Drew University

College of Liberal Arts

The Effects of Natural Supplements on the Growth and Development of Primary Cortical Neurons in a Model of Alzheimer's Disease

A Thesis in Biology

By

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Submitted in Full Fulfillment
of the Requirements
for the Degree of
Bachelor in Arts
With Specialized Honors in Biology
May 2025

Acknowledgements

First and foremost, I would like to express my deepest gratitude to Dr. Knowles, my academic and honors thesis advisor, and the most inspiring mentor I have had at Drew. From the moment he took me under his wing in my sophomore year, he has guided, challenged, and supported me in ways that have shaped not only my academic journey but also my growth as a person. His belief in me made this entire experience possible, and I am profoundly indebted to him for giving me the opportunity to pursue an honors thesis. I am also incredibly thankful to my other thesis advisors, Dr. Dunaway and Professor Rhodes, for generously offering their time, insight, and support throughout this process. Their thoughtful feedback and encouragement helped strengthen my thesis, and the knowledge I gained from their courses pushed me to think critically and expansively. I am grateful for the ways they helped me grow as a student and as a thinker.

A special thanks goes to Amineh Al-Aydi, Julianna Daudelin, and Erika Krukiel for their invaluable help with data collection and their perseverance in continuing our lab work despite the challenges we faced. Mentoring them in their own research and having them contribute to my project was an incredibly rewarding experience that deepened my passion for both research and leadership.

Finally, I would like to thank my parents, my siblings, and my grandmother, Entisar Abdelhack, for being my unwavering support system throughout college and especially during this thesis journey. Their love, encouragement, and compassion have carried me through every step of the way, and I truly could not have done this without them.

Abstract

Alzheimer's Disease (AD) is a progressive neurodegenerative condition driven by several pathological processes, including the accumulation of amyloid-beta plaques, abnormal tau protein hyperphosphorylation, oxidative stress, and synaptic dysfunction. Current pharmacological treatments provide only modest symptomatic relief, as well as heavy financial burden, highlighting the urgent need for alternative and complementary therapeutic approaches. Recent trends towards holistic health have led to increased use of natural supplements for treating various conditions, many of which originate from cultural traditional medicines used for millennia. Although many supplements are praised for their health benefits, they often remain understudied and lack comprehensive research on their mechanistic and molecular effects. This thesis explores the neuroprotective potential of three natural supplements–ashwagandha (*Withania somnifera*), ginkgo biloba, and olive leaf extract–in a cell culture model of AD. Experiments were conducted to determine the safety and efficacy of the supplements and their ability to mitigate the deleterious effects of oxidative stress and amyloid-beta toxicity. Our experimental results revealed that formulations of treatment with ashwagandha, ginkgo biloba, and olive leaf extract were generally tolerable to cells, either maintaining or improving the health of neurons. Preliminary findings also suggest that all three supplements in isolation and in combination could encourage increased cell survival while under AD stress-like conditions.

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Part I

Introduction to Alzheimer's Disease

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder that primarily affects memory, thinking, and behavior. It is the most common cause of dementia, accounting for an estimated 60-80% of all cases worldwide (Olazarán et al., 2023). AD is characterized by the abnormal accumulation of amyloid-beta plaques and neurofibrillary tangles in the brain, which can disrupt neuronal function and lead to cognitive decline (Olazarán et al., 2023). This condition remains a significant challenge amongst aging populations, who are predominantly affected by AD. Furthermore, its impact on both affected individuals and society is immense. For many families, the emotional toll of witnessing a loved one's decline is intensified by the financial and physical burdens of caregiving, with many caregivers reporting high levels of stress, anxiety, and depression (Ameri et al. 2024). More than 50 million people globally have been diagnosed with AD, and that number is expected to triple by 2050 due to population aging (Olazarán et al., 2023). As the median age increases and more of the population becomes susceptible to the disease, the societal burden of AD will become more pronounced, stressing the importance of finding more effective treatments and preventative strategies.

Additionally, the economic burden of AD is profound, imposing substantial financial costs on patients, families, and healthcare systems. In 2019, the total cost of dementia globally, including Alzheimer's disease, exceeded $1 trillion annually and is expected to double by 2030 due to the increasing prevalence of the disease (Wimo et al. 2023). According to the Journal of the Alzheimer's Association, in the United States alone, the total cost of dementia-related care including AD in 2024 was estimated to be at $360 billion annually. With patients requiring

multiple therapies and specialized treatments in order to go about daily life, families are forced to shoulder significant out-of-pocket expenses for caregiving services, home modifications, and medications. The average total annual cost of treating AD in patients 65 or older have been estimated to exceed $40,000 for Medicare beneficiaries (Skaria, 2022). AD medications in particular contribute to further amplifying these costs as they are relatively expensive and require extensive monitoring over the course of treatment. For instance, Leqembi (lecanemab) is an amyloid-targeting intravenous therapy that was approved for early-stage Alzheimer's, and is priced at approximately $26,000 annually (Cubanski & Neuman, 2023). Patients receiving the drug are responsible for more than $5,000 out-of-pocket each year. Similarly, Biogen's Aduhelm (aducanemab), another FDA-approved amyloid-targeting therapy, was initially priced at $56,000 annually but faced widespread criticism, leading to a price reduction of $28,000 per year. These treatments necessitate regular infusions and frequent clinical visits for cognitive assessments and monitoring, adding to the logistical and financial challenges for patients and healthcare systems. The economic realities of treatment underscore the urgent need for more affordable and effective therapeutic options that can reduce both the direct and indirect costs associated with AD while improving the quality of life for patients and their families.

Current Therapeutic Approaches

Until 2021, the therapeutic landscape for Alzheimer's disease was largely focused on managing symptoms rather than addressing the underlying causes of the disease. Pharmacological interventions are the standard treatment, with cholinesterase inhibitors (e.g. donepezil, galantamine) and N-methyl-D-aspartate (NMDA) receptor antagonists (e.g. memantine) being the most widely prescribed drugs (Passeri et al., 2022). These medications aim to temporarily slow the loss of function by maintaining neurotransmitter levels, thereby aiding

memory, attention, and other cognitive abilities (Passeri et al., 2022). Previous research has indicated the presence of a cholinergic deficit in AD brains, and that a cholinergic depletion is associated with the progression of neurodegeneration (Briggs et al., 2016). This, along with the classification of acetylcholine as a neurotransmitter that helps to facilitate memory and learning, prompted the development of cholinesterase inhibitors, which block the activity of the cholinesterase enzyme that breaks down acetylcholine at the synaptic cleft (Briggs et al., 2016).

Another hypothesis relates to the over-stimulation of the NMDA receptors on the gated ion channels, resulting in synaptic or dendritic damage that could lead to the characteristic neurodegeneration seen in AD (Robinson et al., 2006). Memantine is an uncompetitive antagonist that allows for the blocking of the NMDA channels, helping to regulate glutamate activity to prevent excessive neuronal firing and ultimately degradation (Robinson et al., 2006). Both of these treatments provide marginal neuroprotective effects, and they do not halt the progression of the disease. Rather, they provide symptomatic relief for a limited period of time, often delaying more severe cognitive decline by only a few months to a year (Passeri et al., 2022). Studies have suggested that memantine works most effectively in moderate to severe AD, with minimal evidence of improvements found in milder cases (McShane et al., 2019), while only ⅕ of patients taking cholinesterase inhibitors experience a noticeable benefit (Birks et al., 2018). However, even these responders tend to lose the benefits of the drug within the next couple of years. This highlights the need to develop more disease-modifying therapies, such as investigating upstream pathways as a primary target for slowing disease progression.

A major challenge with the current pharmacological treatments is their limited efficacy. The aforementioned drugs are only moderately effective at alleviating symptoms and have no impact on slowing or reversing the disease progression (Passeri et al., 2022). As the disease

advances, the cognitive benefits of the medications diminish, and the side effects exacerbate the challenges of long-term treatment. Common adverse side effects found in these medications include gastrointestinal issues such as nausea and diarrhea, as well as cardiovascular side effects like bradycardia (Briggs et al., 2016). These complications can lead to poor adherence to medication regimens, further reducing treatment effectiveness. Furthermore, one of the most significant hurdles in developing more effective therapies for AD is the blood-brain barrier (BBB). This selective barrier prevents many drugs from reaching the brain in sufficient concentrations in order to have a therapeutic effect (Passeri et al., 2022). As a result, researchers are exploring novel delivery methods, such as nano-particle based systems, to enhance drug bioavailability and ensure that treatments can more effectively target the brain and bypass the BBB (Passeri et al., 2022). This will be critical to broadening therapeutic options available to AD patients.

Recent developments in Alzheimer's treatment have also focused on targeting amyloid-beta plaques, a hallmark of the disease. The approval of new drugs such as aducanumab and lecanemab has marked a shift towards disease modifying therapies aimed at reducing amyloid deposition in the brain (Huang et al., 2023). Clinical trials have demonstrated that these drugs can lower levels of amyloid plaques in the brain. While animal models suggest that this reduction may occur through microglial activation and subsequent phagocytosis of amyloid-beta, the precise mechanisms remain unclear, and other biological processes could be contributing to this effect (Wojcieszak, 2023). However, their effectiveness in improving cognitive function is up for debate. Aducanumab directly binds to amyloid-beta plaques in the brain tissue to promote its breakdown (Sevigny et al., 2016). Mixed results were observed in terms of reduction of cognitive decline and the effectiveness of plaque clearance, thus the FDA approval of

aducanumab in 2021 was met with controversy as the primary endpoints for reducing cognitive decline were not met during clinical trials (Brockmann et al., 2023). Conversely, lecanemab was designed to target earlier stages of amyloid-beta aggregation by binding to amyloid-beta protofibrils and oligomers as opposed to the plaques themselves (Swanson et al., 2021). Clinical trials demonstrated that lecanemab significantly reduced amyloid-beta levels in the brain and led to a statistically significant 27% slowing of cognitive decline (Swanson et al., 2021). This would seem to indicate that, in a typical 12-month period, a patient receiving treatment would experience the equivalent of approximately 9 months of cognitive decline compared to an untreated individual. While this represents a measurable effect, the real-world impact remains uncertain—whether this would be a noticeable effect is still rather debatable. Notably, lecanemab's effectiveness was found to be more pronounced in patient's with the APOE ε4 genotype, a gene known for increasing an individual's risk for AD. While this approach is promising, it is not without considerable risks. Firstly, while these treatments have demonstrated strong efficacy in reducing amyloid-beta plaques, patients continue to experience cognitive decline despite significant plaque clearance. This discrepancy raises concerns about the validity of the amyloid-beta hypothesis, which has been the dominant framework guiding Alzheimer's research for the past three decades. The failure of amyloid-targeting therapies to produce substantial cognitive improvements suggests that other pathological mechanisms, such as tau protein aggregation, neuroinflammation, or vascular dysfunction, may play a more critical role in disease progression than previously thought. Patients treated with these drugs have also reported side effects such as amyloid-related imaging abnormalities (ARIA), including brain swelling and microhemorrhages (Wojcieszak, 2023). The long-term impact of these side effects is still unknown, raising questions about the overall safety and viability of anti-amyloid therapies.

Additionally, these disease-modifying drugs come with substantial costs, with treatments like lecanemab priced at approximately $26,500 per year and aducanumab initially launched at $56,000 annually before being reduced to $28,200. Given these high costs, access to these treatments remains largely restricted to high-income countries such as the U.S., Japan, and Western Europe, making it unlikely that patients in lower-income regions could afford or obtain them through their healthcare systems. As a result, there is growing interest in alternative approaches that may be more accessible and cost-effective.

In addition to pharmacological treatments, non-drug therapies are gaining attention as complementary approaches to managing Alzheimer's disease. Cognitive stimulation therapy (CST) and reminiscence therapy are two such interventions that have shown promise in improving cognitive function and quality of life for individuals with AD (Min et al., 2023). These therapies focus on engaging patients in mental exercises and social activities designed to stimulate memory and cognition. Some studies have suggested that consistent engagement in these programs can improve mood, reduce anxiety, and potentially slow the progression to more severe cognitive impairment (Min et al., 2023). For instance, research suggests that CST provides a modest cognitive benefit compared to standard care or unstructured activities, with its effects comparable to delaying cognitive decline by approximately six months in individuals with mild-to-moderate dementia (Woods et al., 2023). While these non-pharmacological interventions do not alter the disease's course, they provide meaningful benefits for patients and caregivers. Importantly, they emphasize the role of holistic, patient-centered care in Alzheimer's treatment, promoting mental, emotional, and social well being alongside traditional therapies. Regardless, it is important to mention that these therapies can be resource-intensive, often requiring trained professionals, structured programs, and ongoing engagement, which can lead to

significant costs. For example, an 8-week CST program costs approximately $299.96 (~$37.81 per week), with accessibility often limited by the availability of trained facilitators and institutional funding (D'Amico et al., 2015). Maintenance Cognitive Stimulation Therapy (MCST) has shown cost-effectiveness in improving quality of life, with an Incremental Cost-Effectiveness Ratio (ICER) of £266 ($352.37) per 1-point improvement on the QoL-AD scale. However, it was not considered cost-effective for cognitive improvements measured by ADAS-Cog, suggesting that while MCST provides some benefits, its financial burden may not be justified by modest cognitive gains (D'Amico et al., 2015). What's more, while CST is scalable and can be implemented in community centers and care homes, its accessibility remains limited in regions lacking trained facilitators and institutional funding. Similarly, reminiscence therapy (RT) varies in cost depending on the setting and resources used, but its implementation often requires specialized facilitators and materials such as music and photographs, making it less accessible in under-resourced areas. Additionally, tailoring these interventions to individual patients based on their disease stage can be challenging, further limiting their accessibility in broader healthcare settings, particularly in regions with limited healthcare funding or specialized dementia care services. Indeed, while each of the therapies have tangible benefits, it is important to explore alternative strategies for reducing and preventing cognitive decline in all patients.

Part II

Need for Alternative Therapies

Rationale

In recent years, there has been a significant surge in interest surrounding alternative and complementary therapies for Alzheimer's disease. This growing trend can be attributed to several factors, including the increasing popularity of traditional medicine practices known as ayurvedic medicine, the accessibility and affordability of natural supplements, and a general shift towards holistic health approaches (Pandey et al. 2013). Unlike traditional pharmaceuticals, which often come with a range of side effects and high costs, alternative therapies typically offer cost-effective and readily available options for individuals seeking to manage their health proactively (Pandey et al. 2013).

One of the primary motivations for exploring alternative therapies is the urgent need to find treatments that can effectively slow disease progression or enhance the quality of life for patients. Chronic diseases (and particularly complex neurodegenerative diseases such as Alzheimer's Disease), present challenges that conventional treatments often fail to address comprehensively. Current pharmaceutical treatments for Alzheimer's disease include cholinesterase inhibitors (e.g., donepezil, rivastigmine, galantamine) and NMDA receptor antagonists (e.g., memantine) (Wollen 2010). Cholinesterase inhibitors aim to increase the availability of acetylcholine, a neurotransmitter important for memory, while NMDA antagonists work to regulate glutamate activity, which can help prevent excitotoxicity and cell death (Wollen 2010). However, these treatments provide only modest benefits, often addressing symptoms temporarily without significantly altering disease progression. Additionally, side effects such as

nausea, dizziness, and insomnia are common, and some patients experience worsening cognitive symptoms over time, highlighting the need for more effective and sustainable therapies (Kim et al. 2018). More recently, monoclonal antibodies like aducanumab and lecanemab have been developed to target amyloid-beta plaques, a hallmark of Alzheimer's pathology (Söderberg et al. 2022). While promising in their approach, these therapies have shown mixed efficacy and can be prohibitively expensive, with potential adverse effects including brain swelling and bleeding (Söderberg et al. 2022).

The limitations of existing pharmacological interventions such as limited efficacy and adverse side effects underscore the necessity for novel therapeutic approaches (Wollen 2010). Alternative therapies, such as those derived from natural supplements, offer promising avenues for mitigating symptoms, enhancing cognitive function, and potentially altering disease trajectory (Sharma et al. 2019). Moreover, the holistic nature of alternative therapies aligns well with the multifaceted approach required to manage chronic illnesses. Unlike conventional drugs that target isolated biological pathways, alternative therapies often exert broad systemic effects, addressing multiple aspects of disease pathology simultaneously. Many natural compounds, such as polyphenols and adaptogens, have been shown to modulate oxidative stress, neuroinflammation, and amyloid-beta accumulation—key contributors to neurodegeneration. For instance, polyphenols possess antioxidant and anti-inflammatory properties that may ameliorate gut dysbiosis, which is linked to Alzheimer's disease pathology (Shabbir et al., 2021). Additionally, adaptogens like *Schisandra chinensis*, *Eleutherococcus senticosus*, *Rhodiola rosea*, and *Withania somnifera*have demonstrated anti-neuroinflammatory potential by regulating the expression of cytokines and modulating signaling pathways (Wróbel-Biedwara & Podolak, 2024). Furthermore, the integration of alternative treatments into a comprehensive care model

recognizes that AD is not solely a neurological disorder but one that affects overall well-being, necessitating interventions that support cognitive, emotional, and physical health. By addressing the various aspects of health, these therapies can provide a more comprehensive treatment regimen. This integrative approach not only complements conventional medical treatments but also empowers individuals to take an active role in their health management (Sharma et al. 2019). Consequently, the exploration of these therapies is not merely an adjunct to existing treatments but a crucial component in the quest for more effective and sustainable health solutions.

Introduction to Natural Alternatives

Natural supplements encompass a broad category of products that include vitamins, minerals, herbs, botanicals, amino acids, and other substances intended to supplement the diet and promote health (Hassen et al. 2022). Unlike conventional medications, which are typically synthesized in laboratories and rigorously tested for specific pharmacological effects, natural supplements are derived from natural sources and are often perceived as being more harmonious with the body's physiological processes due to many key factors related to their composition and how they interact with the body (Feng et al. 2021). Unlike synthetic medications, which typically contain a single active ingredient, natural supplements include a range of bioactive compounds working together synergistically, enhancing each other's effects and providing broader physiological support without overwhelming or overstimulating one specific pathway (Caesar et. al 2019). One notable case is the use of *Artemisia annua* (sweet wormwood) in treating malaria. The plant's primary active compound, artemisinin, is highly effective against malaria parasites. However, studies have shown that whole plant extracts of *A. annua* can be more effective than isolated artemisinin alone (Suberu et al., 2013). This enhanced efficacy is attributed to the

presence of other flavonoids and terpenoids in the plant that work synergistically with artemisinin, increasing its bioavailability and antimalarial activity.

Research has also highlighted the cost-effectiveness of specific natural interventions largely due to their affordability, widespread availability, and minimal need for medical supervision. Unlike pharmaceutical treatments, which require extensive research, clinical trials, regulatory approval, and ongoing physician oversight, natural supplements are often derived from readily available plant-based compounds that can be mass-produced at a lower cost. Their availability over the counter, without the need for a prescription, further reduces barriers to access, making them a more feasible option for individuals in low-resource settings or those without comprehensive healthcare coverage. For example, a study found that B-vitamin supplementation for individuals with elevated homocysteine levels—a known risk factor for dementia—was highly cost-effective, with estimated savings of £60,021 ($79,520) per quality-adjusted life year (QALY) gained (Tsiachristas & Smith, 2016). This suggests that targeted nutritional interventions could provide a scalable, affordable strategy for neuroprotection, especially when compared to high-cost pharmaceutical interventions. Furthermore, natural supplements typically do not require expensive diagnostic testing, frequent clinical visits, or intensive monitoring for adverse effects, further lowering their overall cost burden. However, while natural supplements offer financial and logistical advantages, concerns regarding standardization, bioavailability, and regulatory oversight persist.

The perception that natural supplements can provide substantial health benefits while being cost-effective is not always accurate. For instance, some herbal compounds, such as St. John's Wort, which has been used to treat depression, has also been shown to interact negatively with prescription medications, reducing their efficacy and potentially causing unwanted side

effects (Henderson et al., 2002). Other natural supplements have been linked to serious health risks. Kava is an herb which, although has been implicated in alleviating anxiety and stress, has also been linked to liver toxicity (Rolf et al., 2012). Additionally, lack of stringent regulation for dietary supplements raises concerns about variability in potency, contamination, and the reliability of health claims. Unlike prescription medications, which undergo rigorous clinical testing and approval by regulatory agencies like the FDA or EMA, dietary supplements are not required to meet the same standards for efficacy, safety, or consistency before reaching the market (Richardson et al., 2022). This regulatory gap can result in significant variations between products, even among those claiming to contain the same active ingredients. Studies have found discrepancies in the actual content of herbal supplements compared to what is listed on their labels, with some products containing lower-than-advertised levels of key compounds or, conversely, dangerously high doses (Crawford et al., 2024). Additionally, contamination with heavy metals, pesticides, or other undisclosed ingredients has been reported in certain supplements, posing potential health risks to consumers. Furthermore, manufacturers are not required to provide robust clinical evidence to support health claims, leading to misleading or exaggerated marketing that can influence consumer decisions without adequate scientific backing (Richardson et al., 2022). Further experimentation on the effects of these supplements are necessary before ascertaining any benefits from usage. Despite these regulatory shortcomings, the interest in natural supplements as accessible therapeutic agents remains strong. In the context of Alzheimer's Disease and other neurodegenerative disorders, several natural supplements have garnered attention for their potential therapeutic effects. Particularly, some of the natural supplements that have been investigated for their potential in AD treatment are ashwagandha, olive leaf extract, and ginkgo biloba.

Ashwagandha

Ashwagandha, also known as *Withania somnifera*, is a medicinal herb widely used in traditional ayurvedic medicine, particularly in India, for its adaptogenic properties that help the body manage stress (Mikulska et al. 2023). Ayurveda, which is an ancient traditional medicine system originating in India, can be traced back to as early as 6000 BC (Singh et al., 2011). Rooted in a holistic approach, ayurvedic medicine practices seek to balance the mind, body, and spirit through diet, herbal medicine, lifestyle practices, and spiritual well-being (Jaiswal et al., 2017). Traditionally classified as a "Rasayana," or rejuvenator, ashwagandha has been revered in ayurveda for millenia due its wide-ranging health benefits (Singh et al., 2011). Its roots and leaves are harvested for medicinal purposes, commonly ground into powders or extracts and ingested as supplements. Historically, ashwagandha has been used to support vitality, improve memory, and promote overall mental clarity, making it a long-standing remedy for cognitive and physical health (Mikulska et al. 2023). Recently, research has shown promising results regarding its neuroprotective effects. Studies suggest that ashwagandha may help to reduce oxidative stress in the brain, a known contributor to AD, by enhancing antioxidant activity (Shah et al. 2015). Animal studies have also shown that it can reduce amyloid-beta plaques, a characteristic of AD pathology, and improve cognitive performance (Kolarsky et al. 2024). Clinical trials in humans have also noted improvements in memory and executive function in patients who took ashwagandha supplements (Gopukumar et al. 2021). While these findings highlight ashwagandha's potential as an adjunct therapy for AD, several limitations must be considered. Many of the promising results come from animal studies, which do not always translate directly to human outcomes due to differences in metabolism and disease progression. For instance, studies in transgenic mouse models of AD have shown significant reductions in amyloid-beta

plaques and tau pathology after ashwagandha administration, but similar effects have not yet been robustly demonstrated in human trials (Sehgal et al., 2012). Additionally, doses used in rodent studies often far exceed those feasible for human consumption, raising concerns about real-world applicability. Moreover, existing clinical trials often involve small sample sizes, short study durations, and variability in supplement formulations, such as the trial conducted in 2017 by Choudhary et al., which included only 50 participants. This limits statistical power and generalizability and makes it difficult to draw definitive conclusions about its long-term efficacy and safety. Short study durations further complicate interpretation—some trials last only 8 to 12 weeks, an insufficient period to assess meaningful changes in AD progression. Furthermore, inconsistencies in supplement formulations make it difficult to draw firm conclusions. For example, some studies use aqueous root extracts, while others use standardized withanolide-rich formulations, each with varying bioavailability and potency (Mikulska et al., 2023). Another critical limitation is the lack of data on potential interactions with standard AD treatments, such as donepezil or memantine. Given ashwagandha's effects on neurotransmitter systems and neuroinflammation, it is unclear whether it could enhance, diminish, or cause adverse reactions when combined with these medications. Without large-scale, placebo-controlled trials, the extent to which ashwagandha can meaningfully impact AD progression remains uncertain. While its traditional use and emerging scientific support are promising, further rigorous research is essential to establish its clinical relevance in AD treatment.

Ginkgo Biloba

Derived from one of the oldest species on Earth, Ginkgo biloba is commonly used in traditional Chinese medicine to enhance memory and cognitive function. Often referred to as a

"living fossil," it has a rich history dating back to at least the 11th century CE (Crane 2013). Ginkgo seeds were commonly administered to alleviate respiratory issues such as asthma and bronchitis, as well as to support kidney and bladder health (Strømgaard et al., 2010). Additionally, members of the royal court were given ginkgo nuts for senility, reflecting its early association with cognitive health (Strømgaard et al., 2010). The fan-shaped leaves of the ginkgo tree are processed into extracts rich in flavonoids and terpenoids, compounds known for their antioxidant properties, which can protect brain cells from oxidative damage (Tabassum et. al 2022). Given its long history as a remedy for age-related cognitive decline, it has garnered intrigue and became studied for its potential in treating AD. Research suggests that ginkgo biloba may improve blood flow to the brain, helping to maintain healthy cognitive function by supplying neurons with essential nutrients and oxygen (Didier et al. 1996). Clinical trials have shown that ginkgo biloba extract can modestly improve memory, attention, and overall cognitive performance in individuals with AD or mild cognitive impairment (Kanowski et al. 1996). However, while some studies highlight significant benefits, others have shown only minimal effects, making its efficacy a topic of ongoing debate.This inconsistency largely stems from variations in study design, dosage, duration of treatment, and patient demographics. Other large-scale studies, such as the Ginkgo Evaluation of Memory (GEM) study, reported no significant difference in cognitive decline between those taking Ginkgo biloba and those given a placebo (Snitz et al., 2010). Additionally, inconsistencies in product quality such as differences in extract standardization and bioavailability may contribute to the mixed findings (Weinmann et al., 2010). These conflicting results highlight the need for further research with rigorous methodology to determine whether Ginkgo biloba is a reliable therapeutic option for Alzheimer’s disease and other forms of cognitive impairment.

Olive Leaf Extract

Olive leaf extract, derived from the leaves of the *Olea europaea* tree, has been recognized in Mediterranean cultures for its health benefits, particularly in treating infections and supporting cardiovascular health. Traditionally, olive leaves have been valued for their high concentration of polyphenols, especially oleuropein, which possesses antioxidant, anti-inflammatory, and neuroprotective properties (Omar et al. 2010). Recent studies have suggested that olive leaf extract may offer therapeutic benefits for Alzheimer's disease due to its ability to reduce inflammation and protect neurons from oxidative stress. Research indicates that oleuropein can help prevent the aggregation of beta-amyloid plaques and reduce tau protein phosphorylation, both of which are central to AD pathology (Romero-Marquez et al. 2022). In animal studies, olive leaf extract has been shown to improve memory and cognitive function (Mikami et al. 2021), while other findings support its use as a neuroprotective agent (Mohagheghi et al. 2011). The mechanisms underlying these effects appear to be interconnected. Oleuropein's antioxidant properties help neutralize reactive oxygen species, thereby reducing oxidative damage to neurons (Romero-Márquez et al., 2023). This reduction in oxidative stress can subsequently decrease neuroinflammation, as oxidative stress often triggers inflammatory responses in the brain. By mitigating these factors, oleuropein may indirectly influence the formation of beta-amyloid plaques and tau phosphorylation. Therefore, the observed effects on plaques and tau phosphorylation could indeed be downstream consequences of oleuropein's antioxidant and anti-inflammatory actions. However, while these findings are promising, more research is necessary to fully elucidate the precise pathways involved and to confirm these effects. Several limitations make the role of olive leaf extract in AD treatment still debatable. Variability in the

composition of olive leaf extract due to differences in oleuropein concentration, extraction methods, and formulation complicates reproducibility and consistency across studies. This lack of standardization makes it difficult to determine the most effective dosage and delivery method for AD patients. Additionally, while oleuropein has demonstrated the ability to cross the blood-brain barrier in animal studies, its bioavailability in humans remains unclear, raising questions about its efficacy in targeting central nervous system pathologies (Nikou et al., 2022). There are many questions that remain about oleuropein's bioavailability in humans. Studies suggest that oleuropein is rapidly metabolized in the gut, and its active metabolites may not reach the brain in sufficient concentrations to exert meaningful neuroprotective effects (Nikou et al., 2022). Clinical trials in humans are also limited, but preliminary studies show that olive leaf extract may contribute to slowing cognitive decline and historical use for health promotion (Loukou et al. 2024), making olive leaf extract an attractive option for further research into alternative AD therapies.

By investigating these supplements, researchers aim to uncover their potential roles in slowing disease progression, improving cognitive function, and ultimately enhancing the quality of life for individuals afflicted with Alzheimer's Disease.The exploration of alternative and complementary therapies represents a promising frontier in the quest to manage and potentially mitigate the effects of Alzheimer's Disease. Beyond managing existing symptoms, an equally critical question is whether these compounds could help delay the onset of Alzheimer's, particularly in individuals at risk for cognitive decline. By leveraging their antioxidant, anti-inflammatory, and neuroprotective properties, these supplements may not only slow disease progression but also enhance the quality of life for individuals living with AD. Continued research, supported by rigorous clinical trials and mechanistic studies, is essential to fully

elucidate the therapeutic potential of these natural compounds and to integrate them effectively into comprehensive treatment regimens. A review of the existing literature on natural remedies and their abundance of beneficial properties supporting cognitive health prompts an important question: could natural supplements such as ashwagandha, ginkgo biloba, and olive leaf extract offer multifaceted mechanisms of action that effectively target the complex pathophysiology of AD? While preliminary evidence suggests potential benefits, the extent to which these compounds can provide meaningful therapeutic effects remains an open question—one that warrants further scientific exploration.

Part III

Mechanisms of Action and Scientific Evidence

Exploring the mechanisms and key processes of natural supplements like ashwagandha, ginkgo biloba, and olive leaf extract is crucial for understanding their potential therapeutic benefits for Alzheimer's disease. These compounds influence key pathological processes, including neuroinflammation, oxidative stress, and the modulation of amyloid-beta and tau proteins, which are central to AD progression. By gaining deeper insight into these mechanisms, researchers can establish the biological plausibility of their potential neuroprotective effects. Furthermore, elucidating these pathways could pave the way for the development of targeted therapies that maximize efficacy while minimizing side effects. These natural compounds may exert their effects at different stages of the disease pathway. Their anti-inflammatory and antioxidant properties could play a preventive role in at-risk individuals by mitigating early pathological changes such as oxidative damage and neuroinflammation. In later stages, their

ability to reduce amyloid-beta aggregation and tau phosphorylation may help slow cognitive decline and neuronal loss. Research in this area has demonstrated promising outcomes, such as reduced amyloid-beta burden, decreased neuroinflammation, and enhanced synaptic function. However, variability in results across studies, stemming from differences in experimental design, dosage, and bioavailability, underscores the need for further exploration. Additionally, the challenges of standardizing natural supplements and understanding their molecular mechanisms remain significant barriers to clinical application. This section provides a foundation for advancing research on ashwagandha, ginkgo biloba, and olive leaf extract in AD treatment.

Ashwagandha Studies

As mentioned previously, ashwagandha is well-known for its adaptogenic properties that help the body to resist stressors. Recent studies suggest that ashwagandha exerts significant anti-inflammatory effects, which may be beneficial in Alzheimer's Disease. Choudhary and Dhingra (2017) demonstrated that ashwagandha administration reduced markers of neuroinflammation in rat models, potentially mitigating neurodegenerative processes that contribute to cognitive decline. In addition, ashwagandha possesses strong antioxidant properties. Kumar and Bansal (2015) found that the herb significantly reduced oxidative stress in neuronal cells, a critical factor in the progression of Alzheimer's disease. The neuroprotective effect of ashwagandha against oxidative damage is attributed to its ability to scavenge free radicals and enhance the body's endogenous antioxidant defense mechanisms. Studies suggest that withanolides behave as potent antioxidants, directly neutralizing reactive oxygen species (ROS) and reactive nitrogen species (RNS), where highly reactive molecules contribute to oxidative damage in neurons (Durg et al., 2015). Additionally, ashwagandha enhances the body's

endogenous antioxidant defense mechanisms by upregulating superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which are enzymes that prevent oxidative damage (Durg et al., 2015). Considering oxidative stress is one of the earliest and most fundamental contributors to neurodegeneration, reducing it can set off a cascade of protective mechanisms. Thus, we can consider its antioxidant action as a potential foundational mechanism from which all other neuroprotective effects arise, however, more research is needed to elucidate these specific interactions.

Emerging literature also indicates that ashwagandha may modulate amyloid-beta and tau protein dynamics. Singh et al. (2019) reported that ashwagandha extract inhibited beta-amyloid-induced toxicity in neuroblastoma cells, suggesting its potential to prevent amyloid-beta accumulation. What is responsible for these effects could be a variety of different pathways. In Alzheimer's disease, amyloid-beta peptides tend to misfold and aggregate into plaques, triggering neuronal toxicity and cognitive decline. Studies have indicated that ashwagandha could play a key role in mitigating this process by upregulating amyloid-beta-degrading enzymes, such as neprilysin and insulin-degrading enzyme (IDE), which help clear amyloid-beta from the brain (Patil et al., 2010). Additionally, its bioactive compound withaferin A has been hypothesized to directly inhibit beta-amyloid fibril formation, reducing the accumulation of toxic aggregates (Das et al., 2021) Beyond amyloid-beta modulation, ashwagandha also affects tau protein dynamics, particularly preventing tau hyperphosphorylation, which is a major contributor to neurofibrillary tangle formation in Alzheimer's disease (Rao et al., 2014). Tau hyperphosphorylation occurs when excessive phosphate groups attach to tau, a microtubule-stabilizing protein found in neurons, at multiple sites, causing dysregulation of its function. This happens when kinases like glycogen synthase

kinase-3 beta (GSK-3β) become overactive or phosphatases (which remove phosphate groups) become impaired. Computational studies suggest that withanolide A, a bioactive compound in ashwagandha, may interact with and inhibit GSK-3β (Joshi et al., 2018). By reducing tau hyperphosphorylation, ashwagandha may further protect against neurodegeneration associated with Alzheimer's disease. While molecular docking analysis supports its potential as a GSK-3β modulator, further in vitro and in vivo studies are needed to confirm its role in reducing tau pathology in Alzheimer's disease. Finally, neuroinflammation exacerbates amyloid-beta and tau pathology, and ashwagandha effectively combats this by reducing levels of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Pandey et al., 2018). Chronic inflammation in Alzheimer's disease is associated with the activation of microglia, the brain's resident immune cells, which in turn contribute to amyloid-beta deposition and neuronal damage (Mikulska et al., 2023). Ashwagandha has been found to shift microglial activation toward an amyloid-clearing phenotype, promoting the phagocytosis of amyloid-beta deposits and reducing inflammation-driven neuronal damage (Gupta & Kaur, 2018). This combined anti-inflammatory and amyloid-clearance effect further strengthens ashwagandha's neuroprotective potential.

The neuroprotective potential of ashwagandha in Alzheimer's disease treatment has been modestly studied for several years now. One of the most recent studies was a study conducted by Gladen-Kolarsky et al., which suggested that ashwagandha root extract reduces amyloid-beta (Aβ) plaque accumulation and improves neuronal health in vitro. Their findings indicated a significant decrease in oxidative stress markers and the restoration of dendritic spine morphology, critical for synaptic function. They attributed these effects to ashwagandha's ability to modulate oxidative stress pathways, emphasizing its potential to reduce neuronal damage caused by amyloid-beta toxicity. In spite of promising results, the researchers were not certain of

the exact mechanism behind the reduction of plaque accumulation, specifically if the ashwagandha constituent had contributed to an alteration in plaque production or the clearance of Aβ plaques altogether (Gladen-Kolarsky et al., 2024). While they cited other studies that presented similar results and proposed unique mechanisms in which Aβ production had been affected, more in-vitro and in-vivo studies are necessary to confirm these conclusions.

Another key ashwagandha study that explored the neuroprotective effects of ashwagandha was conducted by Sehgal et al. (2012). They demonstrated that ashwagandha root extract significantly reduced amyloid-beta plaque accumulation and reversed cognitive deficits in a transgenic mouse model of Alzheimer's disease. Notably, the study revealed a novel mechanism that ashwagandha extract enhances the clearance of amyloid-beta from the brain by upregulating liver production of low-density lipoprotein receptor-related protein 1 (LRP1), a key transporter responsible for facilitating amyloid-beta efflux into the peripheral circulation for degradation (Sehgal et al., 2012). Additionally, the study reported that ashwagandha administration led to improvements in behavioral outcomes, suggesting its potential to mitigate Alzheimer's-related cognitive decline. Although these results are encouraging, it is important to consider the study's limitations. Firstly, while their transgenic mouse model attempts to partially mimic amyloid-beta pathology observed in human AD which makes them useful for studying plaque formation and clearance responses, they fail to replicate tau pathology which is known to be a major driver in the neuronal dysfunction found in AD. The model also lacked the widespread neuronal loss that is associated with human AD pathology. These limitations may have caused a misrepresentation of the data collected which may not realistically reflect what is expected of a human patient who experiences all of the complexities of the disease. With ashwagandha containing multiple active ingredients and affecting multiple pathways, exploring

how the supplement affects all aspects of the disease will be crucial for identifying its value as a therapy. As for the proposed mechanism, while the study found that ashwagandha increased LRP1 expression in cortical microvessels, leading to enhanced amyloid-beta efflux across the blood-brain barrier, this mechanism has not been extensively validated in human subjects (Sehgal et al., 2012). The study also reported that ashwagandha treatment led to a decrease in receptor for advanced glycation end products (RAGE) expression, a key mediator of amyloid-beta influx into the brain. While these findings suggest that ashwagandha may both enhance amyloid-beta clearance and prevent its re-entry into the brain, they do not address whether these changes result in long-term neuroprotection or disease modification. The study also did not explore the potential compensatory effects of altering LRP1 activity, as excessive LRP1 upregulation has been linked to disruptions in lipid metabolism and vascular integrity, both of which could have unintended consequences in AD pathology (Shinohara et al., 2017). If future research confirms that ashwagandha not only reduces amyloid burden but also prevents downstream pathological events, such as tau aggregation and synaptic loss, it could significantly improve AD progression and delay cognitive decline. However, clinical trials are needed to validate these findings in human populations and assess whether ashwagandha can offer meaningful benefits beyond preclinical models.

Further supporting these mechanistic insights, a human clinical study by Gopukumar et al. (2021) investigated the effects of ashwagandha supplementation on cognitive function and stress reduction in adults with mild cognitive impairment (MCI). This double-blind, placebo-controlled study utilized Prolanza™, a specialized ashwagandha extract designed for sustained release, to assess its effects over a 90-day period. The results suggested that Prolanza™ significantly improved memory, executive function, and attention, while also reducing cortisol

levels, a key biomarker of stress. The proposed mechanisms for these benefits include enhanced synaptic plasticity, neuroinflammatory modulation, and improved hypothalamic-pituitary-adrenal (HPA) axis regulation, with additional evidence pointing to increased brain-derived neurotrophic factor (BDNF) levels (Gopukumar et al., 2021). BDNF plays a critical role in neuronal survival, synaptic plasticity, and cognitive function, all of which are impaired in Alzheimer's disease. Elevated BDNF levels could contribute to neuroprotection and synaptic repair, potentially counteracting the loss of neuronal connectivity seen in AD pathology (Gopukumar et al., 2021). These findings provide translational relevance, indicating that the neuroprotective effects observed in preclinical models may extend to human populations. What makes these particular pathways relevant to AD is that they are all implicated in AD progression. Enhanced synaptic plasticity could counteract the synaptic degradation observed in AD, supporting neuronal communication and cognitive function. Neuroinflammatory modulation may help mitigate the chronic inflammation driven by microglial activation and pro-inflammatory cytokines, which contribute to neuronal damage and amyloid-beta accumulation (Onyango et al., 2021). Furthermore, improved HPA axis regulation could reduce the impact of prolonged stress-induced cortisol elevation, which has been linked to accelerated neurodegeneration and increased amyloid-beta and tau pathology (Canet et al., 2019). By addressing these core aspects of AD pathophysiology, ashwagandha may offer a multi-targeted approach to slowing disease progression and preserving cognitive function. At the same time, the study’s lack of direct mechanistic testing on amyloid-beta, tau, neuroinflammation, and synaptic function highlights the need for cell-based and molecular-level studies. Measuring these biomarkers for the disease will provide a better explanation for the observable improvements in cognitive function as well as support for if ashwagandha can affect the progression of the disease in patients.

Ginkgo Biloba Studies

Ginkgo biloba also has a long history of use in traditional medicine, particularly for cognitive enhancement. Recent research has demonstrated its neuroprotective effects, particularly in the context of Alzheimer's disease. Ginkgo biloba contains bioactive flavonoids and terpenoids, which exhibit antioxidant, anti-inflammatory, and neuroprotective properties that may help combat neurodegeneration (Biernacka et al., 2023) They have also been shown to scavenge free radicals, enhance mitochondrial function, and reduce lipid peroxidation in neuronal cells (Di Meo et al., 2020). By preserving mitochondrial integrity, ginkgo biloba may support neuronal survival and energy metabolism, both of which decline in AD patients (Xia et al., 2024). Beyond its antioxidant effects, ginkgo biloba has been studied for its ability to modulate neuroinflammation. Chronic inflammation in the brain, driven by microglial activation and the release of pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α, contributes to neuronal damage and amyloid-beta accumulation. Studies suggest that ginkgo biloba can suppress inflammatory pathways, reduce cytokine levels, and promote an anti-inflammatory microglial phenotype, potentially mitigating the neurotoxic effects of prolonged inflammation (Sun et al., 2024).

To further investigate the clinical benefits of Ginkgo biloba in Alzheimer's disease, a landmark randomized, double-blind, placebo-controlled trial by Le Bars et al. (1997) assessed the efficacy of Ginkgo biloba extract (EGb 761) in patients with Alzheimer's disease and multi-infarct dementia. The study included 309 participants, who received either 120 mg/day of EGb 761 or a placebo over a 52-week period. Results indicated that patients receiving EGb 761 exhibited significant improvements in cognitive performance, as measured by the Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog), compared to the placebo group, in

addition to improvements in activities of daily living. However, more research is necessary to determine whether the biochemical changes observed translate into sustained cognitive improvements and whether EGb 761 influences disease progression beyond symptomatic relief. Furthermore, since the study participants were mild-to-moderate AD patients, its effectiveness in advanced AD remains uncertain. The study did not fully elucidate whether these effects were directly tied to specific neurobiological changes or if they were primarily symptomatic. The proposed mechanisms behind EGb 761's effects include its ability to modulate neurotransmitter systems, particularly serotonin and dopamine, which are often dysregulated in AD (Tan et al., 2015). Additionally, its influence on mitochondrial function and oxidative stress reduction suggests a role in preserving neuronal viability. Further studies are required to establish whether these biochemical effects correspond with structural brain changes, such as reduced neurodegeneration or improved synaptic integrity. Longitudinal neuroimaging studies and biomarker analyses would help clarify whether EGb 761 contributes to slowing disease progression or merely provides temporary symptomatic relief.

Ginkgo biloba is recognized for its potent antioxidant activity which also offers therapeutic potential for AD patients. Huang et al. (2016) also demonstrated that ginkgo biloba extract protects neuronal cells from oxidative stress-induced apoptosis. The flavonoids and terpenoids present in ginkgo biloba contribute to its antioxidant effects, which may help to preserve neuronal integrity in the face of neurodegenerative challenges. The terpenoids in Ginkgo biloba, such as ginkgolides and bilobalide, are also thought to improve circulation by dilating blood vessels and reducing platelet aggregation, which can enhance blood flow (Murray & Nowicki, 2006). Some studies have investigated Ginkgo biloba's effects on cerebral blood flow. For instance, a pilot study using magnetic resonance imaging (MRI) assessed cerebral

blood flow in healthy elderly individuals before and after supplementation with Ginkgo biloba extract (Mashayekh et al., 2011). The results indicated a mild increase in cerebral blood flow in certain brain regions following supplementation. One of the hallmark pathophysiological features of Alzheimer's disease (AD) is cerebrovascular dysfunction, which contributes to neurodegeneration and cognitive decline such as through impaired amyloid-beta clearance and blood-brain barrier breakdown (Eisenmenger et al., 2024). Given these findings, improving cerebral blood flow could play a critical role in mitigating some of the key pathological processes driving AD progression. Research on ginkgo biloba has further revealed its potential to modulate amyloid-beta and tau protein levels. In a study conducted by Wu et al. (2006), the standardized ginkgo biloba extract EGb 761 was found to inhibit amyloid-beta (Aβ) oligomerization and deposition in a transgenic Caenorhabditis elegans model. This inhibition of Aβ aggregation was associated with a reduction in Aβ-induced pathological behaviors, such as paralysis. The study suggests that the protective effects of EGb 761 are mediated primarily by modulating Aβ oligomeric species, rather than by reducing oxidative stress. This modulation could be vital in preventing the pathological hallmarks of Alzheimer's disease and preserving cognitive function. Nonetheless, although the study demonstrated that EGb 761 and its component ginkgolide A can alleviate amyloid-beta-induced pathological behaviors and reduce amyloid-beta oligomerization and deposition, the precise molecular mechanisms underlying these effects remain unclear. Further research is needed to elucidate how EGb 761 interacts with amyloid-beta at the molecular level.

Studies on ginkgo biloba have consistently highlighted its antioxidant and anti-inflammatory properties that could be used to combat Alzheimer's disease pathology. A 2023 study by Singh et al. provided a comprehensive computational analysis of *Ginkgo biloba*

extract (EGb 761), specifically investigating its potential dual-target inhibitory effects on acetylcholinesterase (AChE) and glycogen synthase kinase-3 beta (GSK-3β), two key enzymes implicated in Alzheimer's disease pathology. Using molecular docking and network pharmacology approaches, the researchers identified flavonoids and terpenoids (e.g., quercetin, kaempferol, and isorhamnetin) as bioactive compounds capable of binding to AChE and GSK-3β, thereby exerting potential neuroprotective effects. The study found that EGb 761 may contribute to cognitive improvements by modulating multiple AD-related pathways. This mechanistic analysis suggests that ginkgo biloba may have a multi-target therapeutic potential in Alzheimer's disease by acting at different levels of the neurodegenerative cascade. However, since this study was in silico (computational), further in vitro and in vivo studies are required to validate these findings and establish whether these biochemical interactions translate into tangible cognitive benefits for AD patients. Nonetheless, the results of this research seemed to reveal significant reductions in amyloid-beta plaque accumulation and improvements in synaptic plasticity, which translated into enhanced cognitive function in transgenic mouse models. Similarly, researchers also evaluated ginkgo biloba extract's anti-inflammatory effects and its ability to improve spatial memory in rodent models (Veysanoglu et al. 2023). The study used an intracerebroventricular-streptozotocin (icv-STZ) rat model to simulate sporadic Alzheimer's disease (AD). This model induces cognitive deficits by impairing insulin receptor signaling in the brain, leading to neuronal energy metabolism dysfunction, oxidative stress, and neuroinflammation, all of which contribute to AD-like pathology. The study evaluated the effects of ginkgo biloba extract, along with Rivastigmine and Memantine, delivered via a nanofiber system, on learning and memory deficits in these AD-model rats. The researchers assessed cognitive performance using behavioral tests such as the Morris Water Maze and Novel Object

Recognition Test, while also measuring biochemical markers related to amyloid-beta, tau phosphorylation, and neuroinflammatory cytokines in the hippocampus and cerebral cortex.The study reported decreases in pro-inflammatory cytokines such as IL-1β and IL-6, and enhanced cognitive performance in Y-maze novel object recognition tests. However, differences in extract composition–particularly flavonoid and terpene concentrations–led to variability in efficacy across studies.

Exploring ginkgo biloba extract's effects on the integrity of the blood brain barrier (BBB), Chen et al. (2019) observed the enhanced expression of tight junction proteins, such as claudin-5 and occludin, facilitating amyloid-beta clearance and reducing BBB permeability. The BBB plays a critical role in neuroprotection, preventing harmful substances from entering the brain while regulating the transport of essential molecules. However, in AD, BBB dysfunction leads to increased permeability, allowing neurotoxic compounds such as circulating amyloid-beta (Aβ) and inflammatory cytokines to infiltrate the brain, exacerbating neurodegeneration. Chen et al. found that EGb 761 administration upregulated the expression of tight junction proteins, particularly claudin-5 and occludin, which are essential for maintaining BBB structural integrity. Claudin-5 is a major component of endothelial tight junctions, and its reduced expression in AD has been linked to increased vascular permeability and Aβ accumulation in the brain. By restoring claudin-5 and occludin levels, EGb 761 helped reinforce the BBB, limiting the infiltration of harmful proteins and inflammatory mediators. This could have significant implications for AD treatment, however, variability in EGb 761 dosing, duration of treatment, and methodological differences between studies remain key challenges. Standardized protocols and long-term testing on the molecular level are needed to confirm whether these findings translate into cognitive benefits and long-term neuroprotection in AD patients.

Olive Leaf Extract Studies

Olive leaf extract, derived from the leaves of the olive tree (Olea europaea), has demonstrated several neuroprotective properties, including notable anti-inflammatory effects. A study by Omar et al. (2022) demonstrated that OLE may reduce neuroinflammation by inhibiting the NF-κB pathway and suppressing the activation of NLRP3 inflammasomes and the receptor for advanced glycation end-products (RAGE)/high-mobility group box 1 (HMGB1) pathways. These pathways are critical in the inflammatory response, and their modulation by OLE suggests a potential therapeutic role in alleviating neuroinflammation in AD. The antioxidant properties of olive leaf extract have also been well-documented. González-Sarrías et al. (2017) conducted a systematic review and found that olive leaf extract exhibits strong antioxidant effects, protecting against oxidative damage in vitro and in vivo. This antioxidant activity is crucial in combating the oxidative stress associated with neurodegenerative diseases, including Alzheimer's. Beyond its antioxidant capacity, recent studies have also highlighted its ability to modulate glucose and insulin levels, which may have implications for metabolic disorders such as type 2 diabetes mellitus (T2DM). A randomized clinical trial demonstrated that participants treated with OLE exhibited significantly lower HbA1c and fasting plasma insulin levels, suggesting improved glucose homeostasis (Wainstein et al., 2012).

Additionally, in animal models, OLE has been shown to reduce blood glucose levels and enhance insulin sensitivity, further supporting its potential as a hypoglycemic agent (Abunab et al., 2016). The active compounds in OLE, particularly oleuropein, are believed to contribute to these effects by influencing insulin receptor substrates and glucose transporters, thereby improving insulin action and secretion (De Bock et al., 2013). Given the established link between insulin resistance and Alzheimer's disease, these findings suggest that OLE may offer

dual benefits, not only improving metabolic health but also potentially reducing neurodegenerative risk. Insulin resistance has been associated with increased amyloid-beta accumulation and tau hyperphosphorylation, both hallmarks of AD pathology (Mullins et al., 2017). By enhancing insulin sensitivity and glucose metabolism, OLE could play a role in mitigating some of the metabolic dysfunctions that contribute to cognitive decline. Emerging evidence also indicates that olive leaf extract may influence amyloid-beta and tau protein dynamics. Romero-Márquez et al. (2022) investigated an oleuropein-rich olive leaf extract and its potential therapeutic effects against Alzheimer's disease in *Caenorhabditis elegans*. Their study demonstrated that the extract could reduce oxidative stress and proteotoxicity associated with amyloid-beta (Aβ) and tau aggregation. The oleuropein-rich extract was also shown to delay Aβ-induced paralysis in a transgenic C. elegans model, which expresses human Aβ1-42 in muscle cells. Additionally, the extract reduced the accumulation of Aβ plaques, indicating a decrease in amyloid aggregation. The study also explored tau-related neurotoxicity using a C. elegans strain expressing pro-aggregant human tau protein, revealing that the olive leaf extract improved locomotive behavior and reduced tau-induced motor deficits. The molecular mechanisms underlying these protective effects were linked to the activation of key transcription factors, including DAF-16/FOXO and SKN-1/NRF2, which are involved in oxidative stress response and protein homeostasis regulation (Romero-Marquez et al., 2022). The findings suggest that oleuropein from olive leaf extract offers significant neuroprotective effects by modulating oxidative stress, reducing protein aggregation, and improving tau toxicity, making it a promising candidate for Alzheimer's disease therapy. These results highlight the potential of olive leaf extract to address key pathological features of Alzheimer’s disease.

Olive leaf extract and its active component, oleocanthal, supposedly have a strong potential in addressing multiple aspects of AD pathology. One study conducted by Abdallah et al. (2023) compared OC-low extra virgin olive oil (EVOO) and oleocanthal enriched formulations in AD mouse models, specifically the homozygous 5xFAD mouse model, which is genetically engineered to rapidly develop Alzheimer's disease-like pathology, including amyloid-beta (Aβ) plaque accumulation and cognitive deficits. This model carries five familial Alzheimer's disease (FAD) mutations, leading to early and aggressive amyloid deposition, making it a suitable system for evaluating potential disease-modifying treatments. Both oleocanthal-low EVOO and oleocanthal-enriched formulations significantly reduced amyloid-beta plaque burden and improved synaptic marker expression, such as PSD-95 and SNAP-25. PSD-95 is a critical scaffolding protein involved in maintaining synaptic strength and plasticity, which are essential for learning and memory (Broadhead et al., 2016). Similarly, SNAP-25 is integral to synaptic vesicle fusion and neurotransmitter release, facilitating proper communication between neurons (Antonucci et al., 2016). In AD, the loss of these markers correlates with synaptic dysfunction and cognitive decline. Notably, oleocanthal allegedly exhibited greater efficiency in reducing neuroinflammatory markers, including NLRP3 inflammasomes. This is particularly significant given the growing body of evidence implicating chronic neuroinflammation as a key driver of Alzheimer's disease pathology. The NLRP3 inflammasome is a central component of the innate immune response and plays a crucial role in neuroinflammatory processes. When activated, NLRP3 triggers the release of pro-inflammatory cytokines such as interleukin-1β (IL-1β) and interleukin-18 (IL-18), which contribute to neuronal damage, blood-brain barrier dysfunction, and the exacerbation of amyloid-beta (Aβ) and tau pathology (Bai & Zhang et al., 2021). By dampening chronic neuroinflammation, oleocanthal

may help mitigate the damaging feedback loop between inflammatory cytokines, oxidative stress, and protein aggregation, all of which are factors that accelerate AD progression. Correspondingly, Tajmim et al. (2021) examined olive leaf extract's impact on blood brain barrier integrity and amyloid clearance. The study reported an upregulation of transport proteins such as P-glycoprotein and low-density lipoprotein receptor-related protein 1 (LRP1), which both play critical roles in clearing amyloid-beta from the brain. LRP1, in particular, has been implicated in both amyloid clearance and glucose metabolism, as it interacts with the insulin receptor β in the brain to regulate insulin signaling and glucose uptake (Liu et al., 2015). Deficiency of LRP1 has been associated with impaired insulin signaling, reduced glucose transporter expression, and decreased glucose uptake, which are all factors contributing to metabolic dysfunction in Alzheimer's disease (Liu et al., 2015). Moreover, insulin itself has been shown to facilitate the hepatic clearance of amyloid-beta by promoting LRP1 translocation to the plasma membrane, further reinforcing the link between insulin signaling and amyloid clearance mechanisms (Tamaki et al. 2007).

Additionally, the results also appeared to display that olive leaf extract modulated non-amyloidogenic pathways, increasing soluble amyloid precursor protein alpha (sAPPα) levels and reducing sAPPβ, which decreases amyloidogenic activity. Essentially, amyloid precursor protein (APP) is a transmembrane protein which plays a crucial role in neuronal growth and repair that can be processed via two main pathways: the amyloidogenic pathway and the non-amyloidogenic pathway. In the amyloidogenic pathway, APP is cleaved by beta-secretase (BACE1) followed by gamma-secretase, producing soluble amyloid precursor protein beta (sAPPβ) and amyloid-beta (Aβ) peptides. These Aβ peptides aggregate into amyloid plaques, which are toxic to neurons and contribute to the progression of Alzheimer's disease. In the

non-amyloidogenic pathway, APP is instead cleaved by alpha-secretase, producing soluble amyloid precursor protein alpha (sAPPα) and precluding the formation of amyloid-beta. This pathway is considered neuroprotective because sAPPα can potentially promote neuronal survival, synaptic plasticity, and memory function while reducing oxidative stress and inflammation (Dar & Glazner, 2020).The shift observed in the study between increasing levels of sAPPα and reducing sAPPβ is significant because by shifting APP processing toward the non-amyloidogenic pathway, olive leaf extract appears to reduce the formation of toxic amyloid-beta peptides while simultaneously increasing levels of neuroprotective sAPPα. Given that metabolic dysfunction and insulin resistance are strongly linked to amyloid-beta accumulation and tau pathology, these findings suggest that olive leaf extract may exert neuroprotective effects not only through direct amyloid clearance but also by improving insulin sensitivity and glucose regulation, ultimately supporting both cognitive function and brain metabolism. However, challenges such as oleocanthal's bioavailability and long-term safety profile remain significant barriers to clinical application.The bioavailability of oleocanthal, or at least the proportion of the compound that enters the circulation and can have an active effect, is not well-characterized. To date, only one study has investigated its bioavailability in humans, reporting the presence of oleocanthal metabolites in human urine (García-Villalba et al., 2010). This finding indicates that oleocanthal is metabolized in the human body; however, comprehensive understanding of its absorption, distribution, metabolism, and excretion is lacking. Further studies are required to elucidate these pharmacokinetic parameters to determine the effective dosage and delivery methods necessary for therapeutic efficacy. Moreover, the long-term safety of oleocanthal consumption has not been extensively studied. While its presence in the Mediterranean diet suggests a degree of safety, the specific effects of isolated or

concentrated oleocanthal over prolonged periods remain unknown. Potential concerns include unforeseen interactions with other medications, cumulative toxicity, or adverse effects resulting from high doses. Therefore, rigorous long-term studies are essential to assess its safety profile before oleocanthal can be recommended for clinical use.

Inspiration for Further Research

The neuroprotective effects of ashwagandha, ginkgo biloba, and olive leaf extract may arise from their ability to modulate multiple biological pathways. Each of these supplements exhibits anti-inflammatory and antioxidant properties, which are critical in reducing neuroinflammation and oxidative stress—two key contributors to Alzheimer's disease pathology. Furthermore, the modulation of amyloid-beta and tau proteins suggests that these natural compounds may directly impact the disease's underlying mechanisms. The integrative approach of using these natural supplements could enhance their neuroprotective effects. For instance, combining ashwagandha's ability to reduce inflammation with ginkgo biloba's antioxidant properties may provide a multifaceted strategy for combating the complexities of Alzheimer's disease. As research continues to explore these mechanisms, the potential for developing effective, safe, and holistic treatment options grows. While the findings are encouraging, further research is essential to establish the efficacy and safety of these supplements in treating Alzheimer's disease.

One of the primary challenges in studying natural supplements is the lack of standardization in extract composition. Variability in plant sources, extraction methods, and active compound concentrations can lead to inconsistent results across studies (Shan et al., 2007). For instance, differences in ashwagandha's bioactive components, such as withanolides, have affected outcomes related to cognitive improvement and amyloid-beta reduction. Another

critical challenge is bioavailability. Many bioactive compounds in natural supplements have poor absorption and rapid metabolism, limiting their therapeutic efficacy. For example, oleocanthal's lipid-soluble nature hinders its systemic availability, necessitating the development of innovative delivery systems such as nano-formulations or lipid-based carriers to improve its efficacy (Parkinson & Keast, 2014).

While preclinical studies have shown promising results, several gaps remain in our understanding of these supplements' effects on primary cortical neurons in Alzheimer's disease (AD) models. For example, the specific molecular pathways through which ashwagandha modulates inflammatory and oxidative stress responses in neuronal cells exposed to amyloid-beta are not fully elucidated. Similarly, while ginkgo biloba enhances BBB integrity and reduces oxidative stress, its direct effects on neuronal development and survival in cell culture models remain unclear. Additionally, most studies focus on single-agent therapies, overlooking the potential synergistic effects of combining natural supplements. This thesis investigates both the individual and combined effects of ashwagandha, ginkgo biloba, and olive leaf extract on the growth and development of primary cortical neurons in an AD model simulated by amyloid-beta application. By exploring their combined effects, this research aims to uncover potential interactions that could yield more robust therapeutic outcomes. Future research should prioritize understanding these mechanisms in greater detail to bridge the gap between preclinical and clinical applications.

Part IV

Thesis Statement and Objectives

Alzheimer's Disease (AD) is a devastating neurodegenerative disorder characterized by progressive cognitive decline, synaptic dysfunction, and neuronal death. Current treatments offer only symptomatic relief and fail to address the underlying mechanisms driving the disease. In response to this gap, this thesis explores the effects of natural supplements on a model of primary cortical neurons to determine their potential as therapeutic agents for mitigating cognitive decline in AD patients. By examining the role of these supplements in addressing oxidative stress, amyloid-beta toxicity, and tau hyperphosphorylation, this research aims to contribute to the development of more effective treatment strategies. This thesis aims to investigate the effects of natural supplements on primary cortical neurons under Alzheimer's Disease-like conditions. A key advantage of the experiments is the exclusion of many physiological effects such as blood flow, glucose and insulin production, and immune cell regulation, which will help to determine if the effects of the supplements are directly occurring via interactions between the neurons themselves, or instead are the result of other downstream effects related to these physiological interactions. The goal is to evaluate their potential as therapeutic agents to counteract neurodegeneration, focusing on key pathological mechanisms and neuronal health. My experimental analysis will involve in vitro testing of supplements on primary cortical neurons to measure their effects on neuronal survival, microtubule stability, and synaptic connectivity under AD-like conditions.

Experimental Design and Hypotheses

The experimental design for this study involves exposing primary cortical neurons to Alzheimer's Disease (AD)-like conditions, which include oxidative stress and amyloid-beta (Aβ) toxicity. The neurons will then be treated with selected natural supplements to evaluate their potential neuroprotective effects. Specifically, the study will assess the impact of these supplements on neuronal survival, microtubule stability, and synaptic connectivity. Additionally, it will focus on their potential to mitigate tau hyperphosphorylation and maintain cytoskeletal integrity. A key aspect of the research will also be to analyze how the supplements influence neuronal responses to Aβ-induced toxicity, with particular attention to whether they can counteract the detrimental effects of amyloid-beta on neuronal health.

The scope of this research is deliberately narrowed to focus on the direct effects of natural supplements on neurons, rather than exploring their interactions with glial cells or inflammatory pathways. Previous research in the Knowles lab appears to indicate that roughly 90% of cells that are cultured differentiate into neurons as opposed to glial cells. This is in contrast with typical brain conditions, where approximately 50% of cells in the brain are glial cells. While these aspects play a significant role in AD pathology, the study will specifically assess neuronal responses, providing a foundation for further research into secondary cellular mechanisms that may contribute to the disease's progression. Ashwagandha, ginkgo biloba, and olive leaf extract are each hypothesized to exert their neuroprotective effects through complementary mechanisms. Ashwagandha, an adaptogen known for its ability to reduce oxidative stress and promote neurogenesis, is also believed to stabilize microtubules, potentially mitigating tau-related pathology. Ginkgo biloba, a potent antioxidant, contains flavonoids that are expected to scavenge reactive oxygen species and protect neurons from oxidative damage, a critical factor in the progression of AD. Olive leaf extract, rich in bioactive compounds such as

oleuropein, is thought to exhibit strong anti-inflammatory and antioxidant properties, which may help combat amyloid-beta toxicity and support neuronal health. These supplements will be tested across varying concentrations and time intervals to determine the optimal conditions for neuroprotection. Additionally, potential synergistic effects between the supplements will be explored by combining the supplements in media.

This pilot study is guided by three primary hypotheses. First, it is hypothesized that natural supplements such as ashwagandha, ginkgo biloba, and olive leaf extract will enhance neuronal survival in a concentration- and time-dependent manner under AD-like conditions. Second, it is expected that these supplements will improve microtubule stability and synaptic connectivity, effectively reducing the adverse effects of oxidative stress and amyloid-beta toxicity. Third, the hypothesis posits that these supplements will mitigate tau hyperphosphorylation, thereby stabilizing neuronal structures and promoting cellular health. While these hypotheses cannot be investigated thoroughly due to a lack of foundational research using this particular method of stimulation of neurons, the results collected in this study will provide valuable insights into future directions for experimentation.

Ultimately, the goal of this research is to advance the understanding of natural supplements as potential therapies for Alzheimer's Disease. By evaluating their effects on neuronal survival, microtubule stability, and synaptic connectivity, this study will contribute to the growing body of research on alternative and integrative treatment strategies. The findings may offer valuable insights into the feasibility of incorporating natural compounds into current therapeutic paradigms for AD. Through this exploration, the study seeks to identify promising pathways for combating AD-related neurodegeneration and improving patient outcomes.

Materials & Methods

Primary Cortical Neuronal Culture: Dissection and Incubation

Experimentation was conducted at Drew University's Neurobiology lab under the supervision of Dr. Roger Knowles. 24-well sterile cell culture plates (Greiner Bio-One, Cellstar) and 96-well sterile cell culture plates (Greiner Bio-One, Cellstar) were utilized for culturing neurons. The plates were coated with a Poly-L-lysine Hydrobromide solution for at least 24 hours to improve cell adhesion to the bottom of the plate wells. The Poly-L-lysine was then removed from the wells and plates were washed three times with autoclaved water to remove excess solution. Following the washes, each individual well was filled with plating media (NeurobasalQ Medium - Sigma, fetal bovine serum, Primocin (InvivoGen)) to create a compatible environment to satisfy the nutritional requirements of the cultured neurons.The plates were placed in an incubator which maintained a temperature of 37°C and a carbon dioxide (CO_2) level of 5%. The plates remained in the incubator under these conditions until the dissection was performed.

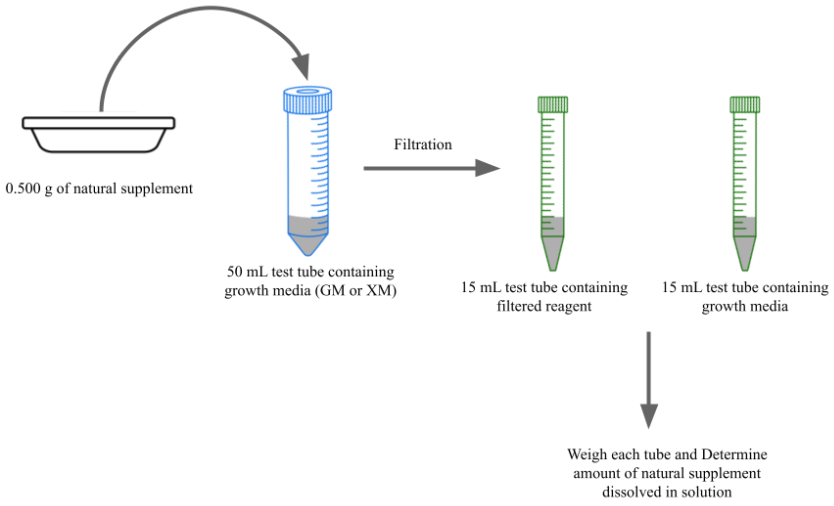
Female Sprague-Dawley rats at approximately 18 days gestation were euthanized in a CO2 chamber, where the animal was readily visible. The animal was monitored continuously as 100% CO2 gas was introduced into the chamber at a controlled rate, inducing rapid unconsciousness while minimizing distress. The rodent was carefully assessed to confirm death, from which then the embryos were isolated and decapitated to collect their brain samples. The extraction of the brain was performed by puncturing the skull and peeling back the skin to expose the two frontal hemispheres, along with the cerebellum and midbrain regions. The intact brain was removed from the skull to separate the forebrain from the cerebellum and midbrain. Alzheimer's Disease is thought to not primarily affect the latter regions, so these were set aside

from the rest of the brain. Following the careful separation of the two hemispheres, which were placed in another dish to prepare for the removal of the meninges. Removing the meninges, which are the thin, red protective membranes that surround and protect the brain, helps to reduce the risk of the cells being exposed to inflammatory responses or other cell secretions that may not be conducive to the culture environment. The cells were teased apart into smaller pieces and placed in an aliquot of trypsin, where it sat in a 37°C water bath for 5 minutes. To help dissociate the cells, trypsin aids in digesting the cellular proteins and creating the cell suspension that will be used for plating. The cells were then transferred to a tube of HBSS and placed in the water bath at the same temperature for 3 minutes, twice. After the washes were completed, the cells were moved into a tube of plating media and mechanically dissociated 50 times using a flame-tipped pipet. The number of cells collected were counted using a hemocytometer. The number of cells calculated determined the volume of media used to prepare the final cell suspension aliquot for plating, such that cells would be plated at 1 x 10^6 cells/mL. Cells were incubated in the prepared cell-culture plates for approximately 1 hour, and the plating media was removed and replaced with serum-free growth media (GM) ((Fetal Bovine Serum, NeurobasalQ Medium - Sigma, Primocin (InvivoGen), B-27 Supplement (50X) (ThermoFisher Scientific)). An alternative, experimental growth media (XM) was also prepared using a B-27 supplement without antibiotics. Every 2-3 days, half of the growth media on the plates would be replaced with fresh media.

Cell Stimulation & Supplement Reagent Preparation

Ashwagandha powder (Mother Nature Organics Organic Ashwagandha Powder), ginkgo biloba powder (Micro Ingredients Raw Organic Ginkgo Biloba Powder), and olive leaf powder (Starwest Botanicals Organic Olive Leaf Powder) were obtained for experimentation, which

were easily accessible for purchase online. Due to the fact that these powders are mostly insoluble in media, a method was developed in order to dissolve some of the powder into solution (Shinde et al., 2023; Liang et al., 2022; Darakijan et al., 2021). Rather than use additional solvents to dissolve the powders and possibly interfere with the action of the media, 0.500 g of powder was weighed and placed in a 50 mL tube of XM, and placed in the hot water bath at 37°C for approximately 10 minutes. Studies suggest that increased temperatures can enhance the solubility and efficacy of herbal compounds (Nadeem et al., 2022). The solution was put through a conical tube filter (CELLTREAT 50 mL Centrifuge Tube Filter) to remove excess powder that did not dissolve in the media. 10 mL of the filtered solution was placed in a 15 mL tube and weighed. This weight was subtracted from the weight of another 15 mL tube containing 10 mL of only growth media. This helped to maintain consistency of the volume for more accurate calculation of the total concentration of the supplement dissolved in media. This filtered media acted as the stock solution for each treatment, which was then further diluted to 1 μg/mL, 5μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL. For the combination reagent of all three supplements, 5μg/mL of each supplement was combined in a test tube. When applying these treatments directly to cells, it is crucial to significantly reduce the concentration of the supplement to account for the absence of metabolic clearance, first-pass metabolism, and systemic elimination that would normally occur in vivo (Huang 2017). These concentrations were tested by stimulating the neurons with the reagent for 72 hours to determine if they were well-tolerated by the neurons.



Schematic 1: Illustration of natural supplement reagent preparation.

Alzheimer's Disease Model: FAB

The ferrous amyloid buthionine (FAB) model is an in vitro model used to simulate Alzheimer's disease-like conditions in neuronal cultures. The FAB solution was prepared by dissolving 7.95 mg of ferrous sulfate ($FeSO_4$), 133.5 mg of L-buthionine-sulfoximine (BSO), and 50 µL of soluble amyloid-beta (Aβ 42) in 50 mL of growth media (GM). This specific formulation was designed to induce oxidative stress in neurons as a model for Alzheimer's disease (AD), providing an alternative to traditional genetic approaches. The development of this model stemmed from previous research that sought to establish a non-genetic method for studying Alzheimer's disease. Historically, many studies relied on genetic mutations associated with AD, which were only present in a small percentage of cases. The FAB approach aimed to

create a more widely applicable model by replicating the oxidative stress conditions observed in sporadic AD rather than focusing on rare genetic variants (Lecanu & Papadopoulos, 2013).

Each component in the solution played a critical role in mimicking the cellular environment of AD. Ferrous sulfate ($FeSO_4$) facilitated the generation of oxidative stress, leading to a loss of cellular integrity over time (Aghajanov et al., 2024). Amyloid-beta (Aβ 42), known for its neurotoxic properties, was included in its soluble form, as this species had been identified as an upstream factor in the formation of neurofibrillary tangles (Aghajanov et al., 2024). Unlike other studies that employed higher concentrations of amyloid-beta, this preparation maintained a physiologically relevant concentration, allowing for a more realistic simulation of its effects on neuronal function. L-Buthionine-sulfoximine (BSO) was incorporated to inhibit the neurons' antioxidant defenses, specifically by blocking glutathione synthesis (Aghajanov et al., 2024). This inhibition left the cells vulnerable to oxidative stress, further simulating age-related neuronal decline.

Cell Viability Measured by MTS Assay

The MTS assay is a colorimetric test used to assess cell viability and metabolic activity. Following cell stimulation, the MTS dye was prepared using 1.2 mL of warmed MTS and 6 mL of warmed growth media. The remaining media in each well was suctioned out and replaced with 100 µL of the prepared dye. An additional 3 wells above the stimulated wells were also filled to act as controls. The plate was then read by the plate reader (SpectraMax M3 M-Series Multi-Mode Microplate Plate Reader, Molecular Devices), and the absorbance of each well was measured at a wavelength of 490 nm. Cell survival was then calculated for each test condition

using SoftMax Pro Microplate Data Acquisition and Analysis Software. The data was analyzed and arranged into bar graphs using Microsoft Excel.

Table 1: Overview of Experiments

Experiment #	Research Question	Treatment Plan	Assay(s)	Related Figure(s)
1	Are Ashwagandha, Ginkgo Biloba (GB), and Olive Leaf Extract (OLE) safe for neurons?	Dose-response of ashwagandha, GB, and OLE at 1, 5, 10, 50, and 100 µg/mL on the 96 well plate for 72 hours, respectively; 1, 10, and 100 µg/mL on the 24 well plate	MTS, ICC	1, 3, 5, 6
2	Can Ashwagandha, GB, and OLE promote the growth and development of neurons?	1, 10, and 100 µg/mL of each supplement applied for 72 hours	ICC	1, 3, 5, 6
3	Can Ashwagandha, GB, OLE, and combination treatment protect neurons from toxicity (Alzheimer's model)?	Neurons exposed to Aβ toxicity and co-treated with 15 µg/mL of each supplement	MTS, ICC	2, 4, 7

Immunocytochemistry & Imaging Software

Immunocytochemistry (ICC) is an assay used to visualize and analyze the stability of microtubules in neurons by labeling specific proteins with antibodies conjugated to fluorescent markers. This allows for assessment of microtubule integrity, structural changes, and potential disruptions in response to various treatments. ICC is particularly useful for studying neurodegenerative diseases, where microtubule disruption is a hallmark of neuronal dysfunction (e.g., in Alzheimer's disease, where tau proteins detach from microtubules and aggregate into tangles). Following stimulation, 24 well plate cells were fixed with 4% paraformaldehyde for 20 minutes to preserve cellular structures. The wells were washed three times with PBS, and filled with Triton for 10 minutes. Triton assists in cell permeabilization by disrupting cell membranes, allowing antibodies to penetrate and access tubulin. Cells were again washed three times with PBS. The primary antibodies used to target acetylated tubulin were placed in each well and placed on an orbital shaker for an hour. After washing once again with PBS, this was repeated using a secondary antibody to enable fluorescence detection. Cells were washed with PBS once more and ready for fluorescent microscope imaging. Using NIS-Elements or the ImageJ program, 15 images were obtained per well. Each image was analyzed based on the fluorescence intensity, which indicated presence or absence of microtubules. Data was collected and arranged into bar graphs using Microsoft Excel.

Results

To determine neuronal tolerance to ashwagandha, a concentration gradient was tested (Figure 1). Cells appeared to tolerate all concentrations well, exhibiting a viability greater than the control condition. Notably, lower and moderate concentrations (1–10 µg/mL) were associated with slightly improved cell survival compared to higher concentrations (50–100 µg/mL), suggesting a potential threshold for neuroprotective effects. Under stress conditions, the effects of ashwagandha on cell viability were less conclusive (Figure 2). The FAB stressor did not appear to induce sufficient cellular stress, making it difficult to assess ashwagandha's neuroprotective potential. Similarly, when microtubule stability was analyzed in response to ashwagandha treatment (Figure 3), lower concentrations (1 µg/mL and 10 µg/mL) maintained cytoskeletal integrity, whereas higher concentrations resulted in reduced stability, implying a possible dose-dependent effect.

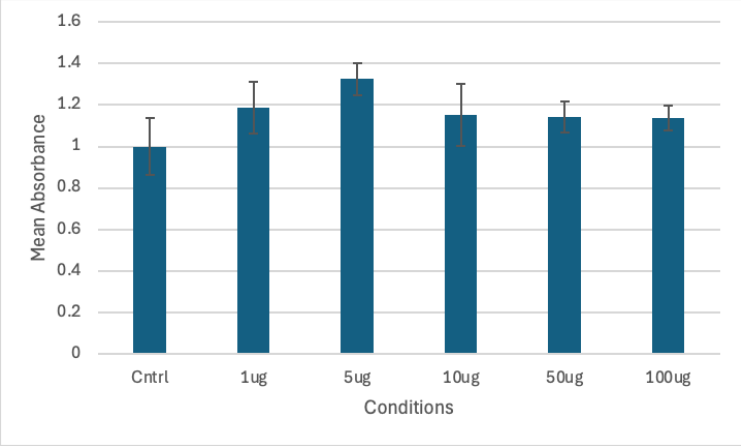


Figure 1. Assessing Neuronal Cell Tolerance by Facilitating a Concentration Gradient of Ashwagandha Supplement

This figure examines how neurons respond to different concentrations of ashwagandha in terms of cell viability. All cells seemed to have tolerated all concentrations of ashwagandha well, achieving a cell

viability greater than the control. Additionally,cells treated with lower and moderate concentrations (1-10µg/mL) appeared to experience greater survival than higher concentrations (50-100µg/mL). Conditions were normalized to the control.

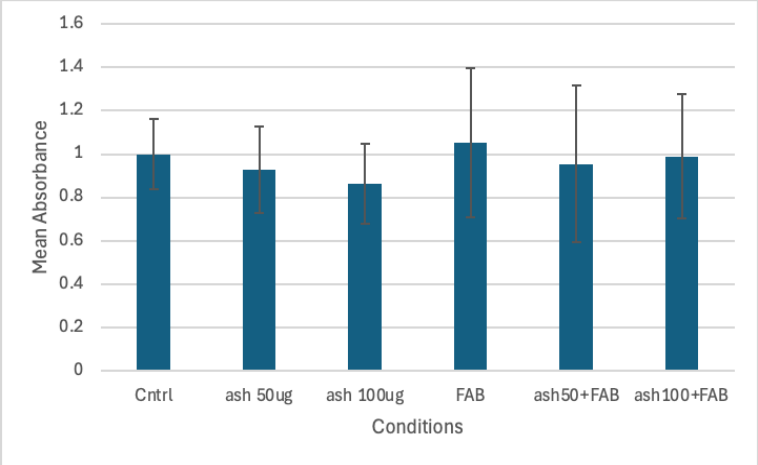


Figure 2. The Effect of Varying Ashwagandha Concentrations on Cell Viability of Neurons Under Stress Conditions

This figure evaluates how ashwagandha influences neuronal survival under stress. FAB did not appear to induce adequate stress on the cells, making it difficult to validate an analysis of the effects of certain concentrations of ashwagandha on the neurons. Conditions were normalized to the control.

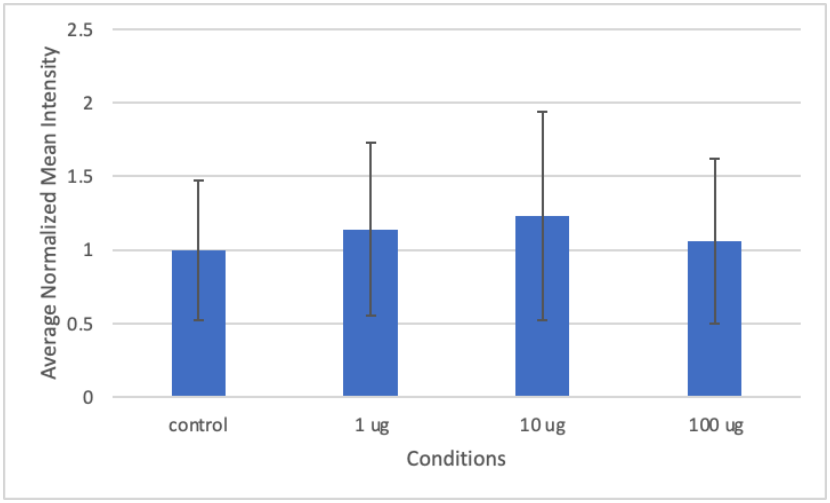


Figure 3. The Effect of Varying Ashwagandha Concentrations on Microtubule Stability

This figure explores the impact of ashwagandha on microtubule integrity. While low and moderate concentrations (1 µg/mL and 10 µg/mL) appeared to maintain stability, neurons exposed to higher concentrations showed reduced microtubule integrity, suggesting a possible dose-dependent effect on cytoskeletal structure. Conditions were normalized to the control.

Additionally, the study examined whether the preparation time of ashwagandha solutions influenced its effects on microtubule stability under stress (Figure 4). Although FAB stress induction was again insufficient for definitive conclusions, data suggested that an older reagent preparation promoted greater microtubule stability than a freshly prepared reagent. This finding underscores the importance of supplement preparation and storage conditions when evaluating their biological effects.

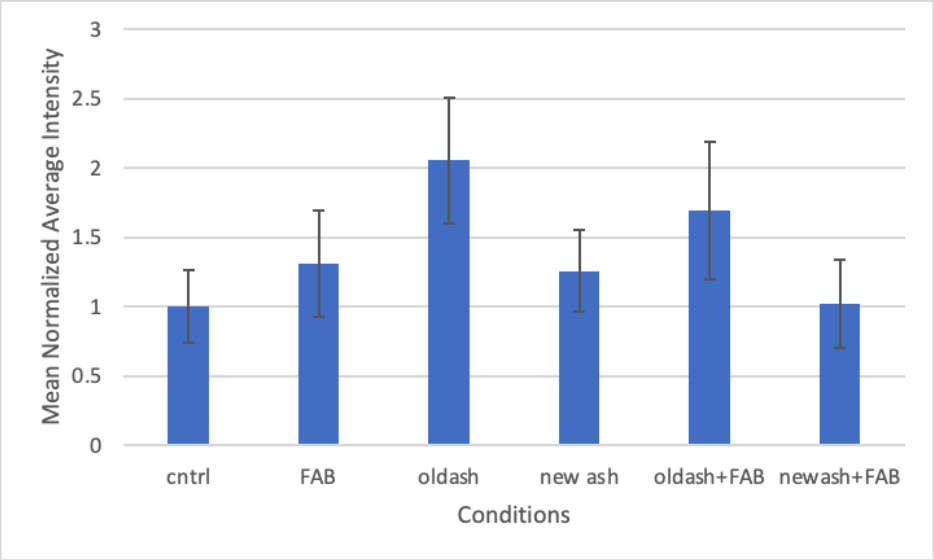


Figure 4. The Effect of Preparations of Ashwagandha Reagent on Microtubule Stability of Neurons Under Stress Conditions

This figure examines how different preparations of ashwagandha affect microtubule stability under stress. Particularly, whether or not a reagent that was prepared a few weeks prior maintained the same effects as a newly prepared reagent. A moderate concentration of ashwagandha reagent (10µg/mL) was used across all conditions. The FAB did not appear to induce adequate stress on the neurons, which made it challenging to determine if ashwagandha had a neuroprotective effect on cells. However, the data suggests that the older reagent promoted greater microtubule stability than the newer reagent. Conditions were normalized to the control.

Olive leaf extract (OLE) was tested across a range of concentrations to determine its impact on neuronal survival (Figure 5). The results indicated that neurons tolerated all tested

concentrations, but no concentration significantly enhanced cell viability beyond the control condition. This suggests that OLE may not have a pronounced effect on neuronal survival under the conditions tested, but more data must be collected drawing further conclusions.

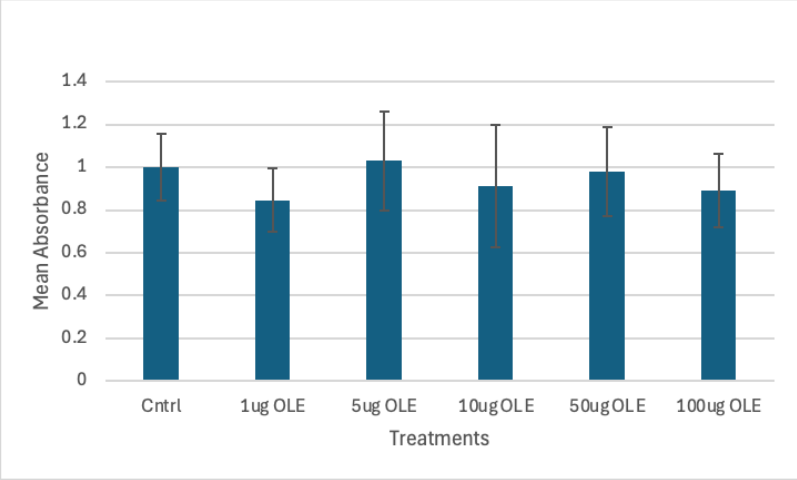


Figure 5. The Effect of Varying Olive Leaf Extract Concentrations on Cell Viability

This figure assesses the effect of olive leaf extract (OLE) on neuronal viability. Results indicate that, while OLE appeared tolerable to the neurons, no particular concentration promoted noticeable enhanced cell survival. Conditions were normalized to the control.

To assess the impact of ginkgo biloba on microtubule integrity, neurons were treated with different concentrations of the extract (Figure 6). All concentrations appeared to promote greater microtubule stability compared to the control. This result indicates that ginkgo biloba may have a stabilizing effect on cytoskeletal structures, warranting further investigation into its potential neuroprotective properties.

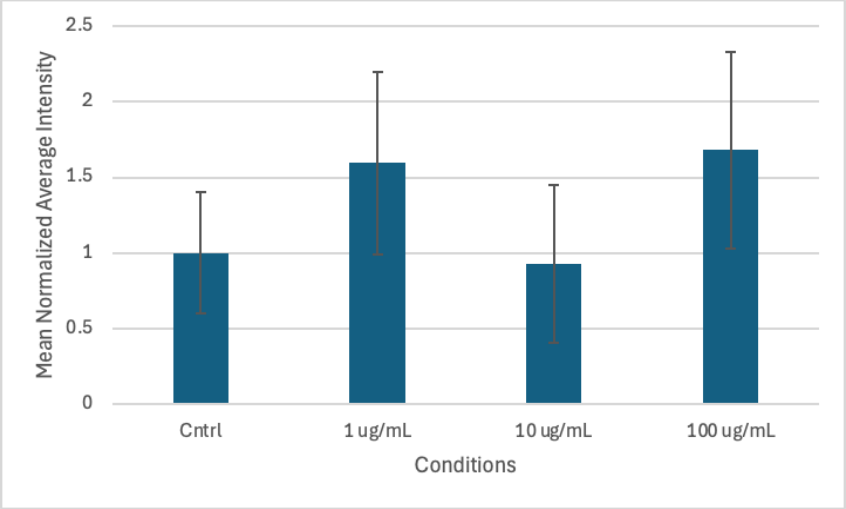


Figure 6. The Effect of Varying Ginkgo Biloba Concentrations on Microtubule Stability

This figure investigates the effects of Ginkgo biloba on microtubule structure. The lowest concentration of GB seemed to promote greater microtubule stability compared to the other two concentrations and the control, indicating a potential dose-dependent effect. Conditions were normalized to the control.

The final set of experiments explored how ashwagandha, olive leaf extract, and ginkgo biloba, both individually and in combination, influenced neuronal viability under stress conditions (Figure 7). When administered separately at a standardized concentration of 15 µg/mL, none of the supplements significantly enhanced survival beyond control levels. However, when stress was induced with FAB, all three supplements appeared to support greater neuronal survival. Notably, the combination of all three supplements was the only condition to promote better viability than the control, both in isolation and under stress conditions. This suggests that these natural compounds may exert synergistic effects when used together, highlighting the potential benefit of combination therapies in mitigating neuronal stress.

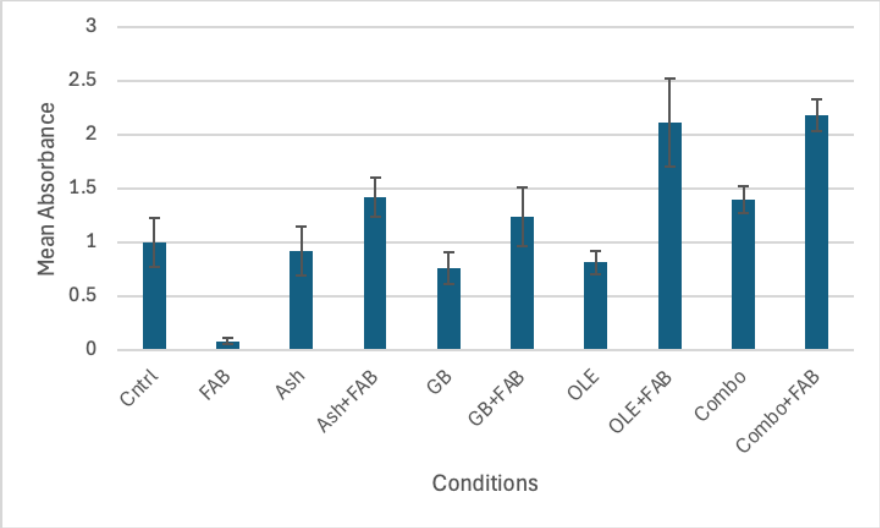


Figure 7. The Effect of Natural Supplements in Isolation and Combined on the Cell Viability of Neurons Under Stress Conditions

This figure examines how ashwagandha, olive leaf extract, and ginkgo biloba, both individually and in combination, influence neuronal viability under stress conditions. All conditions used a standardized concentration of 15 µg/mL. While all three supplements in isolation did not appear to promote better cell survival than the control, the supplements appeared to promote better cell survival when stress was induced with FAB. The combination was the only of the three conditions to promote better cell viability than the control in isolation and under stress. Conditions were normalized to the control.

Discussion

Supplement Safety and Efficacy

This pilot study aimed to evaluate the effects of ashwagandha, olive leaf extract, and ginkgo biloba on neuronal cell viability and microtubule stability under stress conditions. The experiments established baseline concentrations that were used to assess whether these supplements could have neuroprotective or toxic effects. Given the limited research available on optimizing the delivery of these supplements in a biologically relevant form, this study also served as an initial attempt to develop a technique for dissolving and applying the compounds directly onto cultured cells. Establishing an effective method for supplement preparation was a

critical step, as solubility and bioavailability can significantly influence cellular responses. It is important to note that due to the foundational nature of the research conducted, descriptive analysis will be used to interpret the results rather than more advanced statistical modelling. This study, therefore, provides an essential groundwork for future investigations aimed at optimizing supplement delivery and elucidating their potential neuroprotective mechanisms.

Figure 1 illustrates the first objective of this study: to determine safe and adequate concentrations of supplements that could be applied to neuronal cells without overwhelming them. Previous literature suggests that in a typical biological environment, the blood-brain barrier prevents most drugs from entering the brain, with the concentration of a small molecule drug decreasing by approximately 10-fold per millimeter (Pardridge 2012). Given that this cell culture model lacks this filtering mechanism, the reduction in concentration had to be mimicked through the preparation of the reagents. By performing a concentration gradient, appropriate concentrations can be confirmed for future experimentation. Figure 1 depicted the cells having a strong tolerance for the selected concentrations of ashwagandha, with all conditions promoting greater cell survival than the control. Similarly, figure 2 also maintained consistent results, where ashwagandha had elicited a slight increase in microtubule stability across all conditions. In both figures, it was observed that low to moderate concentrations supported neuronal survival, while higher concentrations such as at 50 or 100 μg/mL led to a slight reduction in viability and cytoskeletal integrity. These findings emphasize the importance of dose selection when considering natural supplements as potential neuroprotective agents. The concentration-response experiments also provided insights into the optimal dose ranges of each supplement. Most existing literature on these compounds has focused on general antioxidant effects or cognitive benefits, but little research has been conducted on their direct effects on neuronal microtubule

stability. The results indicated that while moderate doses may offer protective effects against oxidative stress, excessive concentrations could disrupt neuronal homeostasis. While excessive concentration has not been directly studied amongst any of the relevant supplements, previous literature has indicated that moderate doses of other supplements confer protective benefits against oxidative stress and excessive concentrations disrupt neuronal homeostasis. For example, research on edaravone, a synthetic antioxidant, has demonstrated that while it exerts neuroprotective effects by scavenging free radicals and limiting lipid peroxidation, its efficacy and safety are dose-dependent (Ashok et al., 2022). This highlights the need for further investigation into the molecular mechanisms underlying their effects and how dosage adjustments could optimize their therapeutic potential.

Ashwagandha and Neuronal Health

Ashwagandha is widely recognized for its adaptogenic and neuroprotective properties, with research suggesting it may enhance antioxidant defenses and reduce neuroinflammation. However, the present study showed no substantial differences in neuronal viability across varying concentrations of ashwagandha, however a slight decrease was noted for higher concentrations (Figure 1, 3). These findings indicate that ashwagandha may exert its neuroprotective effects in a more nuanced manner, potentially through mechanisms beyond direct enhancement of cell viability. Previous studies have suggested that ashwagandha's key bioactive components, such as withanolides, may influence protein aggregation and oxidative stress pathways (Wang et al., 2021). However, the microtubule destabilization at higher concentrations raises concerns about potential off-target effects or cytoskeletal toxicity at excessive doses. It is also important to note that these observations remained consistent with the

experiments where stress was induced, as seen in Figures 2 and 4, but there appears to be conflicting data. Figures 2 and 4 indicate that the cells under stress conditions survived and maintained their microtubules better than the cells in the control and treatment conditions. This was due to the FAB stressor being unable to encourage the neurodegeneration that is hallmark to Alzheimer's disease. The failure to induce stress properly on the neurons could interfere with the accuracy of the results that were demonstrated in regards to the neuroprotective ability of ashwagandha. Figure 7, however, showed that while ashwagandha-treated cells did not perform better than the control, there was an enhanced improvement in cell viability when applied to properly stressed conditions. The finding that ashwagandha did not improve cell viability compared to the control but enhanced survival under stress suggests that its neuroprotective effects could be more pronounced when neurons experience oxidative or metabolic stress. Withanolides have been shown to activate Nrf2-mediated antioxidant pathways, reduce reactive oxygen species (ROS) accumulation, and modulate inflammatory cytokines such as TNF-α and IL-6 (Shah et al., 2015). However, while the primary culture system that was utilized contained a variety of different neuronal cells, it was presumed that there were very few glial cells present in the culture that would be active in an immune response. These effects may not be as evident in unstressed neurons, where oxidative damage remains minimal, but under stress conditions, ashwagandha may play a critical role in restoring redox balance and cellular function.

A notably unexpected observation in this study was that neurons exposed to FAB in Figures 2 and 4 exhibited higher viability than those in the control condition. While this initially seems counterintuitive given the well-established neurotoxic effects of amyloid-beta aggregates, one possible explanation is that the FAB may have undergone partial disaggregation, leading to the formation of monomeric or low-order oligomeric species. These smaller assemblies, while

potentially harmful in excess, have also been implicated in normal synaptic modulation and neuroprotection at physiologically low concentrations (Puzzo et al., 2008). It is possible that, under certain culture conditions such as incubation time, temperature, or medium composition, FAB does not maintain a fully aggregated state, resulting in a more complex biological effect. This could shift the stressor from being overtly cytotoxic to mildly stimulatory, triggering adaptive responses rather than degeneration. To better characterize these dynamics, future studies should apply a broader range of Aβ concentrations and conformational states (e.g., monomeric, oligomeric, and fibrillar) to test whether neuronal responses follow a biphasic curve, and to determine the thresholds at which Aβ transitions from being protective to toxic. This possible shift in the behavior of FAB provides important context for interpreting the effects of ashwagandha under stress conditions.

Additionally, its adaptogenic properties suggest that it enhances cellular stress resistance, meaning its benefits become more pronounced when neurons experience environmental or metabolic challenges. This could potentially align with the concept of hormesis, where certain compounds exhibit stronger protective effects in the presence of mild to moderate stress, triggering repair mechanisms such as mitochondrial biogenesis, autophagy, and synaptic plasticity (Mattson et al., 2009). The improvement in viability under FAB-induced stress suggests that ashwagandha may be more effective as a neuroprotective agent in diseased or stressed conditions rather than in baseline physiological states. Future studies should focus on understanding the dose-dependent effects of ashwagandha on neuronal structure and function, particularly in models of neurodegeneration.

Olive Leaf Extract and Ginkgo Biloba in Neuronal Models

Olive leaf extract has been associated with anti-inflammatory and antioxidant benefits, primarily due to its high content of polyphenolic compounds. In this study, it was found that all concentrations enhanced or maintained neuronal viability, but higher concentrations led to somewhat decreased cell survival (Figure 5). This biphasic effect is consistent with previous research showing that while polyphenols can be beneficial at low doses, they may induce oxidative stress at higher levels. This was observed in a study which demonstrated that high concentrations of flavonoids could lead to significant oxidative stress in cancer cells, increasing reactive oxygen species (ROS) levels and triggering cell death (Xi et al., 2022). This suggests that while flavonoids possess antioxidant properties at lower concentrations, they may exhibit pro-oxidant effects when administered in higher doses. The findings suggest that dose-dependent regulation of antioxidant pathways may be key in determining the therapeutic potential of olive leaf extract. Nonetheless, considering that this effect was only observed in one trial of data collection, continued experimentation is necessary to confirm its validity.

It is also important to address the performance of olive leaf extract (OLE) in Figure 7, which, similarly to the ashwagandha, seemed to demonstrate that while OLE did not promote better cell survival compared to the control, there was an increase in cell survival when in combination with the FAB stressor. The observed finding that OLE did not promote better cell survival compared to the control but increased survival when combined with the FAB stressor suggests that OLE may exhibit greater neuroprotective effects under stress conditions rather than in baseline conditions. This aligns with the concept that certain bioactive compounds, including polyphenols, may have minimal or negligible effects in healthy cells but become more effective

when cells are exposed to oxidative stress or other neurotoxic environments. Under normal conditions, neurons maintain homeostasis through endogenous antioxidant defenses, which may explain why OLE had little impact. However, FAB-induced stress likely overwhelmed these defenses, leading to increased oxidative damage, mitochondrial dysfunction, and inflammation, all of which are conditions where OLE's antioxidant and anti-inflammatory properties could restore balance and promote survival. Studies have demonstrated that OLE's bioactive compounds, such as oleuropein and hydroxytyrosol, exhibit potent antioxidant and anti-inflammatory effects, particularly under oxidative stress. For instance, research indicates that these compounds can modulate oxidative stress pathways and reduce neuroinflammation, thereby offering neuroprotection in challenged neuronal environments (Iglesias-Gutiérrez et al., 2022).

Ginkgo biloba, widely used for its cognitive-enhancing properties, also demonstrated a dose-dependent effect on microtubule stability. The results indicated that all concentrations generally supported cytoskeletal integrity and microtubule stability (Figure 6). Given that this positive effect was indicated in one trial of experimentation, further trials are needed to verify these findings. Additionally, ginkgo biloba also mimicked the effects seen with ashwagandha and olive leaf extract, where the treatment under stress caused cells to survive better than with the treatment alone, suggesting that its neuroprotective effects are more pronounced during oxidative or metabolic challenges (Figure 7). In these scenarios, GB's bioactive constituents, notably flavonoids and terpenoids, can exert protective effects by scavenging free radicals, preserving mitochondrial function, and inhibiting apoptotic pathways. For instance, studies have demonstrated that GB protects neuroblastoma cells from hydrogen peroxide-induced apoptosis by reducing p53 acetylation, stabilizing mitochondrial membranes, and decreasing the Bax/Bcl-2

ratio, thereby preventing PARP cell death (Di Meo et al., 2020).This indicates that GB's efficacy is heightened under conditions of oxidative stress, aligning with the concept that certain natural compounds activate protective mechanisms primarily when endogenous defenses are compromised. Given that ginkgo biloba contains flavonoids and terpenoids that influence multiple biological pathways, further studies should explore how these bioactive compounds interact with neuronal microtubules at different doses.

Combined Effects of Natural Supplements

One of the more exploratory aspects of this study was the evaluation of supplement combinations as seen in Figure 7, as many natural compounds are used together in dietary supplements or traditional medicine. The results indicated that at moderate concentrations, combining ashwagandha, olive leaf extract, and ginkgo biloba led to improved neuronal viability, suggesting potential synergistic effects. These findings suggest that while natural supplements may offer neuroprotection individually, their interactions need to be carefully considered. The combination treatment also performed better than the combination treatment by itself and all other treatments under stress. Given the previous explanations on how the supplements may have greater efficacy when applied to cells under stress, it would be interesting to investigate how the potential interactions between the supplements could have promoted better cell survival.

However, there are important limitations and questions that require further exploration. To date, there appears to be no published studies that systematically investigates these supplement combinations using clearly defined inclusion and exclusion criteria, making it difficult to interpret such findings across different experimental systems. This lack of standardization may partly explain the variability observed in the data, especially if different

models, cell types, or stress paradigms are used without accounting for disease-specific variables. Moreover, the observed variability may reflect the influence of unmeasured biological pathways. For instance, while this study focused on general markers of neuronal viability and cytoskeletal integrity, it did not examine disease-relevant processes such as autophagy, calcium homeostasis, mitochondrial membrane potential, or tau phosphorylation, all of which could be modulated by these supplements. If these pathways are differentially affected in combination treatments, they could underpin the improved outcomes seen in stressed neurons. Future research should thus prioritize mechanistic studies that delve into molecular interactions among these compounds, particularly in relation to oxidative stress modulation, mitochondrial function, inflammation, and synaptic signaling. It would also be valuable to determine whether the synergistic effects are context-dependent. For example, whether they only emerge under specific stress thresholds, or whether there are thresholds beyond which interactions become cytotoxic. Ultimately, a more rigorous and mechanistically grounded approach will be essential to understand how combination treatments might be harnessed to develop multi-targeted interventions for neurodegenerative diseases like Alzheimer's.

Implications for Alzheimer's Disease Research

The results of this study have direct implications for Alzheimer's disease (AD) and other neurodegenerative disorders, where oxidative stress and cytoskeletal dysfunction play critical roles in disease progression. Many current treatment strategies for AD focus on targeting amyloid-beta plaques or tau tangles, but increasing evidence suggests that preserving neuronal structure and function is equally important. The microtubule stabilization effects observed at moderate doses of the tested supplements indicate that they may have therapeutic potential for

preventing or slowing neurodegeneration. However, the toxicity at higher concentrations raises concerns about long-term use. While many natural supplements are marketed as safe, their effects at high doses or in combination with other compounds remain poorly understood. Given the inconsistencies observed across different doses and conditions, future research should focus on optimizing concentration ranges for therapeutic use and confirming a consistent neuroprotective effect. Additionally, in vivo studies will be crucial to determine whether the dose-dependent effects observed in cell culture models translate to complex biological systems.

Beyond the biological implications, these findings also prompt broader questions about the economic model through which AD is currently treated. As novel pharmacological agents are developed, they often come with exorbitant price tags—sometimes exceeding $25,000 per year per patient—raising concerns about long-term sustainability, especially in aging populations (Alzheimer's Association, 2023). Moreover, access to these treatments is frequently limited by insurance coverage, healthcare infrastructure, and hospital formularies, making them inaccessible to many who need them most. Out-of-pocket costs, coverage delays, and prior authorization requirements often serve as gatekeeping mechanisms that exacerbate existing health disparities, especially among low-income and underinsured patients (Pickern, 2025).

Given that AD affects millions globally, the question of how to scale interventions affordably and equitably is pressing. Natural supplements may offer a more economically viable adjunct or preventive option, especially if they can be shown to offer meaningful protective effects in at-risk populations. While this study does not propose a replacement for conventional therapies, it does underscore the need to rethink the current therapeutic paradigm, which often prioritizes high-cost, narrowly targeted drugs over broader, potentially scalable solutions. In this context, future research should consider how non-pharmaceutical interventions could be

integrated into care models, and how public health institutions, hospitals, and insurance providers might play a role in either supporting or impeding access to these alternatives. Though these systemic questions fall beyond the scope of this project, they are vital to consider as we confront the growing social and economic burden of Alzheimer's disease.

Future Directions

While this study provided important insights into the effects of ashwagandha, olive leaf extract, and ginkgo biloba on neuronal health, further research is necessary to fully understand their mechanisms of action and clinical relevance. One immediate priority is to clarify the molecular mechanisms underlying the observed neuroprotective effects. While this study identified concentration-dependent improvements in cell viability and cytoskeletal stability at moderate doses, particularly under stress, the specific signaling pathways and cellular targets remain speculative. As previously suggested in the paper, one theory is that hormetic responses may be at play, where these compounds exhibit enhanced protective effects under stress conditions. This suggests that the supplements may activate stress-adaptive pathways, such as Nrf2 signaling, mitochondrial repair, or autophagy. Future research should test this hypothesis directly by examining how these compounds influence oxidative stress, mitochondrial function, and apoptotic signaling, especially under carefully calibrated neurotoxic conditions.

Additionally, the results suggested a potential role for these supplements in modulating microtubule integrity, yet the exact relationship between supplement dose and cytoskeletal function requires further examination. Follow-up studies should investigate the involvement of tubulin-associated proteins, tau phosphorylation, and cytoskeletal transport proteins to assess whether these extracts can prevent structural degeneration linked to Alzheimer's pathology.

Another key direction involves the pharmacokinetics and bioavailability of these compounds, which are crucial factors in determining their therapeutic viability. Natural extracts such as ashwagandha, olive leaf extract, and ginkgo biloba contain a complex mixture of bioactive constituents that often exhibit low solubility in aqueous solutions. In this study, challenges in solubility likely contributed to material loss during filtration, where a substantial portion of the supplement precipitated out of solution or was retained by the filter membrane. This not only reduced the effective dose that reached the neuronal cultures, but also may have introduced inconsistencies in actual exposure levels between replicates. Furthermore, the methods used to prepare and administer supplement concentrations relied on manual solubilization and estimation techniques, which, while practical for a pilot study, may lack the precision required to ensure accurate dosing across experimental groups. Variability in how individual compounds were dissolved, mixed, and filtered could have influenced the final concentration that reached the cells, potentially affecting the observed outcomes and limiting reproducibility. These technical limitations highlight the need for standardized and optimized formulation protocols in future studies. To address these issues, future research should explore advanced delivery strategies that improve both solubility and cellular uptake. In parallel, quantitative validation of compound concentrations using techniques like high-performance liquid chromatography (HPLC) or mass spectrometry would be valuable in ensuring accuracy and consistency.

Importantly, the results of combination treatments (Figure 7) indicate that these supplements may act synergistically under stress. Future experiments should evaluate whether these synergistic effects are additive or interactive at the molecular level, with special focus on whether co-treatment produces greater antioxidant responses, improved mitochondrial function,

or modulation of neuroinflammatory markers. To translate these findings toward therapeutic application, in vivo studies using transgenic or toxin-induced models of Alzheimer's disease are essential. These should include behavioral assessments of cognition and motor function to determine whether cellular improvements lead to functional gains. Long-term studies will also be necessary to assess safety, tolerability, and any cumulative effects of chronic supplementation.

Finally, given that neurodegenerative disease management often involves polypharmacy, it's crucial to explore how natural supplements might interact with conventional AD medications, such as acetylcholinesterase inhibitors (e.g., donepezil, galantamine) and NMDA receptor antagonists (e.g., memantine). Despite the widespread use of both pharmacological treatments and natural supplements among AD patients, there's a notable lack of comprehensive studies examining their combined effects. This gap is concerning, especially considering that certain combinations might offer synergistic benefits or, conversely, pose unforeseen risks. For instance, while some studies have explored the combined use of cholinesterase inhibitors and NMDA antagonists, the results have been mixed (Matsunaga et al., 2015), and the addition of natural supplements into this therapeutic regimen remains under-researched. Understanding these interactions requires consideration of pharmacodynamic factors—how the drugs affect the body—as well as pharmacokinetic factors, particularly concerning drug metabolism pathways like the cytochrome P450 (CYP450) enzyme system, which converts drugs into more water-soluble forms for easier excretion. Many herbal supplements can modulate the activity of these enzymes, potentially altering the metabolism of co-administered drugs, leading to changes in efficacy or the risk of adverse effects (Huang et al., 2022). Moreover, the complexity of AD pathology suggests that a multi-targeted approach could be more effective than monotherapy (Wang et al., 2019). Therefore, systematically studying the interactions between these

supplements and conventional drugs could uncover novel therapeutic strategies that harness the benefits of both.

In conclusion, while this study lays critical groundwork in demonstrating the concentration- and stress-dependent neuroprotective potential of ashwagandha, OLE, and GB, the next phase of research must deepen our understanding of their mechanisms, delivery, and clinical relevance. These directions will help bridge the gap between cell culture findings and translational therapies for Alzheimer's disease and related neurodegenerative conditions.

Limitations

While this study provides valuable insights into the effects of ashwagandha, olive leaf extract, and ginkgo biloba on neuronal health, several limitations must be considered when interpreting the findings. Firstly, while there is an abundance of data to substantiate this research, there was some data that was lost as a result of contamination of the plates throughout the course of the research project and technical difficulties when using the microscope. As a result, there are fewer ICC results across all supplements, inconclusive findings on the neuroprotective effect of ashwagandha, and a lack of repeated trials to solidify the findings that were found in regards to safe concentrations for the olive leaf extract and ginkgo biloba treatments. Therefore, it is important that more trials with the latter two treatments as well as in combination are performed in order to improve the accuracy of the results.

Another limitation that was addressed previously was that the cells under stressed conditions somehow survived better than the control and in some cases better than the treatment conditions. This remained consistent for multiple trials throughout the research. This issue did not seem to affect other research plates who were using the same stressor, however, the stressor

may have been prepared improperly for the plates that did demonstrate this trend. While there was also ICC data collected from these same trials, they were unfortunately unable to be analyzed due to the technical difficulties with the microscope. Suggested future research should include continuous testing with the supplements and with the FAB condition to confirm the findings from this study. Alternatively, however, it is possible that the observed increase in viability under FAB exposure may not reflect a true neuroprotective effect but rather an artifact of experimental conditions. For example, the cells in the FAB-treated wells may have been largely non-viable at the time of treatment either due to prolonged stress, premature death during the two-week culture period, or suboptimal initial plating densities (Lincoln & Gabridge, 1998). In such cases, cells may die off more rapidly, and the normalization of raw data per condition could misleadingly show higher survivability percentages, even when the absolute number of viable cells is very low. This could give the appearance of enhanced viability in FAB-treated conditions, when in fact, only a small, marginally healthier subset of cells remains alive. Moreover, amyloid-beta, although toxic in excess, is a naturally occurring peptide in the brain, and mild levels may transiently stimulate certain cellular pathways in stressed cultures, giving a short-lived boost in viability to cells on the verge of death (Puzzo et al., 2008). These possibilities highlight the importance of incorporating tighter experimental controls, repeated stressor calibrations, and complementary viability and morphological assessments in future studies to ensure the reliability of findings and to avoid misinterpretation of artifacts as biological effects.

Conclusion

This research highlights the potential neuroprotective effects of ashwagandha, olive leaf extract, and ginkgo biloba, particularly in relation to neuronal viability and microtubule stability.

The results suggest that moderate concentrations of these supplements may help protect neurons from oxidative stress, while higher concentrations may lead to cytotoxic effects. Additionally, combining these supplements may offer synergistic benefits when used at carefully optimized doses, though improper dosing could reduce efficacy or increase risk. These findings underscore the critical importance of dose optimization and mechanistic understanding when considering natural supplements as therapeutic agents for Alzheimer's disease and other neurodegenerative conditions.

Importantly, this research also speaks to the broader need for accessible, affordable, and scalable treatment strategies. As the global burden of Alzheimer's disease continues to rise, particularly in aging populations, it becomes increasingly urgent to explore therapies that not only improve clinical outcomes but also support the daily functioning, independence, and quality of life of those living with the disease. Natural supplements, if shown to be effective and safe, could serve as low-cost adjunctive options that complement existing therapies and extend support to populations underserved by conventional pharmaceutical approaches.

Thus, continuing to study these compounds is not merely a scientific or medical endeavor—it is a public health and ethical imperative. Further research, particularly in in vivo models and clinical settings, is essential to elucidate the long-term safety, pharmacological interactions, and real-world efficacy of these treatments. In doing so, we move closer to developing more holistic and inclusive strategies for combating neurodegeneration and improving the livelihoods of individuals and families affected by Alzheimer's disease.

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