to the mis-accumulation of soluble proteins.

Jessen et al. (2015) have write an review on the topic The Glymphatic System: A Beginner's Guide (33). Since the concept of the glymphatic system is relatively new, they are here to review its basic structural elements, organization, regulation, and functions. They will also discuss recent studies indicating that glymphatic function is suppressed in various diseases and that failure of glymphatic function in turn might contribute to pathology in neurodegenerative disorders, traumatic brain injury and stroke.

Ren et al. (2017) Have worked on the Omega-3 polyunsaturated fatty acids promote amyloid- β clearance from the brain through mediating the function of the glymphatic system (59). And here they show that n-3 polyunsaturated fatty acids (PUFAs), by use of fat-1 transgenic mice and oral administration of fish oil, significantly promote interstitial A β clearance from the brain and resist A β injury. The results of the impressive study proven the novel mechanism by which n-3 PUFAs exert protective roles in reducing A β accumulation via mediating the glymphatic system function.

Choi et al. (2021) were researched on DSCR1 [(Down syndrome critical region 1), known also as RCAN1 (regulator of calcineurin 1)] upregulation enhances dural meningeal lymphatic drainage to attenuate amyloid pathology of Alzheimer's disease (60). Accumulation of amyloid plaques in the meninges and brain, and causes learning and memory deficit. So, they discovered that DSCR1 facilitates the drainage of waste products by increasing the coverage of dorsal

meningeal lymphatic vessels. In last their data indicate that DSCR1 facilitates the growth of dorsal meningeal lymphatics to improve drainage efficiency and protect against Alzheimer's disease (AD) pathologies.

To improve the clinical diagnostic accuracy of the Alzheimer's disease, around two decades ago Blennow et al. (1995) (19). Have used Cerebrospinal fluid (CSF) biochemical markers for the diagnosis on a title Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer disease. since tau can be detected in CSF, two assays based on several well-defined monoclonal tau antibodies were used to study these proteins in CSF. When the results were obtained for CSF-tau. Not less than 35/40 (88%) of AD patients had a CSFPHFtau value higher than the cutoff level of 1140 pg/mL in controls. By this study, they just demonstrate that elevated tau/PHFtau levels are consistently found in CSF of AD patients.

Baskaran et al. (2020) have done research on INSILICO METHOD FOR PREDICTION OF MAXIMUM BINDING AFFINITY AND LIGAND – PROTEIN INTERACTION STUDIES ON ALZHEIMER'S DISEASE (70). Here they discuss about the interactions of three ligands were compared with one another, in that Hydrogen bond formation of Chlorzoxazone and Donepezil with 4EY6 and 4EY7 interacting the similar aminoacids residues (4EY6-ARG165; 4EY7-ASP74) were studied using insilico studies.

Kanthamma et al. (2018) has researched on the topic 'Insilico approach of interaction studies in Bacopa monnieri compounds targeting multi-proteins for Alzheimer's Disease' (71). In this, they concluded that depicts the Bacopa monnieri derivatives having significant role to design new compounds with these properties for treating Alzheimer's disease.

Takata et al. (2004) have researched on the title High mobility group box protein-1 inhibits microglial Abeta clearance and enhances Abeta neurotoxicity (73). Their results suggest that HMGB1 released from dying neurons may inhibit microglial Abeta42 clearance and enhance the neurotoxicity of Abeta42. HMGB1 may thus be another target in the investigation of a therapeutic strategy for AD.

Rosu et al. (2020) were worked on the heading Inhibition of Aquaporin 4 Decreases Amyloid A β 40 Drainage Around Cerebral Vessels (75). These data

suggest that AQP4 deficiency is directly involved in impaired A β brain clearance via the peri-/para-vascular routes, and AQP4-mediated vascular clearance might counteract blood-brain barrier abnormalities and age-related vascular amyloidopathy.

Li et al. (2004) was worked on the study of “Behavioral and neurochemical effects of the intrahippocampal co-injection of beta-amyloid protein 1-40 and ibotenic acid in rat's” (76). Their results suggest that the co-injection of Abeta1-40 with Ibo may induce an increase of hippocampal damage by peroxidation, and a serious learning and memory impairment of the rats. They result also suggest that the co-injection of Abeta1-40 with Ibo may provide a useful animal model for Alzheimer's disease (AD) research.

Milton (1999) have researched on the topic Amyloid-beta binds catalase with high affinity and inhibits hydrogen peroxide breakdown (77). He elaborates that The Amyloid beta-induced catalase inhibition involved formation of the inactive catalase Compound II and was reversible. Catalase $\leftrightarrow$ Abeta interactions provide rapid functional assays for the cytotoxic domain of Abeta and suggest a mechanism for some of the observed actions of Abeta plus catalase *in vitro*.

Henshaw et al. (2021) have reviewed on the topic The Effects of Cannabinoids on Pro- and Anti-Inflammatory Cytokines: A Systematic Review of *In Vivo* Studies (78). This review shows that CBD, CBG, and CBD+THC combination exert a predominantly anti-inflammatory effect *in vivo*, whereas THC alone does not reduce pro-inflammatory or increase anti-inflammatory cytokines.

Kozela et al. (2010) researched on the Cannabinoids Delta(9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells (79). In the summary, their observations show that CBD and THC vary in their effects on the anti-inflammatory pathways, including the NF-kappaB and IFNbeta-dependent pathways.

Götz et al. (2019) have done research on Structure–Effect Relationships of Novel Semi-Synthetic Cannabinoid Derivatives. They conclude with the CBD- and CBDV-derivatives studied are suitable for targeting CB-receptors. Some may be used as selective CB2 agonists. The length of the aliphatic rest at R² of CBD

(pentyl) and CBDV (propyl) did not correlate with the binding affinity. Higher polarity at R¹ appeared to favor the agonistic activity at CB2-receptors. (80)

CHAPTER 3

AIM AND SCOPE OF THE WORK

Alzheimer's disease has been increasing steadily, and with today's aging population. Alzheimer's disease (AD) is the most frequent cause of dementia in the world. WHO estimates that more than 55 million people (8.1 % of women and

5.4% of men over 65 years) are living with dementia. Currently more than 55 million people live with dementia worldwide, and there are nearly 10 million new cases every year. The assemblage of Amyloid beta ($A\beta$) and tau proteins in the hippocampus region of brain cause Alzheimer's disease (AD). Aggregation of amyloid- β ($A\beta$) peptides into fibrils or other self-assembled states is central to Alzheimer's disease (AD) pathogenesis (5).

The glymphatic system denotes a brain-wide pathway to eliminate substances in the extracellular fluid. The glymphatic system, that is facilitated by water channel aquaporin 4 (AQP4) localized to vascular end feet of astrocytes that facilitates exchange of cerebrospinal fluid (CSF) with interstitial fluid (ISF), may provide a clearance pathway for protein species such as amyloid-beta ($A\beta$) and tau, which accumulate in the brain in Alzheimer's disease (6).

To date only six drugs/ combinations were approved by the FDA for the treatment of AD. These drugs encompass cholinesterase inhibitors: donepezil, rivastigmine, galantamine; NMDA receptor antagonist: memantine; combination of donepezil (cholinesterase inhibitor) plus memantine; anti $A\beta$ monoclonal antibody: aducanumab and Lecanemab. The drugs used for Alzheimer's disease shows some side effects like head ache, dizziness, nausea and diarrhea. But aducanumab shows brain swells, small bleeds (Microhaemorrhage) and brain bleeding along the surface of the brain (superficial siderosis).

In the treatment of glymphatic system in Alzheimer's disease only few drugs have been investigated:

1. L-3-n -butylphthalide show neuroprotective effect and it increases the perivascular drainage and it promotes the perivascular drainage of $A\beta$ via increased cerebral pulsation.
2. N-3 polyunsaturated fatty acids (PUFAS) which promotes interstitial amyloid beta ($A\beta$) clearance and have the ability to decrease amyloid beta ($A\beta$) production.
3. Down syndrome critical region 1 (DSCR1) affects the development of dorsal meningeal lymphatic vessels and facilitates $A\beta$ clearance in a 5XFAD mouse model.
4. The humanized monoclonal antibody solanezumab was designed to increase the clearance of soluble $A\beta$ and peptides that may lead to toxic effects in the synapses and precede the deposition of fibrillary amyloid in the brain (11).

Based on the recent research in this milieu, only few drugs have the ability to ameliorate Alzheimer's disease (AD) via glymphatic clearance mechanism. Considering the pathological relevance of the glymphatic pathway with noxious amyloid beta and tau proteins, the current study is aimed at the evaluation of the interaction of selected natural compounds with various glymphatic molecular parameters including aquaporin-4, amyloid-beta, tau protein, etc. Based on the *in silico* molecular docking analysis, lead cannabinoid compounds from *Cannabis sativa* (namely: cannabidiol, Δ^9 -tetrahydrocannabinol, cannabigerol, cannabidivarin) with substantial target protein interaction will be identified for anti-Alzheimer's activity, and these compounds will be investigated in a suitable cellular AD model by using *in vitro* studies.

CHAPTER-4

EXPERIMENTAL WORK

4.1. *IN SILICO* STUDY:

The *In silico* analysis involved screening cannabinoids such as cannabinal, Δ^9 -tetrahydrocannabinol, cannabigerol and cannabidivarin for their binding and interaction with molecular targets amyloid beta ($A\beta$), tau, aquaporin-4 (AQP4), interleukin-6 (IL-6) and high mobility group box 1 (HMGB1). The ligands were visualized for their physical properties and drug-likeness properties. A detailed description of each part of the methodology is given below:

Retrieval of the ligand molecule and protein structure:

4.1.1 Preparation of protein structure:

The three-dimensional (3D) structure of the target receptors amyloid beta ($A\beta$), tau, aquaporin-4 (AQP4), interleukin-6 (IL-6) and high mobility group box 1 (HMGB1) was retrieved from protein drug bank (PDB). The protein structures were downloaded in PDB format from the protein drug bank. The deposited protein data consisted of water molecules and NAG- and NAM-like molecules to stabilize the crystallographic structure of the proteins. The enzymes were prepared by removing the NAG, NAM, and water molecules from the sequence to be used for further study, assigning bond orders, adding hydrogen atoms, and distributing Kollmann's charge equal to the whole receptor (69).

4.1.2 Preparation of ligand molecule:

The chemical structure of four potential ligands were identified from cannabinoids such as cannabinal, Δ^9 -tetrahydrocannabinol, cannabigerol and cannabidivarin were retrieved from the PubChem and Chempider databases in SDF format. During preparation, Gasteiger charge were added in the ligands using MGL Tool (Auto dock 4) (70).

4.1.3 Molecular docking:

Molecular docking was performed to predict the maximum binding affinity between the ligands (inhibitors) and the protein crystal structures and the type of amino acids residues interaction with the ligand using BIOVIA Discovery Studio software. The drugs were docked with the proteins. Various types of interactions

occur such as Hydrogen bond, Electrostatic, Hydrophobic, Vander waals force interactions between the Ligand-Protein. The Lamarckian Genetic Algorithm and empirical free energy scoring function will provide docking results for ligands with approximately 10 conformations based on the cluster root mean square deviation and orientation (71).

4.1.4 Visualization:

Discovery Studio Visualizer 4.1 is a free, molecular modelling environment, for both small and macromolecule applications. It is developed by accelrys which specializes in scientific software products. The BIOVIA discovery studio visualizer 4.1 software is used in visualization of the docked molecules.

4.2. *IN VITRO* STUDY

4.2.1 Cell culture:

U251 cells were obtained from the BeNa Culture Collection (No. BNCC337874) (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Huzhou, China), CBDV was dissolved in 100% ethanol. At a concentration of 30 nM CBDV was added to the cell culture. 0.01% of ethanol is added to control culture and kept in a humidified 5% CO₂ incubator at 37 °C.

4.2.2 Cell viability assay:

U251 cells were seeded into 96-well plates at a density of 8×10^3 cells per well. After 12 h of incubation, cells were pretreated with Cannabidiol (CBDV) for 3 h at doses of 10nM and 30nM, and then co-incubated with or without 25mM and 65mM of A β 1-42 and Ibotenic acid (IA) at 37 °C for a further 24 h. Twenty microliters of 3-(4,5-dimethylthiazole-2-yl)-2,5- diphenyl tetrazolium bromide (5 mg/mL) was added to each well, and the cells were further incubated for 4 h at 37 °C in darkness. After removing the supernatant, 150 μ L of dimethyl sulfoxide were added to dissolve crystalline formazan. The absorbance at 490 nm was measured using a microplate reader (Synergy 4, Omega Bio-tek, Inc., Norcross, GA, USA).

4.2.3 Reactive oxygen species (ROS):

U251 cells were seeded into 6-well plates at a density of 2×10^5 cells per well, pretreated with 10nM and 30nM of CBDV for 3 h, and co-incubated with or without 25mM and 65mM of A β 1-42 and IA at 37 °C for 12 h. The intracellular concentrations of ROS were measured using an ROS assay kit (E004, Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The intracellular concentrations of Ca²⁺ were measured using Fluo-4 AM dye (F14201, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The fluorescence intensity of cells under light excitation was observed using a fluorescent inverted microscope (CKX31, Olympus, Tokyo, Japan).

4.2.4 ELISA:

The collected cells were quantified using a Pierce™ BCA Assay Kit. The concentrations of the following proteins were analysed using the corresponding commercial ELISA kits (Jiangsu Kete Biological Technology Co., Ltd, Jiangsu, China): TNF-alpha, IL--6, IL-1-beta. The absorbance at 450 nM was detected using a microplate reader (Synergy 4, Omega BioTek, Inc., Norcross, GA, USA).

4.2.5 Western blot:

U251 cells were seeded into 6-well plates at a density of 2×10^5 cells per well, pretreated with 10nM and 30nM of CBDV for 3 h, and then co-incubated with or without 25mM and 65mM of A β 1-42 and IA at 37 °C for 24 h. The brain tissues collected from experimental mice and treated cells were lysed using RIPA buffer containing PMSF, stored on ice, and quantified using the Pierce™ BCA Protein Assay Kit. As in our previous study, 40 μ g of protein and 4 μ L of marker (26616, Thermo scientific, Waltham, MA, USA) were separated using sodium dodecyl sulfatepolyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride membranes (0.45 μ m) (GE Healthcare Life Science, Beijing, China). The loaded membranes were blocked in 5% bovine serum albumin at 4 °C for 5 h and then incubated separately with the following primary antibodies at 4 °C overnight: HMGB1 and NF- κ B. After washing with tris-buffered saline containing 0.1% Tween 20, the membranes were incubated separately with the following secondary antibodies: goat anti-mouse IgG (H+L) (peroxidase/HRP conjugated) and goat anti-rabbit IgG (H+L) (peroxidase/HRP conjugated). The membranes were then

exposed using an electrochemiluminescence kit (Merck, Darmstadt, Germany) to obtain blots, which were visualized using an automatic chemiluminescence image analysis system (Tanon 5200, Tanon Science and Technology Co., Ltd., Shanghai, China) and quantified using Image-pro Plus 6.0 analysis software (Media Cybernetics, Bethesda, MD, USA).

4.2.6 Statistical analysis:

Data were expressed as means $\pm$ standard errors of the mean (SEM). Statistical significance was determined using one-way analysis of variance with SPSS 16.0 software (IBM Corp., Armonk, NY, USA). Post hoc analysis was performed using multiple comparisons (Dunn's test). A p value < 0.05 was considered to indicate a statistically significant difference. All of the graphs were produced using GraphPad Prism 7.0.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 *IN SILICO* ANALYSIS:

5.1.1 Δ^9 -TETRAHYDROCANNABINOL (THC):

HMGB1:

Pi-alkyl interactions can play an important role in drug-receptor interactions and can contribute to the specificity and affinity of the interaction between the drug and the **Lysine 86** (Lys 86) is located in the acidic tail of HMGB1, which is a highly negatively charged region that plays a critical role in many of the protein's functions (72). The acidic tail of HMGB1 contains several acidic amino acid residues that can interact with positively charged molecules, such as DNA, and can influence the binding and bending of DNA. **Lysine 86** is located near the C-terminus of the acidic tail, which also contains several other lysine residues that can undergo post-translational modifications such as acetylation or ubiquitination. These modifications can alter the charge and function of the acidic tail and thus affect the protein's interactions with DNA and other proteins. Overall, the position of Lys 86 within the acidic tail of HMGB1 suggests that it may play a role in the protein's interactions with DNA or other proteins and may be subject to post-translational modifications that can influence these interactions.

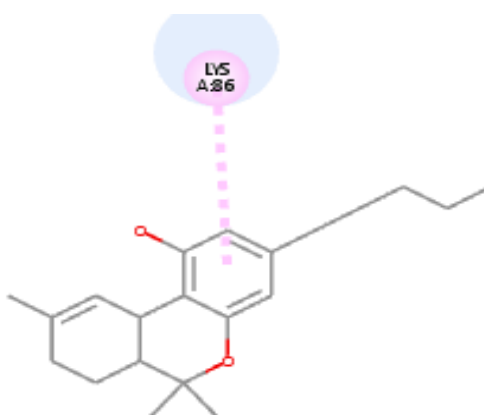


Fig 5.1 Interaction of THC with various amino acids residues in HMGB1

TAU:

The tau protein is a microtubule-associated protein that is abundant in the neurons of the central nervous system. Tau protein is known for its involvement in various neurodegenerative diseases, including Alzheimer's disease, where it forms insoluble aggregates called neurofibrillary tangles (NFTs). The exact role of lysine 340 (Lys340) in tau fibril formation is not well understood. However, some studies

suggest that **Lys340** may be involved in stabilizing the structure of tau fibrils. For instance, a recent study using solid-state NMR spectroscopy showed that Lys340 forms a salt bridge with glutamic acid at position 342 (Glu342) in the tau protein, and this interaction plays a crucial role in stabilizing the fibril structure. Moreover, mutations at Lys340 have been shown to affect tau fibril formation. For example, a study published in the Journal of Biological Chemistry demonstrated that mutations at Lys340 disrupted the formation of tau fibrils, leading to the formation of amorphous aggregates. Overall, the evidence suggests that Lys340 plays a role in stabilizing the structure of tau fibrils, although further research is needed to fully understand its specific mechanism of action.

Another key amino acid residues in the tau protein that has been implicated in fibril formation is glutamic acid at position 338 (Glu338). This residue is located within the microtubule-binding repeat region of the tau protein and is known to be involved in the stabilization of microtubules. Studies have shown that mutations or modifications of Glu338 can affect the aggregation of tau into fibrils. For example, phosphorylation of Glu338 has been shown to promote the formation of tau fibrils, while mutations of Glu338 to alanine or lysine residues can prevent or reduce tau fibril formation. Moreover, recent studies using advanced biophysical techniques such as solid-state NMR spectroscopy have revealed that Glu338 plays an important role in the formation of intermolecular contacts between tau molecules, which are necessary for the formation of tau fibrils.

Studies have shown that His329 can undergo protonation/deprotonation under physiological conditions, which may affect the electrostatic interactions between tau molecules and thereby influence tau aggregation and fibril formation. For instance, a study published in the Journal of Biological Chemistry showed that **His329** plays a crucial role in the early stages of tau fibril formation. The authors demonstrated that the protonation state of His329 affects the conformation of tau and its ability to form intermolecular contacts with other tau molecules. Furthermore, mutations of His329 to alanine or arginine residues have been shown to affect tau fibril formation. In particular, the H329A mutation was found to reduce the propensity of tau to form fibrils, while the H329R mutation was found to promote tau fibril formation.

Studies have shown that Lys331 can be involved in the electrostatic interactions between tau molecules, which are important for the formation of intermolecular

contacts that lead to the formation of tau fibrils. For instance, a study published in the Journal of Biological Chemistry showed that the substitution of Lys331 with alanine or glutamic acid residues inhibited tau fibril formation in vitro. This suggests that Lys331 is involved in the early stages of tau aggregation.

Moreover, recent studies using advanced biophysical techniques such as solid-state NMR spectroscopy have revealed that Lys331 forms salt bridges with glutamic acid residues at positions 332 and 335 in the tau protein, and these interactions play an important role in the formation of intermolecular contacts between tau molecules.

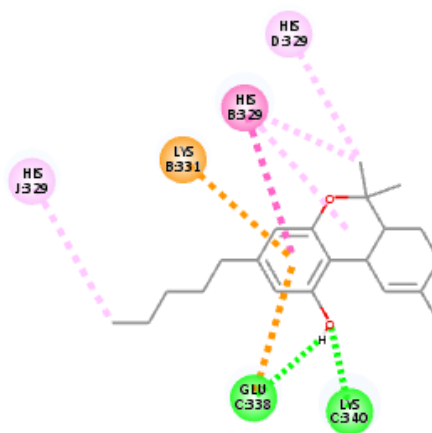


Fig 5.2 Interaction of THC with various amino acids residues in Tau

IL-6:

Leucine at position 181 (Leu181) is located in the IL-6 protein sequence and has been studied for its potential effects on IL-6 function. Research suggests that mutations in Leu181 can affect the activity of IL-6. For example, a study published in the Journal of Biological Chemistry showed that substitution of Leu181 with alanine or arginine residues reduced the ability of IL-6 to bind to its receptor and activate downstream signaling pathways. Additionally, a study published in the Journal of Immunology showed that a mutation in Leu181 (L181P) is associated with reduced levels of IL-6 production in response to certain stimuli. The authors suggest that this mutation may affect the stability of the IL-6 protein, leading to decreased levels of functional IL-6.

Research suggests that mutations in Ile25 can affect the activity of IL-6. For example, a study published in the Journal of Biological Chemistry showed that

substitution of Ile25 with alanine or leucine residues reduced the ability of IL-6 to bind to its receptor and activate downstream signaling pathways.

Moreover, the same study also showed that the I25A mutation disrupted the tertiary structure of the IL-6 protein, resulting in decreased stability and reduced activity. Another study published in the Journal of Immunology suggested that a mutation in Ile25 (I25T) was associated with decreased IL-6 production in response to certain stimuli, although the mechanism behind this effect is not fully understood.

Research suggests that Arg182 is involved in the binding of IL-6 to its receptor and the subsequent activation of downstream signaling pathways. For instance, a study published in the Journal of Biological Chemistry showed that substitution of Arg182 with alanine or lysine residues reduced the ability of IL-6 to bind to its receptor and activate downstream signaling pathways. Another study published in the Journal of Immunology suggested that a mutation in Arg182 (R182C) was associated with reduced levels of IL-6 production in response to certain stimuli. The authors suggest that this mutation may affect the stability of the IL-6 protein or its ability to interact with other proteins, leading to decreased levels of functional IL-6.

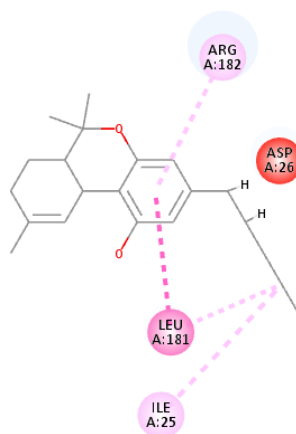


Fig 5.3 Interaction of THC with various amino acids residues in IL-6

AQUAPORIN-4:

Asn181: This amino acid is located in the pore region of the protein and plays a critical role in regulating water transport through the channel. Mutations in Asn181 have been shown to significantly impair AQP4 function.

Arg216: This amino acid is also located in the pore region of the protein and forms a hydrogen bond with water molecules passing through the channel. It plays an important role in regulating water transport.

Ser111: This amino acid is located in the extracellular loop of the protein and has been implicated in the regulation of AQP4 activity. Mutations in Ser111 have been shown to affect the localization of AQP4 in cells.

Ser180: This amino acid is located near the N-terminus of the protein and is thought to play a role in stabilizing the protein structure.

Trp287: This amino acid is located in the C-terminal helix of the protein and is involved in stabilizing the tetrameric structure of AQP4.

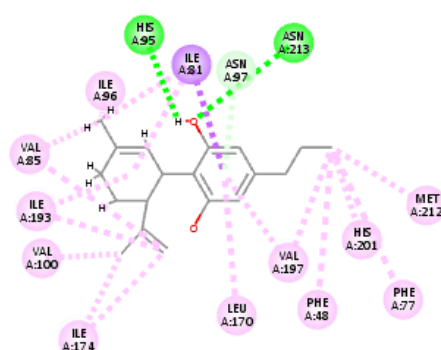


Fig 5.4 Interaction of THC with various amino acids residues in AQP4

5.1.2 CANNABIGEROL:

HMGB1:

Pi-alkyl interactions can play an important role in drug-receptor interactions and can contribute to the specificity and affinity of the interaction between the drug and the receptor

- Lysine 86 (Lys 86) is located in the acidic tail of HMGB1, which is a highly negatively charged region that plays a critical role in many of the protein's functions. The acidic tail of HMGB1 contains several acidic amino acid residues that can interact with positively charged molecules, such as DNA, and can influence the binding and bending of DNA.
- Lysine 86 is located near the C-terminus of the acidic tail, which also contains several other lysine residues that can undergo post-translational modifications such as acetylation or ubiquitination. These modifications can

alter the charge and function of the acidic tail and thus affect the protein's interactions with DNA and other proteins.

- Overall, the position of Lys 86 within the acidic tail of HMGB1 suggests that it may play a role in the protein's interactions with DNA or other proteins and may be subject to post-translational modifications that can influence these interactions.

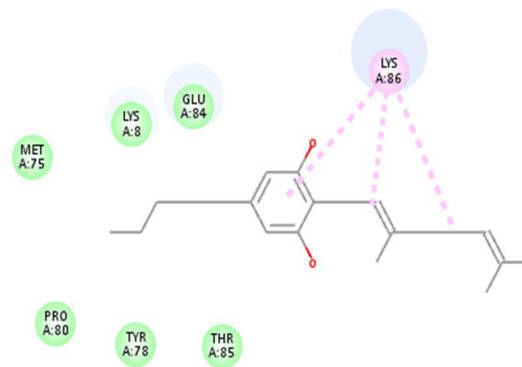


Fig 5.5 Interaction of CBG with various amino acids residues in HMGB1

TAU:

The formation of tau aggregates is facilitated by the presence of specific amino acids that can promote the formation of beta-sheet structures. These amino acids include proline, glycine, and glutamate, which are abundant in the repeat domain of tau protein.

Charge-pairing is a well-known stabilizing feature of amyloid fibrils, and its disruption can lead to charge repulsion by like charges stacked in adjacent fibril layers.

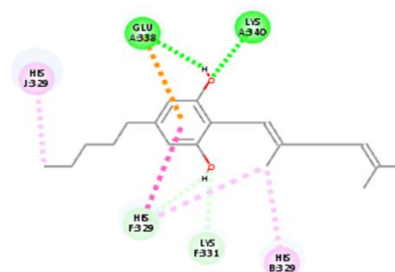


Fig 5.6 Interaction of CBG with various amino acids residues in TAU

IL-6:

Research suggests that Arg182 is involved in the binding of IL-6 to its receptor and the subsequent activation of downstream signaling pathways. For

instance, a study published in the Journal of Biological Chemistry showed that substitution of Arg182 with alanine or lysine residues reduced the ability of IL-6 to bind to its receptor and activate downstream signaling pathways.

Another study published in the Journal of Immunology suggested that a mutation in Arg182 (R182C) was associated with reduced levels of IL-6 production in response to certain stimuli. The authors suggest that this mutation may affect the stability of the IL-6 protein or its ability to interact with other proteins, leading to decreased levels of functional IL-6.

Research suggests that mutations in Ile25 can affect the activity of IL-6. For example, a study published in the Journal of Biological Chemistry showed that substitution of Ile25 with alanine or leucine residues reduced the ability of IL-6 to bind to its receptor and activate downstream signaling pathways.

Moreover, the same study also showed that the I25A mutation disrupted the tertiary structure of the IL-6 protein, resulting in decreased stability and reduced activity. Another study published in the Journal of Immunology suggested that a mutation in Ile25 (I25T) was associated with decreased IL-6 production in response to certain stimuli, although the mechanism behind this effect is not fully understood.

Research suggests that mutations in Leu181 can affect the activity of IL-6. For example, a study published in the Journal of Biological Chemistry showed that substitution of Leu181 with alanine or arginine residues reduced the ability of IL-6 to bind to its receptor and activate downstream signaling pathways.

Additionally, a study published in the Journal of Immunology showed that a mutation in Leu181 (L181P) is associated with reduced levels of IL-6 production in response to certain stimuli. The authors suggest that this mutation may affect the stability of the IL-6 protein, leading to decreased levels of functional IL-6.

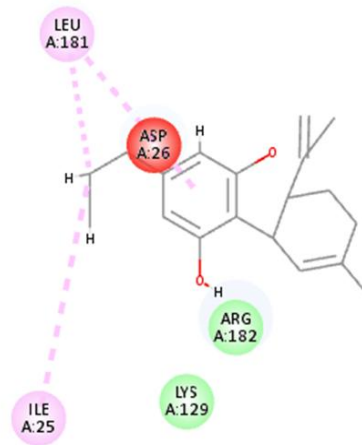


Fig 5.7 Interaction of CBG with various amino acids residues in IL-6

BETA- AMYLOID:

Lys28 and Lys16: The amino acid lysine at positions 28 and 16 are important because they can be modified by acetylation, which has been shown to affect the aggregation and toxicity of beta amyloid. Acetylation of Lys28 has been shown to reduce beta amyloid aggregation and toxicity, while acetylation of Lys16 has been shown to increase aggregation and toxicity.

LEU 17 position is of interest because it is located within the hydrophobic core of the peptide, and mutations or alterations in this region can affect the stability and aggregation of the beta amyloid peptide. Substitutions of leucine with other amino acids at position 17 can affect the aggregation propensity and neurotoxicity of beta amyloid peptide. For example, replacing Leu 17 with alanine has been shown to decrease beta amyloid aggregation, while replacing it with methionine or valine can increase aggregation.

Ala 21 position is also located within the hydrophobic core of the peptide, and mutations or alterations at this position can affect the aggregation and toxicity of the beta amyloid peptide.

Research has shown that substitutions of alanine with other amino acids at position 21 can affect the aggregation propensity and neurotoxicity of beta amyloid peptide. For example, replacing Ala 21 with glycine or serine has been shown to increase beta amyloid aggregation and toxicity, while replacing it with valine or isoleucine has been shown to decrease aggregation and toxicity.

Interestingly, certain mutations at position 21, such as Ala21Gly or Ala21Thr, have been associated with early-onset familial Alzheimer's disease. These mutations may affect the stability or aggregation of beta amyloid peptide, leading to the accumulation of amyloid plaques in the brain and the development of Alzheimer's disease symptoms.

Met 35 position is located near the C-terminus of the peptide and is involved in the formation of intermolecular disulfide bonds between beta amyloid peptides.

Research has shown that the presence of a disulfide bond between Met 35 and Cys 31 can affect the aggregation and toxicity of beta amyloid peptide. When a disulfide bond is formed between these two residues, it can promote the formation of stable oligomers that are less toxic than the soluble oligomers that are typically formed in the absence of disulfide bonds.

However, the formation of disulfide bonds between Met 35 and Cys 31 is not always beneficial. Under certain conditions, such as oxidative stress, the disulfide bond can become unstable and break, leading to the formation of more toxic beta amyloid aggregates.

In addition, certain mutations at position 35, such as Met35Val or Met35Thr, have been associated with increased beta amyloid aggregation and toxicity. These mutations may affect the conformation or stability of the beta amyloid peptide, leading to the formation of more toxic aggregates.

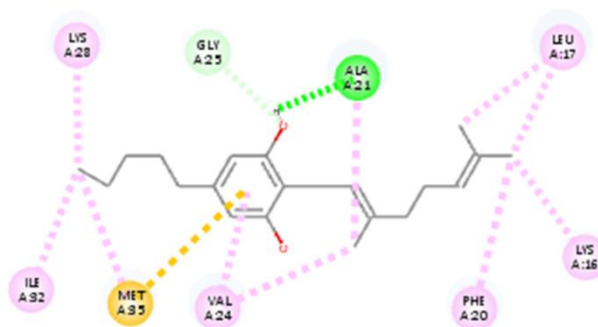


Fig 5.8 Interaction of CBG with various amino acids residues in β - Amyloid

AQUAPORIN-4:

Val 100 position in AQP4 plays an important role in maintaining normal water homeostasis in the central nervous system, and alterations at this position can have significant implications for neurological health and disease.

The Val 100 position in AQP4 has been studied as a potential drug target for the treatment of certain neurological disorders, particularly neuromyelitis optica (NMO), which is characterized by inflammation and damage to the optic nerves and spinal cord.

One strategy for targeting the Val 100 position involves the development of small molecules that can selectively inhibit the function of AQP4. One example is the drug candidate AER-271, which has been shown to inhibit AQP4 function by binding to a hydrophobic pocket near the Val 100 position in the protein. AER-271 has demonstrated efficacy in preclinical models of NMO and is currently being evaluated in clinical trials.

Another strategy for targeting the Val 100 position is through the use of monoclonal antibodies that can specifically bind to AQP4 and block its function. One such antibody is eculizumab, which has been shown to reduce the severity and frequency of NMO attacks in clinical trials. Eculizumab targets a region of AQP4 that includes the Val 100 position and has been shown to inhibit AQP4 function by reducing its expression and altering its localization.

Cys 178 position is located in the fourth extracellular loop of AQP4 and is involved in the formation of disulfide bonds between AQP4 monomers.

Research has shown that certain mutations or alterations at the Cys 178 position can affect the expression, localization, and function of AQP4. For example, mutations that disrupt the formation of disulfide bonds at this position can lead to misfolding and degradation of AQP4, resulting in reduced expression and localization at the plasma membrane.

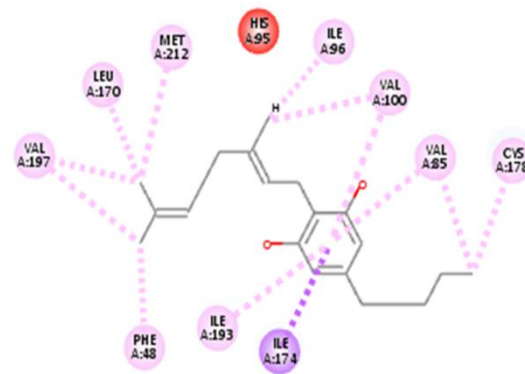


Fig 5.9 Interaction of CBG with various amino acids residues in AQP4

5.1.3 CANNABINOL:

HMGB1:

Position of lys 87 within the acidic tail of HMGB1 suggests that it may play a critical role in the protein's interactions with DNA and other proteins, and that post-translational modifications of lys 87 may be important for regulating the function of HMGB1.

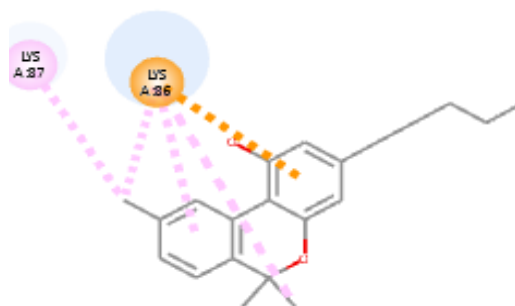


Fig 5.10 Interaction of CANNABINOL with various amino acids residues in HMGB1

TAU:

The formation of tau aggregates is facilitated by the presence of specific amino acids that can promote the formation of beta-sheet structures. These amino acids include proline, glycine, and glutamate, which are abundant in the repeat domain of tau protein.

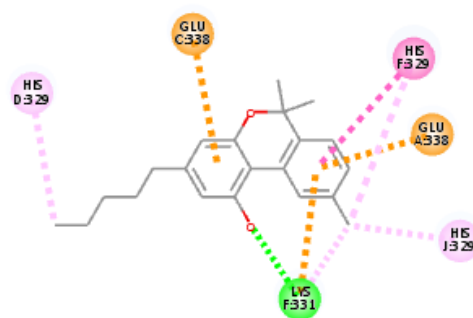


Fig 5.11 Interaction of CANNABINOL with various amino acids residues in *Tau*

IL-6:

The Asp 26 residue is thought to be important for the biological activity of IL-6, as it is involved in binding to the IL-6 receptor and initiating downstream signaling pathways.

In particular, Asp 26 is important for the binding of interleukin to its receptor, which initiates downstream signaling pathways that ultimately lead to the regulation of various immune and inflammatory responses.

Additionally, mutations at this position have been linked to certain diseases and disorders, such as amyloidosis and rheumatoid arthritis.

Asp 26 position is an important structural and functional component of interleukin, and further research into its role in disease and immunity may provide insights into the development of novel therapeutic interventions.

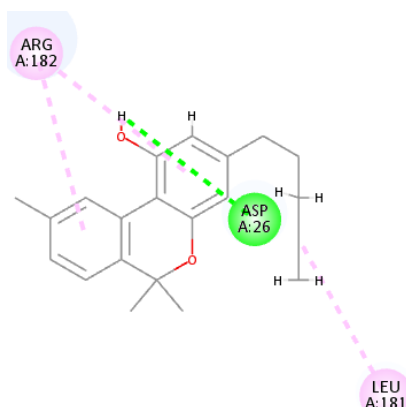


Fig 5.12 Interaction of CANNABINOL with various amino acids residues in *IL-6*

BETA- AMYLOID:

Beta amyloid is a protein fragment that is known to accumulate in the brains of individuals with Alzheimer's disease, and the Ala 21 position is located in the N-terminal region of the beta amyloid protein. Rest of the positions significance discussed in the earlier.

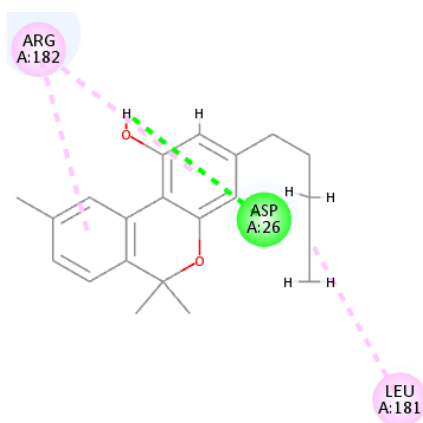


Fig 5.13 Interaction of CANNABINOL with various amino acids residues in β - Amyloid

AQP4:

The Cys 178 position in aquaporin-4 (AQP4) refers to the 178th amino acid residue in the protein sequence of AQP4, which is a water channel protein that is primarily expressed in the brain and spinal cord. Cys 178 is an important amino acid residue in AQP4, as it is involved in the formation of disulfide bonds that help to stabilize the protein's structure. Disulfide bonds are covalent bonds that form between two cysteine residues, and they are important for maintaining the three-dimensional structure and stability of many proteins.

His 95 is an important amino acid residue in AQP4, as it is involved in coordinating the binding of zinc ions to the protein. Zinc ions are essential for the proper function of AQP4 and are thought to play a role in stabilizing the protein's structure.

Mutations or modifications to His 95 or other nearby amino acid residues in AQP4 can disrupt the binding of zinc ions and lead to changes in the protein's structure and function. This can result in the development of autoimmune disorders such as neuromyelitis optica, which is characterized by inflammation and damage to the central nervous system.

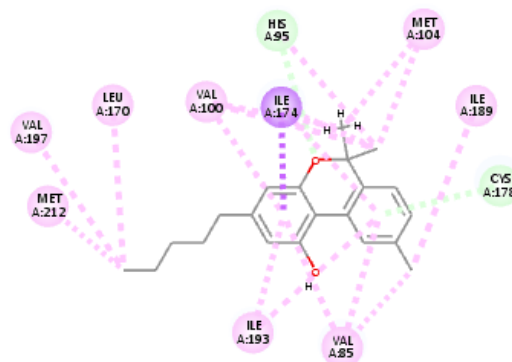


Fig 5.14 Interaction of CANNABINOL with various amino acids residues in AQP4

5.1.4 CANNABIDIVARIN (CANNABIDIVAROL):

HMGB1:

Compounds that target enzymes or receptors contain functional groups that can form hydrogen bonds with amino acid residues in the active site of the enzyme or the binding pocket of the receptor. This can help to stabilize the binding of the drug and enhance its affinity for the target molecule

- Lysine 86 (Lys 86) is located in the acidic tail of HMGB1, which is a highly negatively charged region that plays a critical role in many of the protein's functions. The acidic tail of HMGB1 contains several acidic amino acid residues that can interact with positively charged molecules, such as DNA, and can influence the binding and bending of DNA.
- Lysine 86 is located near the C-terminus of the acidic tail, which also contains several other lysine residues that can undergo post-translational modifications such as acetylation or ubiquitination. These modifications can alter the charge and function of the acidic tail and thus affect the protein's interactions with DNA and other proteins.
- Overall, the position of Lys 86 within the acidic tail of HMGB1 suggests that it may play a role in the protein's interactions with DNA or other proteins and may be subject to post-translational modifications that can influence these interactions.

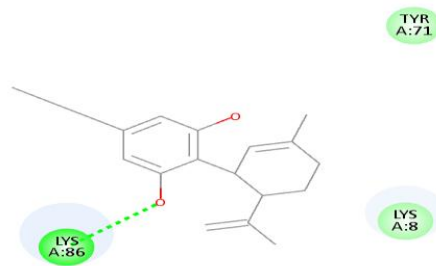


Fig 5.15 Interaction of CBDV with various amino acids residues in HMGB1

TAU:

Lysine (Lys) 340 is an important amino acid position in the tau protein. This residue is located within one of the four microtubule-binding repeat domains of tau, specifically in the third repeat domain (R3).

Studies have shown that Lys 340 plays a critical role in the binding of tau to microtubules. This residue is thought to form an electrostatic interaction with negatively charged residues on the surface of microtubules, helping to anchor tau to the microtubule lattice. Mutations at this position can disrupt tau-microtubule interactions and lead to tau aggregation and neurodegeneration.

Additionally, Lys 340 is also a potential site for post-translational modifications, such as acetylation or ubiquitination, which may affect tau's interaction with microtubules and/or its propensity to form aggregates.

Glutamic acid (Glu) 338 is an important amino acid position in the tau protein. This residue is located within the microtubule-binding repeat domain R3 of tau, adjacent to Lysine (Lys) 340.

Glu 338 is thought to play a critical role in the binding of tau to microtubules. It has been suggested that this residue forms a salt bridge with a lysine residue on the microtubule surface, helping to anchor tau to the microtubule lattice. Mutations or modifications at this position can disrupt tau-microtubule interactions and lead to tau aggregation and neurodegeneration.

Interestingly, Glu 338 is also a potential site for post-translational modifications, such as acetylation or phosphorylation, which may affect tau's interaction with microtubules and/or its propensity to form aggregates.

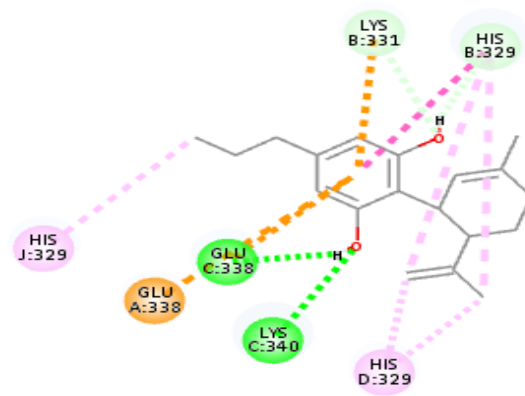


Fig 5.16 Interaction of CBDV with various amino acids residues in Tau

IL-6:

Interleukin-6 (IL-6) is a cytokine that plays a critical role in the immune response and inflammation. The protein is composed of four alpha-helices and is held together by disulfide bonds.

The serine residue at position 22 of the IL-6 protein is located in the first alpha-helix and is involved in stabilizing the helical structure. Specifically, Ser22 forms a hydrogen bond with Arg28 in the same helix, which helps to stabilize the helix by fixing the position of Arg28.

Mutation of Ser22 can disrupt the hydrogen bond with Arg28 and lead to destabilization of the first alpha-helix. This can affect the overall structure of the protein and potentially alter its biological activity.

There is some evidence that Ser22 phosphorylation can also affect the biological activity of IL-6. Phosphorylation of Ser22 has been shown to reduce the ability of IL-6 to activate signaling pathways in cells, suggesting that this modification may play a role in regulating IL-6 function.

Overall, the Ser22 position in the IL-6 protein is significant for maintaining the protein structure and function, and alterations to this position can have important biological consequences.

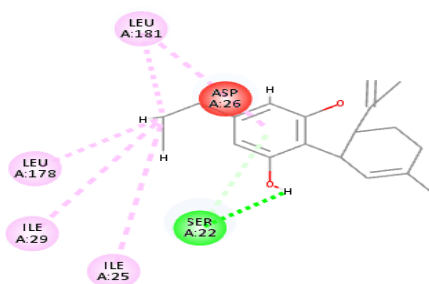


Fig 5.17 Interaction of CBDV with various amino acids residues in IL-6

BETA- AMYLOID:

Beta amyloid is a protein fragment that is known to accumulate in the brains of individuals with Alzheimer's disease, and the Ala 21 position is located in the N-terminal region of the beta amyloid protein.

Leu 34 position is located in the middle region of the beta amyloid protein. Studies have shown that mutations or changes in the amino acid sequence at the Leu 34 position can affect the aggregation and toxicity of beta amyloid. For example, some research has found that changing the leucine residue to another amino acid, such as proline, can decrease the aggregation and toxicity of beta amyloid, potentially reducing the risk of Alzheimer's disease. Other studies have suggested that the Leu 34 position may play a role in the interaction of beta amyloid with other proteins in the brain, such as apolipoprotein E (APOE), which is known to be a risk factor for Alzheimer's disease.

Research has found that substituting the methionine residue with another amino acid, such as leucine or valine, can increase the aggregation and toxicity of the amyloid peptide, potentially contributing to the development and progression of Alzheimer's disease. Other studies have suggested that the Met 35 position may play a role in the interaction of the amyloid peptide with other proteins in the brain, such as apolipoprotein E (APOE), which is known to be a risk factor for Alzheimer's disease.

Val 24 is of particular interest because it is located near the N-terminus (beginning) of beta amyloid and is part of a hydrophobic core that stabilizes the protein's structure. This hydrophobic core is thought to play a role in the aggregation of beta amyloid into toxic oligomers and fibrils that are associated with Alzheimer's disease. Mutations or modifications to Val 24 have been studied as

potential targets for therapeutic interventions aimed at preventing the aggregation of beta amyloid. For example, researchers have investigated the effects of substituting Val 24 with other amino acids, or modifying it chemically, to disrupt the hydrophobic core and prevent aggregation. However, more research is needed to determine whether these approaches will be effective in treating Alzheimer's disease.

Ile 21 is of particular interest because it is located near the N-terminus (beginning) of beta amyloid and is part of a hydrophobic core that stabilizes the protein's structure. This hydrophobic core is thought to play a role in the aggregation of beta amyloid into toxic oligomers and fibrils that are associated with Alzheimer's disease. Mutations or modifications to Ile 21 have been studied as potential targets for therapeutic interventions aimed at preventing the aggregation of beta amyloid. For example, researchers have investigated the effects of substituting Ile 21 with other amino acids, or modifying it chemically, to disrupt the hydrophobic core and prevent aggregation. However, more research is needed to determine whether these approaches will be effective in treating Alzheimer's disease.

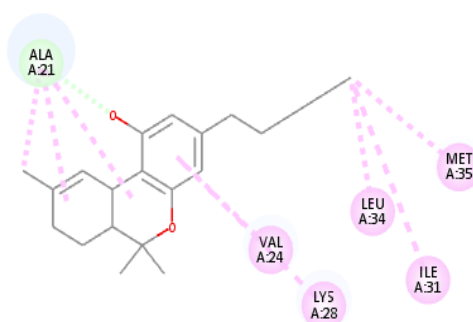


Fig 5.18 Interaction of CBDV with various amino acids residues in β -Amyloid

AQP4:

Cys178 is located in the extracellular loop C of AQP4, which is involved in the formation of the water channel pore. This cysteine residue is not involved in the direct interaction with water molecules, but it may play a role in the regulation of the channel's activity through the formation of disulfide bonds with other cysteine residues or other proteins.

Histidine 95 (His95) is also an amino acid residue in the primary structure of aquaporin-4 (AQP4). It is located in the transmembrane helix H3, which is one of the six transmembrane helices that span the cell membrane and form the water channel pore.

His95 is thought to be involved in the regulation of the channel's activity, possibly by acting as a pH sensor. At neutral pH, His95 is protonated, and its positive charge may interact with neighboring amino acids to stabilize the channel structure. However, at acidic pH, His95 becomes deprotonated, and this may induce conformational changes in the protein, leading to altered channel activity.

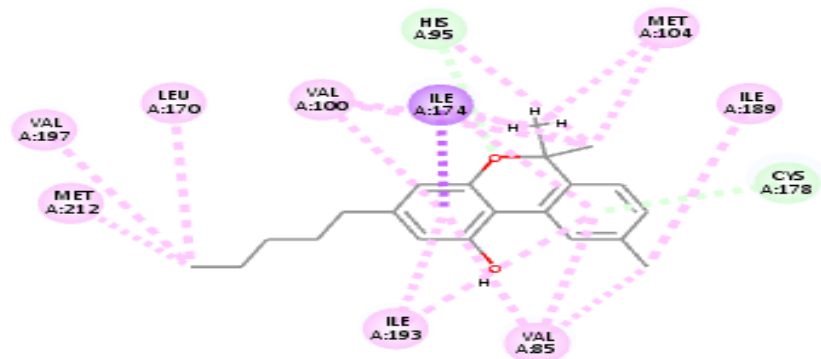


Fig 5.19 Interaction of CBDV with various amino acids residues in AQP4

5.2 IN VITRO ANALYSIS:

5.2.1. Effect of CBDV (low and high doses) and Aβ1A on the relative SOD and CAT protein levels on U251 cells.

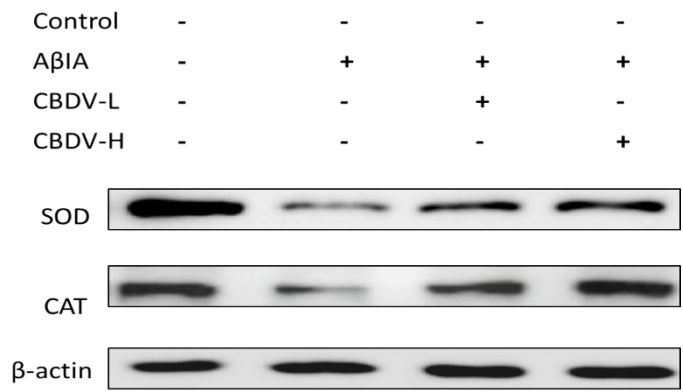


Fig 5.20 (A) Representative Western blots of SOD and CAT expressions

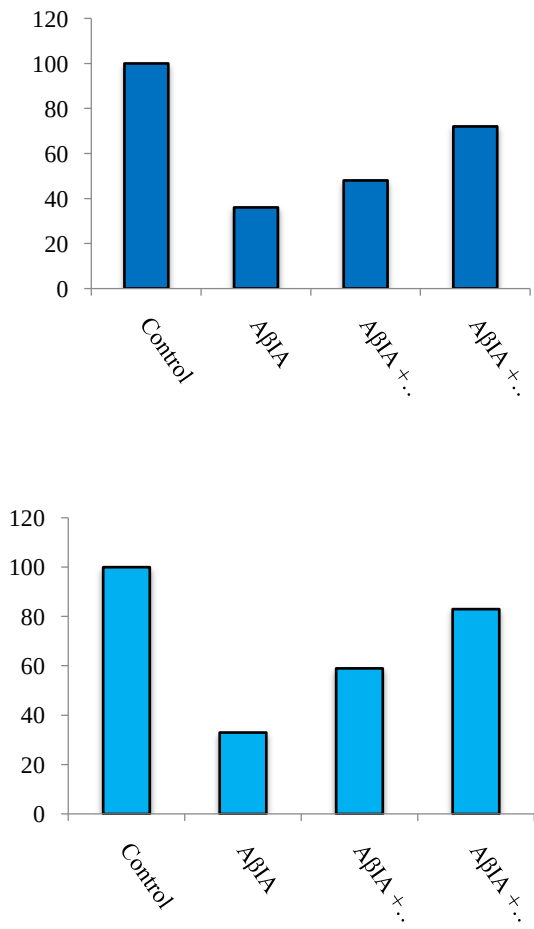


Fig 5.20 (B) Relative protein level of SOD and CAT in different treatments.

Cannabidivarin ameliorated the antioxidant status of U251 cells against Aβ1A provoked neurotoxic challenge

When we probed the U251 cells for the pivotal antioxidants SOD and CAT, we observed that SOD and CAT levels were significantly ($P<0.05$) diminished and elevated by 64% and 67% respectively in the Aβ1A challenged cells, as compared to that of the controls. Nevertheless, CBDV-L and CBDV-H treated cells depicted near normal SOD and CAT levels in the cell lysates. Especially, high-dose CBDV (CBDV-H) treated was more effective in replenishing the antioxidant levels in the Aβ1A challenged cells.

Aβ1-40 with Ibo-treated rats showed a significant decrease in the mitochondrial membrane fluidity and a significant decrease the activity of SOD of the hippocampus, as well as a marked increase in the content of MDA of the hippocampus (67). The observation that blockade of catalase Compound II formation prevents Aβ inhibition of catalase suggests that Aβ may be involved in oxidizing the enzyme, a mechanism already proposed for Aβ inactivation of other enzymes, and this process appears to involve Aβ binding to catalase (68).

5.2.2. Effect of CBDV (low and high doses) and Aβ1A on the relative HMGB1 protein levels on U251 cells.

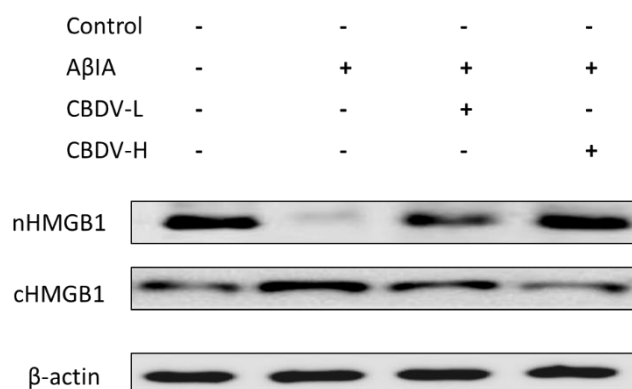


Fig 5.21 (A) Representative Western blots of HMGB1 expressions

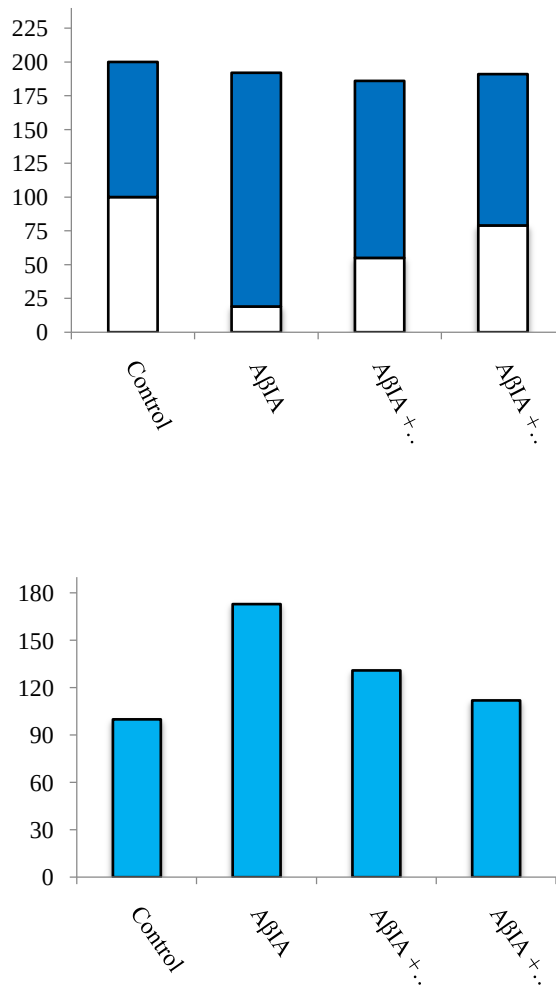


Fig 5.21 (B) Relative protein level of HMGB1 in different treatments.

Shuttling of HMGB1 from nucleus to cytosol is decreased in the cannabidivarin treated Aβ1A-challenged cells

Translocation of HMGB1 from the nucleus to cytosol is increased in the cells due to the neurotoxic assault provoked by Aβ1A. We observed that treatment with both low-dose and high-dose of CBDV reinstated the normal levels of nuclear and cytosolic HMGB1 levels. Of note, CBDV-H treatment displayed better activity when compared to CBDV-L treatment.

HMGB1 decreased the A42- immunoreactive area in the cytoplasm and increased that on the cell surface where HMGB1 was colocalized with A42. These results suggest that HMGB1 may be released from dying cells such as neurons under neurotoxic conditions (73).

CB2R agonist treatment decreased LPS-induced HMGB1 secretion but not influencing HMGB1 mRNA in peritoneal macrophages. The autophagy-lysosome pathway but not ubiquitination pathway was involved in CB2R-mediated HMGB1 degradation. CB2R could promote the intracellular degradation of HMGB1 via the autophagy-lysosome pathway in macrophage (74).

5.2.3. Effect of CBDV (low and high doses) and Aβ1A on the relative NF- κB protein levels on U251 cells.

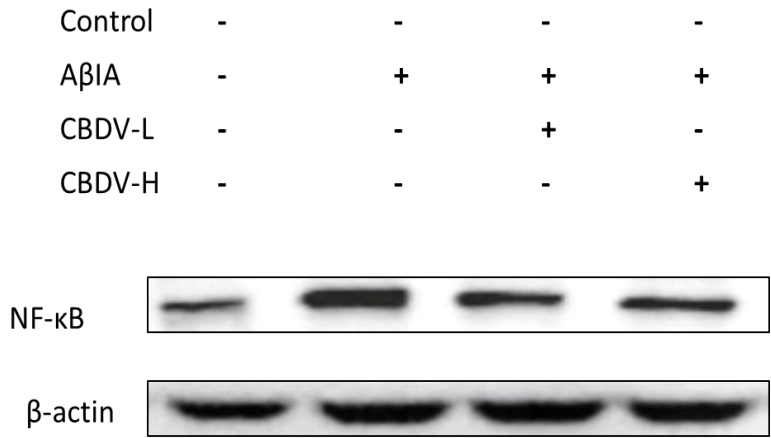


Fig 5.22 (A) Representative Western blots of NF-κB expressions

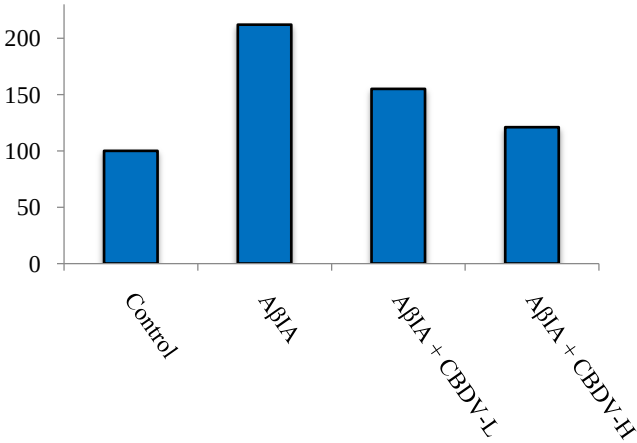


Fig 5.22 (B) Relative protein level of NF-κB in different treatments.

Cannabidivarin mitigated the expression of NF-κB against the Aβ1A-challenge

In this study, about 2.1-fold increase in the protein expression of NF- κ B was observed in the A β 1A-challenged cells. However, treatment with the CBDV (both CBDV-L and CBDV-H) ameliorated the abnormal increase in the protein expression of NF-Kb.

CBD, but not THC, reduces the activity of the NF-kappaB pathway, a primary pathway regulating the expression of proinflammatory genes. Moreover, CBD, but not THC, up-regulates the activation of the STAT3 transcription factor, an element of homeostatic mechanism(s) inducing anti-inflammatory events (75). The transcription factors NFAT and NF-KB in T-cells strongly indicate that the tested CB2-agonists are more likely to inhibit the NFAT signalling pathway than the NF-KB pathway (76).

5.2.4. Effect of CBDV (low and high doses) and A β 1A on the relative AQP4 protein levels on U251 cells.

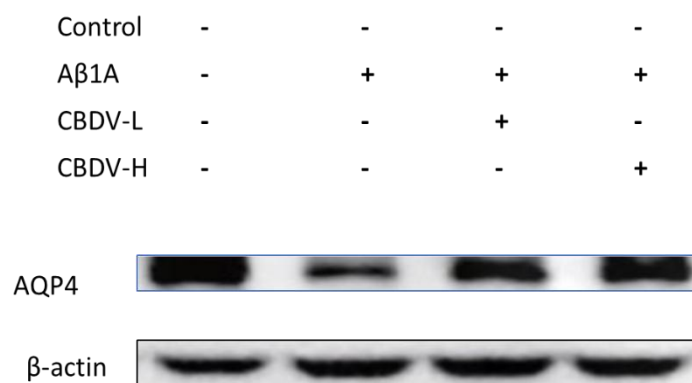
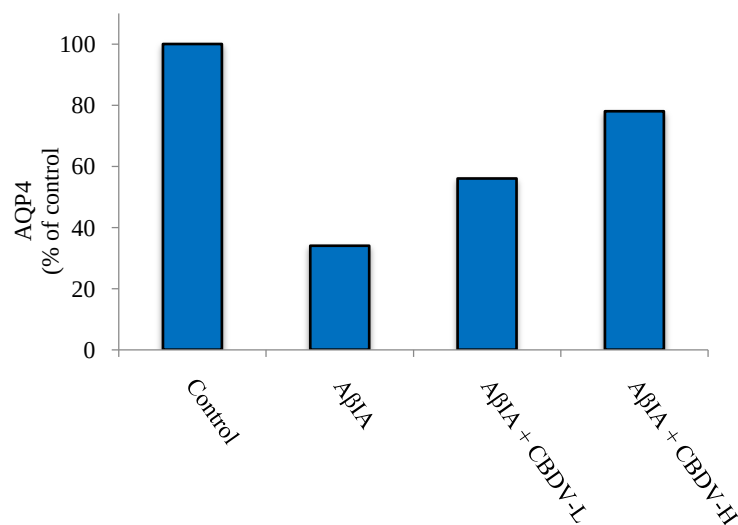


Fig 5.23 (A) Representative Western blots of AQP4 expressions

Fig 5.23 (B) Relative protein level of AQP4 in different treatments.

When we probed the cells for the AQP4, we observed that AQP4 levels were significantly ($P<0.05$) diminished 66% in the A β IA challenged cells, as compared to that of the controls. Nevertheless, CBDV-L and CBDV-H treated cells depicted near normal AQP4 levels in the cell lysates. Especially, high-dose CBDV (CBDV-H) treated was more effective in replenishing the AQP4 levels in the A β IA challenged cells.

AQP4 deficiency is directly involved in impaired A β brain clearance via the peri-/para-vascular routes, and AQP4-mediated vascular clearance might counteract blood-brain barrier abnormalities and age-related vascular



amyloidopathy (77).

5.2.5. Effect of CBDV (low and high doses) and AβIA on the relative TNFα and IL-6 protein levels on U251 cells.

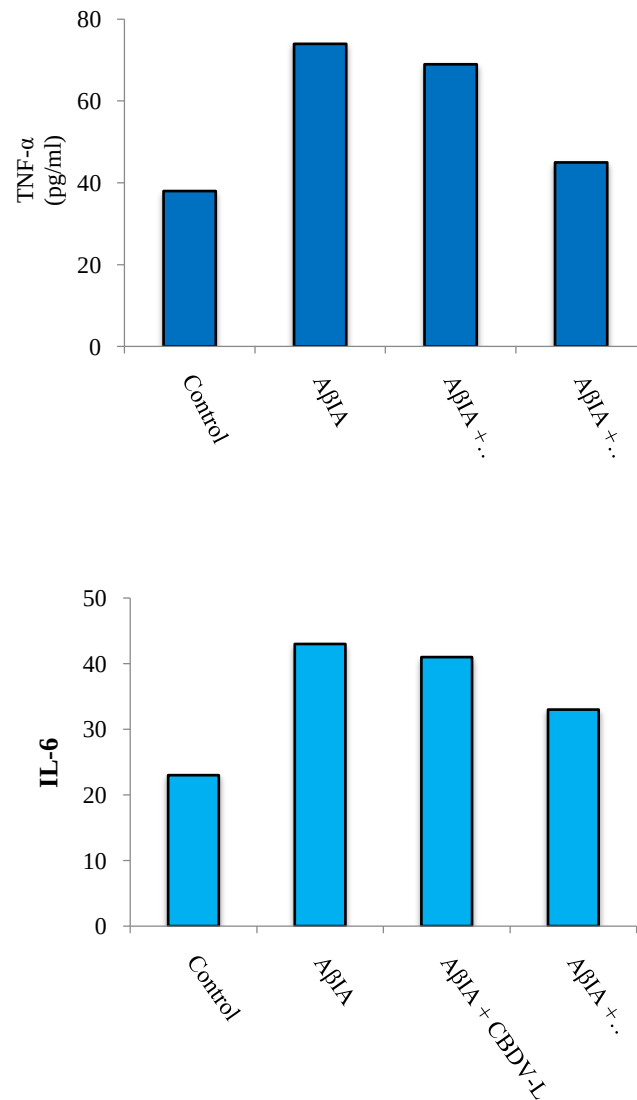


Fig 5.24 (A) Relative protein level of TNFα and IL-6 in different treatments.

Secretion of the pro-inflammatory cytokines including TNFα and IL-6 is an immediate effector function of the cells challenged with the neurotoxic cocktail of AβIA. We observed nearly two-fold increase in the levels of TNFα and IL-6 in the AβIA challenged cells, while high-dose CBDV (CBDV-H) treatment more effectively counteracted the inflammatory insult kicked-off by the exposure of cells

to A β IA cocktail. However, treatment with low-dose CBDV (CBDV-L) did not show any significant anti-inflammatory effect against the neurotoxicants.

A pivotal molecule in inflammation is the pro-inflammatory cytokine TNF- α . Several lines of evidence using genetic and pharmacological manipulations indicate that TNF- α signaling exacerbates both A β and tau pathologies in vivo (78). The proinflammatory cytokine interleukin-6 (IL-6) is upregulated in human AD and believed to promote amyloid plaque deposition (79).

Tumor necrosis factor alpha, interleukin IL-6 and interferon gamma were the most commonly studied pro-inflammatory cytokines and their levels were consistently reduced after treatment with CBD, CBG, or CBD + THC, but not with THC alone (80).

SUMMARY

The neuroprotective effects of *C. sativa* as well as its active constituents, mainly cannabinoids, have been already reported. However, their role in glymphatic clearance pathway in AD has not been analyzed to assess their interaction with various targets related to the glymphatic clearance mechanism.

Based on molecular docking study we found that THC has medium - high interaction with two molecular targets (tau and aquaporin 4). Cannabigerol has medium – high interaction with two molecular targets (beta-amyloid and tau). Cannabinol has medium – high interaction with three molecular targets (beta-amyloid, tau and interleukin-6). Cannabidivarin has medium – high interaction with four molecular targets (tau, aquaporin-4, interleukin-6 and HMGB1).

Overall interaction of CBDV with all the 5 molecular targets is better when compared to the other drugs. Hence, CBDV has been chosen for further analysis using *in-vitro* studies.

For the *in-vitro* studies U251 cell line was used to study the effect of CBDV on various molecular targets including antioxidant's (SOD and CAT), inflammatory mediators (HMGB1, NF-KB, TNF- α and IL-6) and pivotal target in the glymphatic clearance (AQP4).

We found that the expression of antioxidant's (SOD and CAT) were significantly diminished in the A β IA challenged cells (vs control). Nevertheless CBDV (especially CBDV-H) treated cells depicted near normal SOD and CAT levels in the cell lysate.

Translocation of HMGB1 from the nucleus to cytosol is increased in the cells due to the neurotoxic assault provoked by A β IA. We observed that treatment with high-dose of CBDV reinstated the normal levels of nuclear and cytosolic HMGB1 levels.

We also found that the expression of anti-inflammatory agents (NF-KB) were significantly increased in the A β IA challenged cells (vs control). The treatment with CBDV (mainly CBDV-H) ameliorated the abnormal increase in the protein expression of NF-KB.

The expression of anti-inflammatory agents (TNF- α and IL-6) were shown some significantly increase in the A β IA challenged cells (vs control). High-dose CBDV (CBDV-H) treatment was more effectively counteracted the inflammatory insult kicked-off by the exposure of cells to A β IA cocktail.

CONCLUSION

To summarize precisely CBDV showed better interaction with various glymphatic system targets.

Further analysis using *in-vitro* studies clearly indicate the antioxidant, anti-inflammatory and glymphatic clearance facilitating properties of CBDV.

Hence, CBDV might be a putative neurotherapeutic agent for the treatment of Alzheimer's Disease. However, further studies are warranted to elucidate the comprehensive effect of CBDV on various upstream and downstream targets in the glymphatic clearance pathway. Besides the effects of CBDV on cognitive dysfunction in AD need to be studied.

REFERENCES:

1. Oboudiyat C, Glazer H, Seifan A, Greer C, Isaacson RS. Alzheimer's disease. *Semin Neurol*. 2013 Sep;33(4):313-29. DOI: 10.1055/s-0033-1359319. ISSN: 0271-8235; Epub 2013 Nov 14. PMID: 24234352.
2. Tiwari S, Atluri V, Kaushik A, Yndart A, Nair M. Alzheimer's disease: pathogenesis, diagnostics, and therapeutics. *Int J Nanomedicine*. 2019 Jul 19;14:5541-5554. ISSN: 1178-2013; DOI: 10.2147/IJN.S200490. PMID: 31410002; PMCID: PMC6650620.
3. Ishida K, Misawa K, Nishimura H, Hirata T, Yamamoto M, Ota N. 5-Caffeoylquinic Acid Ameliorates Cognitive Decline and Reduces A β Deposition by Modulating A β Clearance Pathways in APP/PS2 Transgenic Mice. *Nutrients*. 2020 Feb 14;12(2):494. ISSN: 2072-6643; DOI: 10.3390/nu12020494. PMID: 32075202; PMCID: PMC7071270.
4. Yang C, Huang X, Huang X, Mai H, Li J, Jiang T, et al. Aquaporin-4 and Alzheimer's Disease. *J Alzheimers Dis*. 2016 Mar 25;52(2):391-402. ISSN 1387-2877; DOI: 10.3233/JAD-150949. PMID: 27031475.
5. Nasrabad SE, Rizvi B, Goldman JE, Brickman AM. White matter changes in Alzheimer's disease: a focus on myelin and oligodendrocytes. *Acta Neuropathol Commun*. 2018 Mar 2;6(1):22. ISSN: 2051-5960; DOI: 10.1186/s40478-018-0515-3. PMID: 29499767; PMCID: PMC5834839.

6. Hu N, Yu JT, Tan L, Wang YL, Sun L, Tan L. Nutrition and the risk of Alzheimer's disease. *Biomed Res Int*. 2013;2013:524820. ISSN: 2314-6141; DOI: 10.1155/2013/524820. Epub 2013 Jun 20. PMID: 23865055; PMCID: PMC3705810.
7. Santos CY, Snyder PJ, Wu WC, Zhang M, Echeverria A, Alber J. Pathophysiologic relationship between Alzheimer's disease, cerebrovascular disease, and cardiovascular risk: A review and synthesis. *Alzheimers Dement (Amst)*. 2017 Feb 9;7:69-87. ISSN:1552-5279; DOI: 10.1016/j.dadm.2017.01.005. PMID: 28275702; PMCID: PMC5328683.
8. Qiang W, Yau WM, Lu JX, Collinge J, Tycko R. Structural variation in amyloid- β fibrils from Alzheimer's disease clinical subtypes. *Nature*. 2017 Jan 12;541(7636):217-221. ISSN: 1476-4687; DOI: 10.1038/nature20814. Epub 2017 Jan 4. PMID: 28052060; PMCID: PMC5233555.
9. Cipriani G, Dolciotti C, Picchi L, Bonuccelli U. Alzheimer and his disease: a brief history. *Neurol Sci*. 2011 Apr;32(2):275-9. ISSN: 1590-3478; DOI: 10.1007/s10072-010-0454-7. Epub 2010 Dec 11. PMID: 21153601.
10. Mota BC, Ashburner N, Abelleira-Hervas L, Liu L, Aleksynas R, Rovati LC, et al. I2-Imidazoline Ligand CR4056 Improves Memory, Increases ApoE Expression and Reduces BBB Leakage in 5xFAD Mice. *Int J Mol Sci*. 2022 Jun 30;23(13):7320. ISSN: 1422-0067; DOI: 10.3390/ijms23137320. PMID: 35806327; PMCID: PMC9266435.
11. Reiman EM. Alzheimer's disease: Attack on amyloid- β protein. *Nature*. 2016 Sep 1;537(7618):36-7. ISSN: 1476-4687 DOI: 10.1038/537036a. PMID: 27582214.
12. Shokri-Kojori E, Wang GJ, Wiers CE, Demiral SB, Guo M, Kim SW, et al. β -Amyloid accumulation in the human brain after one night of sleep deprivation. *Proc Natl Acad Sci U S A*. 2018 Apr 24;115(17):4483-4488. ISSN: 1091-6490; DOI: 10.1073/pnas.1721694115. Epub 2018 Apr 9. PMID: 29632177; PMCID: PMC5924922.
13. Nedergaard M. Neuroscience. Garbage truck of the brain. *Science*. 2013 Jun 28;340(6140):1529-30. ISSN: 1095-9203; DOI: 10.1126/science.1240514. PMID: 23812703; PMCID: PMC3749839.
14. Jack CR Jr, Knopman DS, Jagust WJ, Shaw LM, Aisen PS, Weiner MW, et al. Hypothetical model of dynamic biomarkers of the Alzheimer's

- pathological cascade. *Lancet Neurol.* 2010 Jan;9(1):119-28. ISSN: 1474-4422; DOI: 10.1016/S1474-4422(09)70299-6. PMID: 20083042; PMCID: PMC2819840.
15. Zhang B, Li W, Zhuo Y, Xiang H, Li W, Liu H, et al. L-3-n-Butylphthalide Effectively Improves the Glymphatic Clearance and Reduce Amyloid- β Deposition in Alzheimer's Transgenic Mice. *J Mol Neurosci.* 2021 Jun;71(6):1266-1274. ISSN: 1559-1166; DOI: 10.1007/s12031-020-01752-z. Epub 2020 Nov 13. PMID: 33188502.
 16. Walker LC, Diamond MI, Duff KE, Hyman BT. Mechanisms of protein seeding in neurodegenerative diseases. *JAMA Neurol.* 2013 Mar 1;70(3):304-10. ISSN: 2168-6157; DOI: 10.1001/jamaneurol.2013.1453. PMID: 23599928; PMCID: PMC3665718.
 17. Serý O, Povová J, Míšek I, Pešák L, Janout V. Molecular mechanisms of neuropathological changes in Alzheimer's disease: a review. *Folia Neuropathol.* 2013;51(1):1-9. ISSN: 1641-4640; DOI: 10.5114/fn.2013. 341 90. PMID: 23553131.
 18. Tapiola T, Overmyer M, Lehtovirta M, Helisalmi S, Ramberg J, Alafuzoff I, et al. The level of cerebrospinal fluid tau correlates with neurofibrillary tangles in Alzheimer's disease. *Neuroreport.* 1997 Dec 22;8(18):3961-3. ISSN: 0959-4965; DOI: 10.1097/00001756-199712220-00022. PMID: 9462474.
 19. Blennow K, Wallin A, Agren H, Spenger C, Siegfried J, Vanmechelen E. Tau protein in cerebrospinal fluid: a biochemical marker for axonal degeneration in Alzheimer disease? *Mol Chem Neuropathol.* 1995 Dec;26(3):231-45. ISSN:1044-7393; DOI: 10.1007/BF02815140. PMID: 8748926.
 20. Tarasoff-Conway JM, Carare RO, Osorio RS, Glodzik L, Butler T, Fieremans E, et al. Clearance systems in the brain-implications for Alzheimer disease. *Nat Rev Neurol.* 2015 Aug;11(8):457-70. ISSN: 1759-4766 DOI: 10.1038/nrneurol.2015.119. Epub 2015 Jul 21. Erratum in: *Nat Rev Neurol.* 2016 Apr;12(4):248. PMID: 26195256; PMCID: PMC4694579.
 21. Da Mesquita S, Fu Z, Kipnis J. The Meningeal Lymphatic System: A New Player in Neurophysiology. *Neuron.* 2018 Oct 24;100(2):375-388. ISSN:

- 1097-4199; DOI: 10.1016/j.neuron.2018.09.022. PMID: 30359603; PMCID: PMC6268162.
22. Dunn N, Mullee M, Perry VH, Holmes C. Association between dementia and infectious disease: evidence from a case-control study. *Alzheimer Dis Assoc Disord*. 2005 Apr-Jun;19(2):91-4. ISSN: 1546-4156; DOI: 10.1097/01.wad.0000165511.52746.1f. PMID: 15942327.
23. Lyman M, Lloyd DG, Ji X, Vizcaychipi MP, Ma D. Neuroinflammation: the role and consequences. *Neurosci Res*. 2014 Feb;79:1-12. ISSN: 0168-0102 DOI: 10.1016/j.neures.2013.10.004. Epub 2013 Oct 19. PMID: 24144733.
24. Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci*. 2007 Nov;10(11):1387-94. ISSN: 1546-1726; DOI: 10.1038/nn1997. PMID: 17965659.
25. Plog BA, Lou N, Pierre CA, Cove A, Kenney HM, Hitomi E, et al. When the air hits your brain: decreased arterial pulsatility after craniectomy leading to impaired glymphatic flow. *J Neurosurg*. 2019 May 17:1-14. ISSN: 2694-1902; DOI: 10.3171/2019.2.JNS182675. Epub ahead of print. PMID: 31100725; PMCID: PMC7331946.
26. Erickson MA, Hartvigson PE, Morofuji Y, Owen JB, Butterfield DA, Banks WA. Lipopolysaccharide impairs amyloid β efflux from brain: altered vascular sequestration, cerebrospinal fluid reabsorption, peripheral clearance and transporter function at the blood-brain barrier. *J Neuroinflammation*. 2012 Jun 29;9:150. ISSN: 1742-2094; DOI: 10.1186/1742-2094-9-150. PMID: 22747709; PMCID: PMC3410805.
27. Oboudiyat C, Glazer H, Seifan A, Greer C, Isaacson RS. Alzheimer's disease. *Semin Neurol*. 2013 Sep;33(4):313-29. ISSN: 0271-8235; DOI: 10.1055/s-0033-1359319. Epub 2013 Nov 14. PMID: 24234352.
28. Brickman AM. Contemplating Alzheimer's disease and the contribution of white matter hyperintensities. *Curr Neurol Neurosci Rep*. 2013 Dec;13(12):415. ISSN: 1528-4042; DOI: 10.1007/s11910-013-0415-7. PMID: 24190781; PMCID: PMC3874404.
29. Lanoiselée HM, Nicolas G, Wallon D, Rovelet-Lecrux A, Lacour M, Rousseau S, et al. collaborators of the CNR-MAJ project. APP, PSEN1, and PSEN2 mutations in early-onset Alzheimer disease: A genetic

- screening study of familial and sporadic cases. *PLoS Med.* 2017 Mar 28;14(3):e1002270. ISSN:1549-1676; DOI: 10.1371/journal.pmed.1002270. PMID: 28350801; PMCID: PMC5370101.
30. Henderson AS. The risk factors for Alzheimer's disease: a review and a hypothesis. *Acta Psychiatr Scand.* 1988 Sep;78(3):257-75. ISSN: 1600-0447; DOI: 10.1111/j.1600-0447.1988.tb06336.x. PMID: 3057813.
31. Anttila T, Helkala EL, Viitanen M, Kåreholt I, Fratiglioni L, Winblad B, et al. Alcohol drinking in middle age and subsequent risk of mild cognitive impairment and dementia in old age: a prospective population based study. *BMJ.* 2004 Sep 4;329(7465):539. ISSN: 1756-1833; DOI: 10.1136/bmj.38181.418958.BE. Epub 2004 Aug 10. PMID: 15304383; PMCID: PMC516103.
32. Qiu C, Kivipelto M, von Strauss E. Epidemiology of Alzheimer's disease: occurrence, determinants, and strategies toward intervention. *Dialogues Clin Neurosci.* 2009;11(2):111-28. ISSN No: 1294-8322; DOI: 10.31887/DCNS.2009.11.2/cqiu. PMID: 19585947; PMCID: PMC3181909.
33. Jessen NA, Munk AS, Lundgaard I, Nedergaard M. The Glymphatic System: A Beginner's Guide. *Neurochem Res.* 2015 Dec;40(12):2583-99. ISSN: 0022-3042; DOI: 10.1007/s11064-015-1581-6. Epub 2015 May 7. PMID: 25947369; PMCID: PMC4636982.
34. Kerjaschki D. The lymphatic vasculature revisited. *J Clin Invest.* 2014 Mar;124(3):874-7. ISSN: 1558-8238; DOI: 10.1172/JCI74854. Epub 2014 Mar 3. PMID: 24590271; PMCID: PMC3938252.
35. Reeves BC, Karimy JK, Kundishora AJ, Mestre H, Cerci HM, Matouk C, et al. Glymphatic System Impairment in Alzheimer's Disease and Idiopathic Normal Pressure Hydrocephalus. *Trends Mol Med.* 2020 Mar;26(3):285-295. ISSN No: 1471-499X; DOI: 10.1016/j.molmed.2019.11.008. Epub 2020 Jan 18. PMID: 31959516; PMCID: PMC7489754.
36. Kamagata K, Andica C, Takabayashi K, Saito Y, Taoka T, Nozaki H, et al. for the Alzheimer's Disease Neuroimaging Initiative. Association of MRI Indices of Glymphatic System With Amyloid Deposition and Cognition in Mild Cognitive Impairment and Alzheimer Disease. *Neurology.* 2022 Sep 19;99(24):e2648–60. ISSN: 2648-2660; DOI: 10.1212/WNL.0000000000201300. Epub ahead of print. PMID: 36123122; PMCID: PMC9757870.

37. Mawuenyega KG, Sigurdson W, Ovod V, Munsell L, Kasten T, Morris JC, et al. Decreased clearance of CNS beta-amyloid in Alzheimer's disease. *Science*. 2010 Dec 24;330(6012):1774. ISSN: 1095-9203 DOI: 10.1126/science.1197623. Epub 2010 Dec 9. PMID: 21148344; PMCID: PMC3073454.
38. Lundgaard I, Li B, Xie L, Kang H, Sanggaard S, Haswell JD, et al. Direct neuronal glucose uptake heralds activity-dependent increases in cerebral metabolism. *Nat Commun*. 2015 Apr 23;6:6807. ISSN: 2041-1723 DOI: 10.1038/ncomms7807. PMID: 25904018; PMCID: PMC4410436.
39. Benveniste H, Liu X, Koundal S, Sanggaard S, Lee H, Wardlaw J. The Glymphatic System and Waste Clearance with Brain Aging: A Review. *Gerontology*. 2019;65(2):106-119. ISSN: 2381-8662 DOI: 10.1159/000490349. Epub 2018 Jul 11. PMID: 29996134; PMCID: PMC6329683.
40. Iliff JJ, Wang M, Liao Y, Plogg BA, Peng W, Gundersen GA, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid β . *Sci Transl Med*. 2012 Aug 15;4(147):147ra111. ISSN: 1946-6242 DOI: 10.1126/scitranslmed.3003748. PMID: 22896675; PMCID: PMC3551275.
41. Zhang B, Li W, Zhuo Y, Xiang H, Li W, Liu H, et al. L-3-n-Butylphthalide Effectively Improves the Glymphatic Clearance and Reduce Amyloid- β Deposition in Alzheimer's Transgenic Mice. *J Mol Neurosci*. 2021 Jun;71(6):1266-1274. ISSN: 1559-1166 DOI: 10.1007/s12031-020-01752-z. Epub 2020 Nov 13. PMID: 33188502.
42. Oshio K. What Is the "Glymphatic System"? *Magn Reson Med Sci*. 2023 Jan 1;22(1):137-141. ISSN: 1347-3182; DOI: 10.2463/mrms.bc.2021-0059. Epub 2021 Oct 13. PMID: 34645739; PMCID: PMC9849416.
43. Grøntvedt GR, Schröder TN, Sando SB, White L, Bråthen G, Doeller CF. Alzheimer's disease. *Curr Biol*. 2018 Jun 4;28(11):R645-R649. ISSN: 0960-9822; DOI: 10.1016/j.cub.2018.04.080. PMID: 29870699.
44. Rasmussen MK, Mestre H, Nedergaard M. The glymphatic pathway in neurological disorders. *Lancet Neurol*. 2018 Nov;17(11):1016-1024. ISSN: 1474-4422; DOI: 10.1016/S1474-4422(18)30318-1. PMID: 30353860; PMCID: PMC6261373.

45. Nagelhus EA, Ottersen OP. Physiological roles of aquaporin-4 in brain. *Physiol Rev.* 2013 Oct;93(4):1543-62. ISSN: 1522-1210; DOI: 10.1152/physrev.00011.2013.; PMID: 24137016; PMCID: PMC3858210.
46. Gleiser C, Wagner A, Fallier-Becker P, Wolburg H, Hirt B, Mack AF. Aquaporin-4 in Astroglial Cells in the CNS and Supporting Cells of Sensory Organs-A Comparative Perspective. *Int J Mol Sci.* 2016 Aug 26;17(9):1411. ISSN: 1422-0067; DOI: 10.3390/ijms17091411. PMID: 27571065; PMCID: PMC5037691.
47. Nagelhus EA, Mathiisen TM, Ottersen OP. Aquaporin-4 in the central nervous system: cellular and subcellular distribution and coexpression with KIR4.1. *Neuroscience.* 2004;129(4):905-13. ISSN: 0306-4522; DOI: 10.1016/j.neuroscience.2004.08.053. PMID: 15561407.
48. Fagan AM, Holtzman DM, Munson G, Mathur T, Schneider D, Chang LK, et al. Unique lipoproteins secreted by primary astrocytes from wild type, apoE (-/-), and human apoE transgenic mice. *J Biol Chem.* 1999 Oct 15;274(42):30001-7. ISSN: 0021-9258; DOI: 10.1074/jbc.274.42.30001. PMID: 10514484.
49. Rosenberg GA, Kyner WT, Estrada E. Bulk flow of brain interstitial fluid under normal and hyperosmolar conditions. *Am J Physiol.* 1980 Jan;238(1):F42-9. ISSN: 0363-6119; DOI: 10.1152/ajprenal.1980.238.1.F42. PMID: 7356021.
50. Zhang ET, Inman CB, Weller RO. Interrelationships of the pia mater and the perivascular (Virchow-Robin) spaces in the human cerebrum. *J Anat.* 1990 Jun;170:111-23. ISSN No: 1469-7580; PMID: 2254158; PMCID: PMC1257067.
51. Harrison IF, Ismail O, Machhada A, Colgan N, Ohene Y, Nahavandi P, et al. Impaired glymphatic function and clearance of tau in an Alzheimer's disease model. *Brain.* 2020 Aug 1;143(8):2576-2593. ISSN: 2576-2593; DOI: 10.1093/brain/awaa179. PMID: 32705145; PMCID: PMC7447521.
52. Parums DV. Editorial: Targets for Disease-Modifying Therapies in Alzheimer's Disease, Including Amyloid β and Tau Protein. *Med Sci Monit.* 2021 Jul 26;27:e934077. ISSN: 1643-3750; DOI: 10.12659/MSM.934077. PMID: 34305135; PMCID: PMC8323472.

53. Knapp M, King D, Romeo R, Adams J, Baldwin A, Ballard C, et al. Cost-effectiveness of donepezil and memantine in moderate to severe Alzheimer's disease (the DOMINO-AD trial). *Int J Geriatr Psychiatry*. 2017 Dec;32(12):1205-1216. ISSN: 08856230; DOI: 10.1002/gps.4583. Epub 2016 Oct 13. ISSN No: 08856230; PMID: 27739182; PMCID: PMC5724694.
54. Adler G, Brassen S, Chwalek K, Dieter B, Teufel M. Prediction of treatment response to rivastigmine in Alzheimer's dementia. *J Neurol Neurosurg Psychiatry*. 2004 Feb;75(2):292-4. ISSN: 0022-3050; PMID: 14742608; PMCID: PMC1738912.
55. Samochocki M, Höffle A, Fehrenbacher A, Jostock R, Ludwig J, Christner C, et al. Galantamine is an allosterically potentiating ligand of neuronal nicotinic but not of muscarinic acetylcholine receptors. *J Pharmacol Exp Ther*. 2003 Jun;305(3):1024-36. ISSN: 00223565; DOI: 10.1124/jpet.102.045773. Epub 2003 Mar 20. PMID: 12649296.
56. Reisberg B, Doody R, Stöffler A, Schmitt F, Ferris S, Möbius HJ; Memantine Study Group. Memantine in moderate-to-severe Alzheimer's disease. *N Engl J Med*. 2003 Apr 3;348(14):1333-41. ISSN: 0028-4793; DOI: 10.1056/NEJMoA013128. PMID: 12672860.
57. Howard R, McShane R, Lindesay J, Ritchie C, Baldwin A, Barber R, et al. Donepezil and memantine for moderate-to-severe Alzheimer's disease. *N Engl J Med*. 2012 Mar 8;366(10):893-903. ISSN: 0028-4793; DOI: 10.1056/NEJMoA1106668. PMID: 22397651.
58. Zhang B, Li W, Zhuo Y, Xiang H, Li W, Liu H, et al. L-3-n-Butylphthalide Effectively Improves the Glymphatic Clearance and Reduce Amyloid- β Deposition in Alzheimer's Transgenic Mice. *J Mol Neurosci*. 2021 Jun;71(6):1266-1274. ISSN No: 08958696; DOI: 10.1007/s12031-020-01752-z. Epub 2020 Nov 13. PMID: 33188502.
59. Ren H, Luo C, Feng Y, Yao X, Shi Z, Liang F, et al. Omega-3 polyunsaturated fatty acids promote amyloid- β clearance from the brain through mediating the function of the glymphatic system. *FASEB J*. 2017 Jan;31(1):282-293. ISSN: 0892-6638; DOI: 10.1096/fj.201600896. Epub 2016 Oct 7. PMID: 27789520.

60. Choi C, Park J, Kim H, Chang KT, Park J, Min KT. DSCR1 upregulation enhances dural meningeal lymphatic drainage to attenuate amyloid pathology of Alzheimer's disease. *J Pathol.* 2021 Nov;255(3):296-310. ISSN: 0022-3417; DOI: 10.1002/path.5767. Epub 2021 Aug 20. PMID: 34312845.
61. Zheng X, Lin W, Jiang Y, Lu K, Wei W, Huo Q, et al. Electroacupuncture ameliorates beta-amyloid pathology and cognitive impairment in Alzheimer disease via a novel mechanism involving activation of TFEB (transcription factor EB). *Autophagy.* 2021 Nov;17(11):3833-3847. ISSN: 2769-4127; DOI: 10.1080/15548627.2021.1886720. Epub 2021 Feb 23. PMID: 33622188; PMCID: PMC8632298.
62. De la Rosa A, Olaso-Gonzalez G, Arc-Chagnaud C, Millan F, Salvador-Pascual A, García-Lucerga C, et al. Physical exercise in the prevention and treatment of Alzheimer's disease. *J Sport Health Sci.* 2020 Sep;9(5):394-404. ISSN: 2095-2546; DOI: 10.1016/j.jshs.2020.01.004. Epub 2020 Feb 4. PMID: 32780691; PMCID: PMC7498620.
63. Mahdi O, Baharuldin MTH, Nor NHM, Chiroma SM, Jagadeesan S, Moklas MAM. The Neuroprotective Properties, Functions, and Roles of Cannabis sativa in Selected Diseases Related to the Nervous System. *Cent Nerv Syst Agents Med Chem.* 2021;21(1):20-38. ISSN: 18715249; DOI: 10.2174/1871524921666210127110028. PMID: 33504317.
64. Sampson PB. Phytocannabinoid Pharmacology: Medicinal Properties of *Cannabis sativa* Constituents Aside from the "Big Two". *J Nat Prod.* 2021 Jan 22;84(1):142-160. ISSN No: 0163-3864; DOI: 10.1021/acs.jnatprod.0c00965. Epub 2020 Dec 23. PMID: 33356248.
65. PubChem [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Compound Summary for CID 2543, Cannabinol; [cited 2023 Apr. 3]. Available from: <https://pubchem.ncbi.nlm.nih.gov/compound/Cannabinol>.
66. PubChem [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Substance Record for SID 349991117, (-)-DELTA.1-Tetrahydrocannabinol, Source: Therapeutic Target Database (TTD); [cited 2023 Apr. 3]. Available from: <https://pubchem.ncbi.nlm.nih.gov/substance/349991117>.

67. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Compound Summary for CID 5315659, Cannabigerol; [cited 2023 Apr. 3]
68. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Compound Summary for CID 11601669, Cannabidivarin; [cited 2023 Apr. 3.
69. Patil N, Chandel V, Rana A, Jain M, Kaushik P. Investigation of *Cannabis sativa* Phytochemicals as Anti-Alzheimer's Agents: An *In Silico* Study. *Plants (Basel)*. 2023 Jan 22;12(3):510. ISSN: 2223-7747; DOI: 10.3390/plants12030510. PMID: 36771595; PMCID: PMC9919841.
70. Baskaran KP, Arumugam A, Kandasamy R, Alagarsamy S. Insilico method for prediction of maximum binding affinity and ligand—protein interaction studies on Alzheimer's disease. *Int J Res Granthaalayah*. 2020 Dec 19;8(11):362-70. ISSN: 2350-0530; DOI:10.29121/granthaalayah.v8.i11.2020.2472.
71. Kanthamma SL, Kriszthnamurthi J, Hemalathaa CN, Aanandhi MV. Insilico approach of interaction studies in *Bacopa monnieri* compounds targeting multi-proteins for Alzheimer's Disease. *Research Journal of Pharmacy and Technology*. 2018 Apr 1;11(4):1522-6. ISSN: 0974-360x; DOI:10.5958/0974-360X.2018.00283.4
72. Tripathi A, Shrinet K, Singh VK, Kumar A. Molecular modelling and docking of *Mus musculus* HMGB1 inflammatory protein with CGA. *Bioinformation*. 2019 Jul 31;15(7):467-473. ISSN: 0973-2063; DOI: 10.6026/97320630015467. PMID: 31485132; PMCID: PMC6704330.
73. Takata K, Kitamura Y, Tsuchiya D, Kawasaki T, Taniguchi T, Shimohama S. High mobility group box protein-1 inhibits microglial Abeta clearance and enhances Abeta neurotoxicity. *J Neurosci Res*. 2004 Dec 15;78(6):880-91. ISSN: 1613-6810; DOI: 10.1002/jnr.20340. PMID: 15499593.
74. Zhou H, Du R, Li G, Bai Z, Ma J, Mao C, et al. Cannabinoid receptor 2 promotes the intracellular degradation of HMGB1 via the autophagy-lysosome pathway in macrophage. *Int Immunopharmacol*. 2020 Jan;78:106007. ISSN: 1878-1705; DOI: 10.1016/j.intimp.2019.106007. Epub 2019 Dec 2. PMID: 31806570.

75. Rosu GC, Catalin B, Balseanu TA, Laurentiu M, Claudiu M, Kumar-Singh S, et al. Inhibition of Aquaporin 4 Decreases Amyloid A β 40 Drainage Around Cerebral Vessels. *Mol Neurobiol*. 2020 Nov;57(11):4720-4734. ISSN: 1559-1182; DOI: 10.1007/s12035-020-02044-8. Epub 2020 Aug 11. PMID: 32783141; PMCID: PMC7515968.
76. Li Y, Qin HQ, Chen QS, Wang JJ. Behavioral and neurochemical effects of the intrahippocampal co-injection of beta-amyloid protein 1-40 and ibotenic acid in rats. *Int J Neurosci*. 2004 Dec;114(12): 1521-31. ISSN: 1563-5279; DOI: 10.1080/00207450490509159. PMID: 15512836.
77. Milton NG. Amyloid-beta binds catalase with high affinity and inhibits hydrogen peroxide breakdown. *Biochem J*. 1999 Dec 1;344 Pt 2(Pt 2):293-6. ISSN: 1470-8728; PMID: 10567208; PMCID: PMC1220643.
78. Henshaw FR, Dewsbury LS, Lim CK, Steiner GZ. The Effects of Cannabinoids on Pro- and Anti-Inflammatory Cytokines: A Systematic Review of *In Vivo* Studies. *Cannabis Cannabinoid Res*. 2021 Jun;6(3):177-195. ISSN: 2578-5125; DOI: 10.1089/can.2020.0105. Epub 2021 Apr 28. PMID: 33998900; PMCID: PMC8266561.
79. Kozela E, Pietr M, Juknat A, Rimmerman N, Levy R, Vogel Z. Cannabinoids Delta (9)-tetrahydrocannabinol and cannabidiol differentially inhibit the lipopolysaccharide-activated NF-kappaB and interferon-beta/STAT proinflammatory pathways in BV-2 microglial cells. *J Biol Chem*. 2010 Jan 15;285(3):1616-26. ISSN: 1083-351x; DOI: 10.1074/jbc.M109.069294. Epub 2009 Nov 12. PMID: 19910459; PMCID: PMC2804319.
80. Götz MR, Collado JA, Fernández-Ruiz J, Fiebich BL, García-Toscano L, Gómez-Cañas M, et al. Structure-Effect Relationships of Novel Semi-Synthetic Cannabinoid Derivatives. *Front Pharmacol*. 2019 Nov 20;10:1284. ISSN: 1663-9812; DOI: 10.3389/fphar.2019.01284. PMID: 31824305; PMCID: PMC6880192.