CHARACTERIZATION OF NEW BIOACTIVE MOLECULES FOR THE TREATMENT OF NEUROINFLAMMATORY AND NEURODEGENERATIVE DISEASES

UNIVERSITY OF VERONA

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Dedication

I dedicate this work to my son, **Erik Majumder**, who is my greatest source of strength and inspiration.

I dedicate it equally to my beloved wife, **Haimanti Biswas**. I could not have completed this journey without your unwavering support. Your love, patience, and constant encouragement have sustained me through every challenge.

This achievement is as much yours as it is mine.

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a significant global health concern. It is characterized by the accumulation of extracellular amyloid-β (Aβ) plaques, intracellular neurofibrillary tangles of hyperphosphorylated tau, and progressive neuronal and synaptic degeneration. These pathological changes lead to cognitive decline and dementia. A key feature of AD is the neuroinflammation, where chronic microglial activation promotes the release of pro-inflammatory cytokines and neurotoxic mediators, which exacerbate neuronal injury and enhance neurodegeneration. Due to the lack of effective treatments in AD, research efforts are increasingly focusing also on natural compounds as potential alternative therapeutics strategies to address AD pathology. The present study is part of National Biodiversity Future Center project, which aims to identify and characterize novel bioactive plant-derived extracts from Italian flora, evaluating their potential to modulate neuroinflammation and provide neuroprotection. Twenty-three natural extracts were first tested *in vitro* for cytotoxicity and biological activity using the murine SIM-A9 microglial cell line, primary microglia isolated from neonatal 3xTg-AD mice, and N2A neuroblastoma cells. Particularly, cytotoxicity was evaluated using the MTT assay, whereas the anti-inflammatory activity was assessed in LPS-stimulated microglia by measuring nitric oxide (NO) production using the Griess assay and determining the release of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) using ELISA. The promising anti-inflammatory natural extracts were subsequently evaluated for their ability to reduce the cytokine release in primary microglia stimulated by Aβ peptides. Among the natural extracts tested, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis exhibited the most potent inhibitory effects on the production of TNF-α and IL-6 in microglia stimulated by both LPS and Aβ. Additionally, the neuroprotective efficacy of these extracts was evaluated in

N2A cells exposed to glutamate-induced excitotoxicity. Althaea officinalis and Dianthus superbus significantly enhanced cell viability in glutamate-exposed N2A neuroblastoma cells, indicating their potential to mitigate excitotoxic neuronal damage. These findings highlight new properties for Althaea officinalis and Dianthus superbus that have not been previously studied, showing a significant reduction in the production of pro-inflammatory mediators induced by LPS and Aβ, along with notable neuroprotective effects. Although further research is needed to confirm the effects of Althaea officinalis and Dianthus superbus in *in vivo* studies, our *in vitro* findings suggest that these plants may be promising therapeutic approaches to attenuate neuroinflammation and neuronal damage, particularly relevant for neurodegenerative disorders, like AD.

Keywords: Alzheimer's disease; Neuroinflammation; Microglia; Plant-derived extracts; Neuroprotection.

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List of Commonly Used Abbreviations

AD	*Alzheimer's Disease*
MCI	*Mild Cognitive Impairment*
MMSE	*Mini-Mental State Examination*
GA	*Glutamic Acid*
MTT	*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide*
LPS	*Lipopolysaccharide*
TNF-α	*Tumor Necrosis Factor-alpha*
IL-6	*Interleukin-6*
NO	*Nitric Oxide*
ELISA	*Enzyme-Linked Immunosorbent Assay*
GWAS	*Genome-Wide Association Studies*
CSF	*Cerebrospinal Fluid*
NFT	*Neurofibrillary Tangle*
PET	*Positron Emission Tomography*
MRI	*Magnetic Resonance Imaging*
SPECT	*Single-Photon Emission Computed Tomography*
EEG	*Electroencephalography*
SIM	*SIM-A9 (a murine microglial cell line)*
RNA	*Ribonucleic Acid*
IL	*Interleukin*
MEG	*Magnetoencephalography*
EOFAD	*Early-Onset Familial Alzheimer's Disease*
LOAD	*Late-Onset Alzheimer's Disease*
EOAD	*Early-Onset Alzheimer's Disease*
APP	*Amyloid Precursor Protein*
APOE	*Apolipoprotein E*
AICD	*Amyloid Intracellular Domain*
NMDA	*N-methyl-D-aspartate*
AMPA	*α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid*
CAA	*Cerebral Amyloid Angiopathy*
MAP	*Microtubule-Associated Protein*
CNS	*Central Nervous System*
ROS	*Reactive Oxygen Species*
BBB	*Blood-Brain Barrier*
GFAP	*Glial Fibrillary Acidic Protein*
BDNF	*Brain-Derived Neurotrophic Factor*
TGF	*Transforming Growth Factor*
VEGF	*Vascular Endothelial Growth Factor*
DAM	*Disease-Associated Microglia*
MGnD	*Microglia Neurodegenerative*
NBFC	*National Biodiversity Future Center*

PNRR	*Piano Nazionale di Ripresa e Resilienza*
FDA	*U.S. Food and Drug Administration*
MAPK	*Mitogen-Activated Protein Kinase*
COX	*Cyclooxygenase*
JNK	*c-Jun N-terminal Kinase*
MCP-1	*Monocyte Chemoattractant Protein-1*
iNOS	*Inducible Nitric Oxide Synthase*
NF-kB	*Nuclear Factor kappa-light-chain-enhancer of activated B cells*
AO	*Althaea officinalis*
AL	*Adenophora lilifolia*
EASL	*Eupatorium adenophorum Spreng leaves*
BHP	*Tert-butyl hydroperoxide*
CCGA	*Cryptochlorogenic Acid*
DS	*Dianthus superbus*
SP	*Succisa pratensis*
GBM	*Glioblastoma*
ATCC	*American Type Culture Collection*
CRL	*Catalog Reference Number (e.g., CRL-3265TM)*
USA	*United States of America*
DMEM	*Dulbecco's Modified Eagle Medium*
CAT	*Catalog*
CI	*Catalog Item / Identification*
DMSO	*Dimethyl Sulfoxide*
FBS	*Fetal Bovine Serum*
EDTA	*Ethylenediaminetetraacetic acid*
LC	*Liquid Chromatography*
MS	*Mass Spectrometry*
UPLC	*Ultra-Performance Liquid Chromatography*
ESI	*Electrospray Ionization*
HRMS	*High-Resolution Mass Spectrometry*
CO	*Carbon Dioxide (CO_2)*
PBS	*Phosphate-Buffered Saline*
SDS	*Sodium Dodecyl Sulfate*
PAGE	*Polyacrylamide Gel Electrophoresis*
MES	*2-(N-morpholino)ethanesulfonic acid*
PVDF	*Polyvinylidene Difluoride*
TBS	*Tris-Buffered Saline*
HRP	*Horseradish Peroxidase*
ECL	*Enhanced Chemiluminescence*
DAPI	*4',6-diamidino-2-phenylindole*
DNA	*Deoxyribonucleic Acid*
IBA	*Ionized calcium-Binding Adaptor molecule 1 (IBA-1)*
GFAP	*Glial Fibrillary Acidic Protein*
Aβ	*Amyloid-beta (Aβ)*

BSA	*Bovine Serum Albumin*
RT	*Room Temperature*
TMB	*3,3',5,5'-Tetramethylbenzidine*
SEM	*Standard Error of the Mean*
ANOVA	*Analysis of Variance*
CA	*California*
YL	*Yellow Luteolin (often refers to specific sulfate-conjugated molecules)*
IF	*Immunofluorescence*
RNS	*Reactive Nitrogen Species*
IWG	*International Working Group*
DC	*District of Columbia*
NE	*Northeast*
RSC	*Royal Society of Chemistry*
JAD	*Journal of Alzheimer's Disease*
RAW	*RAW 264.7 (murine macrophage cell line)*

INTRODUCTION

1. Alzheimer’s disease

Alzheimer's disease (AD) is a devastating and progressive neurodegenerative disorder, affecting over 50 million individuals worldwide. AD is notably prevalent within the elderly demographic, affecting over 10% of individuals aged 65 and approximately 50% of those aged 85 and older, with women constituting two-thirds of reported cases (Nichols et al., 2022). It is estimated that the number will exceed 140 million by 2050, aggravating the burden on healthcare systems (S. Chen et al., 2024). AD is increasingly understood as a continuous biological process characterized by distinct neurobiochemical hallmarks, most notably the extracellular deposition of amyloid-beta plaques (Aβ), the presence of intracellular neurofibrillary tangles (NFTs), concurrent neuroinflammation, and progressive neuronal loss. When this underlying pathology reaches advanced stages of neurodegeneration, it manifests clinically as Alzheimer’s dementia, which stands as the most prevalent form of clinical dementia globally, severely disrupting memory, reasoning, and daily activities (Knopman et al., 2021). The slow progression of this disease occurs over several years or even decades, resulting in a significant decline in memory and cognitive functions. This deterioration severely impacts an individual’s daily activities, mainly due to the gradual buildup of neuropathological features in the brain. The exact etiology of AD is still unclear, and a cure or adequate clinical treatment remains unavailable for patients. Therapeutic efforts are still struggling to identify molecular targets to change the clinical course for AD patients significantly.

Alzheimer’s disease can be classified in three distinct stages:

- Preclinical stage
- Mild cognitive impairment (MCI)
- Alzheimer's dementia (clinical stage)

1.1 Preclinical stage

This initial phase of AD is characterized by detectable neurological alterations, including the accumulation of amyloid plaques and tau tangles. Although there are no noticeable cognitive symptoms in daily life, these pathological changes can manifest as early as two decades before the emergence of any apparent symptoms (Dubois et al., 2016).

1.2 Mild Cognitive Impairment (MCI)

MCI is considered as the longest stage of AD, characterized by the initial cognitive changes. Besides the detection of AD-related brain changes, individuals experience mild alterations in memory and thinking abilities. This often includes difficulties in learning and retaining new information, although patients are still able to perform everyday tasks (Porsteinsson et al., 2021).

1.3 Alzheimer's dementia (clinical stage)

Dementia is typically divided into three distinct stages: mild, moderate, and severe. In the mild stage, patients can live independently but require assistance with some activities. During the moderate stage, they experience changes in personality and behavior and need help with most of their daily activities. In the severe stage, patients

lose the ability to perform daily activities due to the significant impact of the disease on their physical and motor skills, which is often accompanied by pronounced behavioral symptoms. Verbal communication is minimal, and the patients require 24/7 care (Ward et al., 2002).

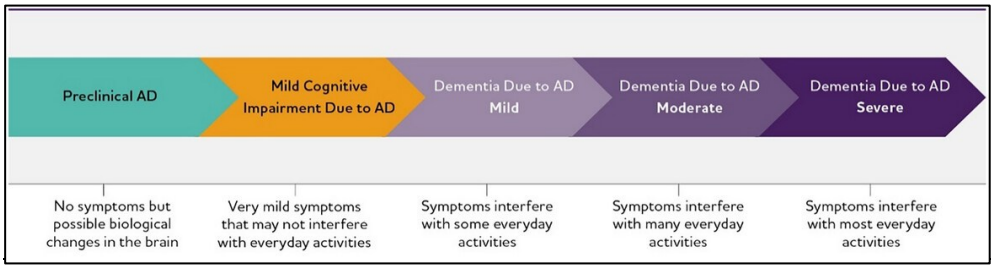


Figure 1. Clinical stage of AD. This figure shows the gradual AD progression from presymptomatic stages to severe dementia. These five consecutive stages represent the evolution of AD pathology and its clinical manifestations ('2024 Alzheimer's Disease Facts and Figures', 2024).

2. Clinical diagnosis of Alzheimer's disease

Whereas in the past, neuropathological changes of AD were definitively diagnosed only post-mortem through histopathological examinations, nowadays, the diagnosis of AD involves a multifaceted approach, incorporating clinical evaluations, neuropsychological assessments, biomarker analysis, and neuroimaging techniques (Turner et al., 2020).

2.1 Clinical symptoms

Memory impairment is the key feature of the cognitive symptoms associated with AD. Although memory deficits are the classic initial cognitive symptoms, patients may also exhibit significant language, executive, or visuospatial impairments (López, 2008). Conventionally, the diagnosis of AD is based on a combination of medical

background, clinical manifestations, and memory evaluations. The Mini-Mental State Examination (MMSE) is commonly used in both research and clinical settings as a brief screening tool for global cognition. MMSE assesses various cognitive domains, including orientation, delayed recall, attention, language, and construction skills. Scores on the MMSE range from 0 to 30 points, with lower scores suggesting the presence of cognitive issues (Mayo & Peavy, 2019). The pathological process related to AD spans decades, and during this time, the lesions develop according to a predictable sequence. A staging system for the intraneuronal lesions allows to differentiate between initial, intermediate, and late phases of the disease process in symptomatic individuals. In the past, Braak staging was mainly used to determine the NFTs pathology in postmortem individuals. Braak stages I and II indicate that NFTs are primarily confined to the entorhinal region of the brain. Braak stages III and IV show involvement of the limbic regions, particularly the hippocampus. Braak stages V and VI indicate moderate to severe involvement of the neocortex (Braak & Braak, 1991). The Braak neuropathological staging relies on predefined sections of specific brain regions, which may restrict the identification of alternative patterns of NFT accumulation. Nowadays, the recent advances in radiochemistry have enabled the application of the postmortem Braak staging framework to tau-PET (Positron Emission Tomography) images obtained in vivo. PET imaging allows the accurate mapping of tau progression across the entire human brain, providing a significant improvement in AD diagnosis (Macedo et al., 2024).

2.2 Neuroimaging techniques

Advances neuroimaging techniques offer valuable insights into the structural and functional changes in the brain. Neuroimaging is essential for evaluating patients suspected of having AD. Magnetic Resonance Imaging (MRI) and Positron Emission Tomography (PET) are the two primary imaging modalities used for diagnosing AD.

MRI provides structural and functional information about the brain by allowing detailed characterization of regional tissue. This imaging technique clearly distinguishes between cerebral gray matter and white matter, displaying brain tissue in a three-dimensional format. On the other hand, PET can reveal metabolic and molecular changes mapping the whole brain. PET imaging utilizing radiotracers, such as Pittsburgh Compound B (PiB) for amyloid and tracers for tau, enables the direct visualization of these pathological protein deposits. Additionally, PET can quantify glucose metabolism, revealing regions with diminished metabolic activity, which correlates with neuronal dysfunction characteristic of AD (J. Wang et al., 2023) (Chapleau et al., 2022). Functional magnetic resonance imaging (fMRI) is a non-invasive imaging technique that offers several advantages, because it does not require contrast agents or radiation exposure. fMRI provides high spatial resolution and reasonable temporal resolution, and it can be performed in the same session as structural MRI. Most importantly, fMRI can offer valuable insights into the functional integrity of brain networks related to memory and other cognitive abilities. It can also reveal the neural correlations of specific behavioral events, such as successful versus unsuccessful memory formation (Sperling, 2011). Single-Photon Emission Computed Tomography (SPECT) quantifies regional cerebral blood flow, and the distinctive hypoperfusion patterns observed in AD confirm its effectiveness in distinguishing AD from other neurodegenerative dementias, with evidence demonstrating considerable

diagnostic sensitivity and specificity. Concurrently, electrophysiological methodologies, including Electroencephalography (EEG) and Magnetoencephalography (MEG), provide recordings of electrical and magnetic brain activity with high temporal resolution, facilitating the identification of instantaneous changes in neural synchronization, network dynamics, and functional connectivity, thus providing essential complementary insights into the pathophysiology of AD (Babiloni et al., 2020; Davison & O'Brien, 2014). These imaging modalities not only facilitate qualitative diagnoses of AD but also enable the quantification of sensitivity thresholds identified throughout the normal phase and various stages of the disease.

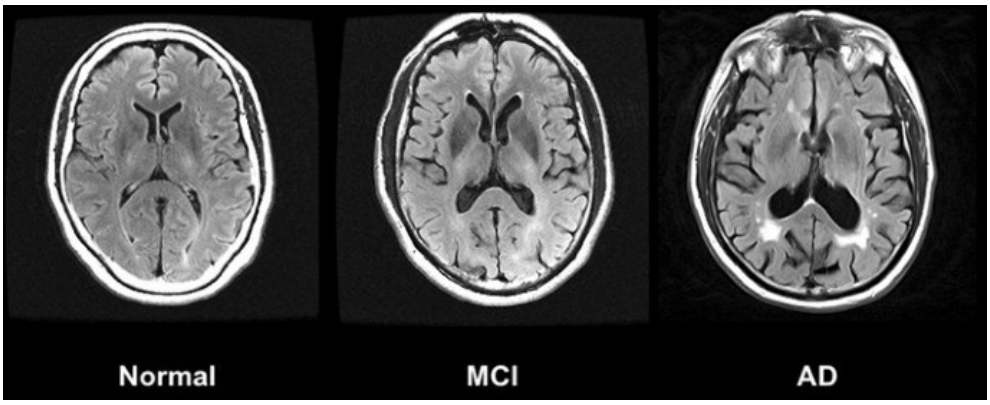


Figure 2. Representative MRI scans in Normal, MCI, and AD Individuals. The image shows MRI scan of a healthy control (on the left) with a normal brain morphology. In the middle, an individual displaying slight hippocampal atrophy. On the right, the AD patient exhibits severe hippocampal atrophy and widened sulci (Chandra et al., 2019).

2.3 CSF biomarkers

Cerebrospinal fluid (CSF) occupies the subarachnoid space and the ventricular system surrounding the brain. It provides essential mechanical and immunological protection to the brain inside the skull. CSF can be collected through a procedure known as lumbar puncture. Although lumbar puncture is an invasive procedure and potentially painful for the patient, CSF is one of the most informative fluids for discovering

biomarkers related to the prognosis of neurodegenerative diseases (Blennow et al., 2010). CSF is considered an ideal source of biomarkers in AD being more in physical contact with the cerebral tissue than any other fluids. Accordingly, the concentration of CSF several proteins reflects with good diagnostic accuracy the pathophysiological features of the disease. In the early stages of AD, such as MCI, accurately diagnosing the condition can be challenging. In this context, cerebrospinal fluid biomarkers would be particularly valuable. Tau and Aβ in cerebrospinal fluid (CSF) are considered the earliest and most extensively studied biomarkers. The CSF of AD patients is characterized by a marked decrease in the levels of Aβ42. It is hypothesized that the aggregation and deposition of Aβ in the AD brain result in decreased CSF levels of this peptide (Blennow & Zetterberg, 2018). The decreased Aβ42/Aβ40 ratio is more pronounced in AD diagnosis than the reduction of Aβ42 alone. Therefore, Aβ42/Aβ40 ratio may be more useful in AD diagnosis in the early as well as the clinical phases of the disease. Upon significant disruption of neuronal architecture, the tau protein could be released into CSF. Consequently, elevated levels of tau and hyperphosphorylated tau in the CSF may correlate with the onset of neurodegeneration in AD (Janelidze et al., 2016). Synaptic deterioration represents an early pathological manifestation of AD and is associated with cognitive impairment. The dendritic protein neurogranin (Ng), a CSF biomarker for synaptic injury, exhibits elevated levels in AD and correlates with increased t (total)- and p (phosphorylated)-tau proteins levels as well as cognitive decline (Portelius et al., 2015). The combination of these biomarkers enables more accurate diagnosis, helping healthcare professionals differentiate AD from other types of dementia. In clinical settings, CSF biomarker profiles are used to confirm suspected cases and guide clinical decision-

making. These biomarkers often indicate disease progression long before symptoms become fully manifest (Papaliagkas et al., 2023).

3. Alzheimer's disease risk factors

The development and progression of AD are influenced by a combination of genetic, environmental, and lifestyle factors. Although the exact cause of AD remains unclear, numerous risk factors have been identified.

3.1 Genetic factors

AD can generally be classified into two categories: early-onset familial AD (EOFAD) and late-onset AD (LOAD). EOFAD is typically diagnosed in individuals who exhibit symptoms before the age of 65. It is estimated that EOFAD accounts for about 5-6% of all AD cases. This familial form of EOAD is caused by highly penetrant mutations in three specific genes: *Amyloid Precursor Protein (APP), Presenilin 1 (PSEN1), and Presenilin 2 (PSEN2)*. These mutations lead to the differential cleavage of APP and the subsequent production of various forms of Aβ peptide. The *APP* gene is located on chromosome 21. Genetic linkage studies and observational data indicate that individuals with Down syndrome, who have triplication of the *APP* gene, develop cognitive impairments associated with biological signatures of AD (Hampel et al., 2021). Several pathogenic mutations in *PSEN1* and *PSEN2* gene cause a partial loss of APP γ-secretase-dependent cleavage function. This change is associated with a shift in cleavage position towards Aβ42, resulting in a decrease in the production of both Aβ42 and Aβ40 (L. Sun et al., 2017). No causal genetic mutations have been

identified in association with LOAD. LOAD is believed to be a multifactorial disease with a complex genetic background. Through large-scale genome-wide association studies (GWAS), several important genetic risk factors linked to susceptibility to AD have been identified, with more than 50 genes or loci associated with the risk of developing LOAD (Sims et al., 2020). The *apolipoprotein E (APOE) ε4 allele*, located on chromosome 19q13.2, is the most significant risk gene for LOAD. *ApoE* exists in three polymorphic alleles: ε2, ε3, and ε4, which result from two-point mutations within exon 4. Individuals carrying one copy of the ε4 allele (ApoE ε3/ε4) have a three-fold increased risk of developing AD compared to those with two ε3 alleles. The ε3 isoform is the most common and is considered the neutral allele for the general population. On the other hand, individuals who are homozygous for the ε4 allele (*ApoE ε4/ε4*) face a twelve-fold increased risk of developing the disease. In contrast, the ε2 allele is the rarest and appears to provide a small protective effect. Despite extensive research on ApoE contribution to the disease, the underlying mechanisms connecting this gene to AD remain poorly understood (Abrego-Guandique et al., 2024). *Triggering Receptor Expressed on Myeloid Cells 2 (TREM2)* is another important genetic risk factor for late-onset AD which encoded on chromosome 6p21. TREM2 is predominantly expressed by microglia, the brain's resident immune cells, where it critically modulates microglial activation, phagocytosis, and responses to amyloid pathology. Rare heterozygous variations in *TREM2*, especially the R47H mutation, elevate the chance of developing AD by 2 to 4 times, a level like that associated with a single *APOE ε4* allele. Functional analyses indicate that these variations impair microglial clearance of amyloid-beta and influence neuroinflammatory responses, thus associating TREM2 dysfunction with neurodegenerative progression. Though rarer than *APOE ε4*, *TREM2* variants

emphasize the involvement of innate immunity and microglial activity in AD pathogenesis (Zgorzynska, 2024). Another genetic risk factor for LOAD is *CD33* gene. *CD33* gene is located on chromosome 19q13.3 and is predominantly expressed by microglia in the brain, and its immunoregulatory functions are crucial in the context of AD pathogenesis. CD33 is a transmembrane receptor that belongs to the sialic acid-binding immunoglobulin-like lectin family, and acts as an inhibitory immune receptor on microglial cells. At the physiological conditions, CD33 regulates innate immune responses by suppressing excessive microglial activation and phagocytosis through intracellular inhibitory signaling motifs. Although this regulatory activity is important for preserving immune homeostasis, upregulated expression of CD33 in AD is thought to inhibit microglial clearance of amyloid-β, resulting in increased amyloid accumulation and worsening of disease. In contrast, CD33 inhibition or genetic deletion improves microglial phagocytic activity, promotes Aβ clearance and attenuates neuroinflammatory responses, highlighting CD33 as a promising therapeutic target for AD (Akinluyi et al., 2026; Griciuc et al., 2019). Several studies have examined the significant role of the *CD33* rs3865444 polymorphism in AD. The main *CD33* allele is associated with increased susceptibility to AD compared to a minor *CD33* allele, which confers protection against AD. The overexpression of CD33 plays a role in the pathogenesis of AD by reducing the internalization of Aβ, which results in the accumulation of neuritic amyloid pathology and affects microglial levels (Griciuc et al., 2013). Eskandari-Sedighi et al. studied the impact of the human CD33 isoforms (main and minor) on microglia cell function by utilizing a transgenic mouse model in which either the major (CD33M) or minor (CD33m) human CD33 isoform expressed along with 5xFAD AD mouse model. This breeding strategy was imperative due to the functional

divergence between mouse and human CD33, allowing the direct introduction of human isoforms into an *in vivo* context of amyloid pathology. The double-transgenic offspring expressed both human CD33 isoforms and the 5xFAD mutations associated with Aβ pathology, allowing the team to study *in vivo* how each isoform regulates microglial responses to Aβ pathology. Notably, opposite effects were found on the response of microglia to Aβ deposition. Mice expressing the main *CD33* isoform showed higher Aβ levels, more diffuse plaques and an increased number of dystrophic neurites compared to 5xFAD control mice. Contrarily, the minor *CD33* isoform promotes plaque compaction and enhances microglia-plaque interaction, minimizing neuritic plaque pathology (Eskandari-Sedighi et al., 2024). These data collectively suggest that *CD33* is a major genetic risk factor for AD, modulating microglia functions.

3.2 External factors

An array of environmental elements contributes significantly to the onset and progression of AD. Prolonged exposure to environmental factors such as air pollution, tobacco use, dietary patterns, heavy metal accumulation, and infectious agents can trigger oxidative stress and inflammatory processes, potentially elevating the risk of developing AD and other neurodegenerative disorders (Weaver, 2023). A diet heavy in highly processed, nutrient-poor foods, combined with a sedentary lifestyle, may elevate the risk of adverse health outcomes. Alterations to the gut microbiome composition can promote inflammation, disrupt glucose regulation, and contribute to the progression of neurodegenerative diseases like Alzheimer's (Cryan et al., 2019). Studies show that AD individual and animal models exhibit decreased gut

microbiome diversity, which can lead to inflammation, brain vascular damage, amyloid-beta aggregation, and tau pathology - all indicators of the gut microbiome's direct impact on Alzheimer's pathology (Murray et al., 2022). Additional factors, including poor sleep, head injuries, and limited mental stimulation increase the risk of AD. Adequate sleep supports the clearance of amyloid-beta, while injuries can cause brain inflammation, and inactive lifestyles reduce the brain's resilience against the disease (Green et al., 2020).

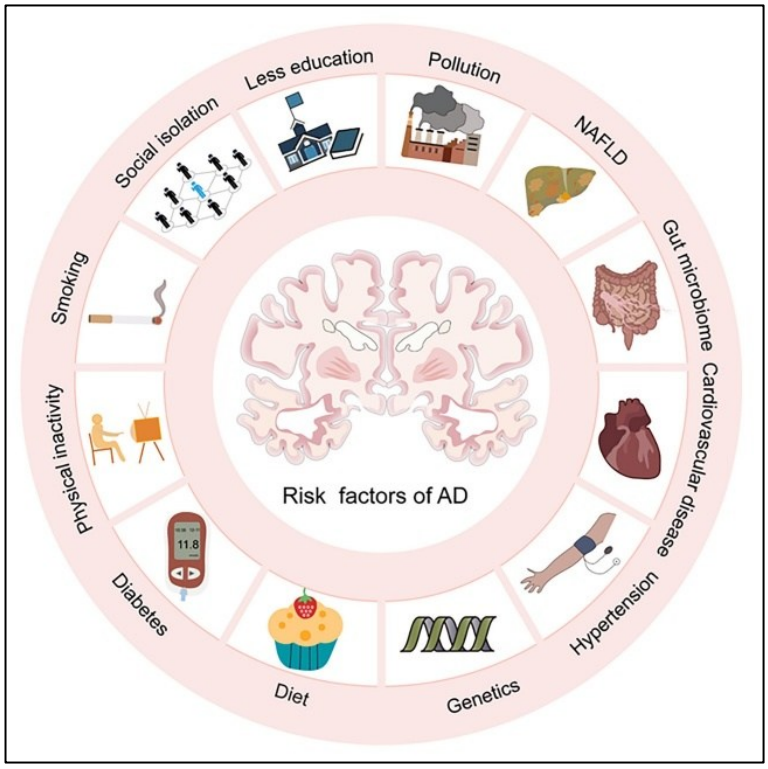


Figure 3. Risk factors of AD. The figure shows the multifaceted nature of AD risk factors, contributing to the development and progression of the condition. These factors are classified into lifestyle, environmental, health-related, and genetic categories. Lifestyle elements encompass smoking, physical inactivity, and dietary habits; environmental factors highlight pollution and social isolation; health-related factors include diabetes, hypertension, cardiovascular diseases, and alterations in the gut microbiome; while genetic factors signify the inherited predispositions associated with AD (from Nasb M. et al., 2024).

4. The neuropathological hallmarks of Alzheimer's disease

The classical neuropathological alterations of AD include the accumulation of extracellular amyloid plaques, the deposition of intracellular neurofibrillary tangles (NFTs), synaptic and neuronal loss, and neuroinflammation (Querfurth & LaFerla, 2010; Serrano-Pozo et al., 2011). Besides these classical neuropathological changes, mitochondrial dysfunction is increasingly considered as another important cause in the pathogenesis of AD. Mitochondrial dysfunction precipitates compromised ATP synthesis, increased production of ROS, disrupted calcium homeostasis, and neuronal susceptibility, all of which enhance amyloid-β and tau pathology as well as neurodegeneration (Swerdlow, 2018; W. Wang et al., 2020). Together, these pathological changes result in brain atrophy and contribute to cognitive impairments associated with AD.

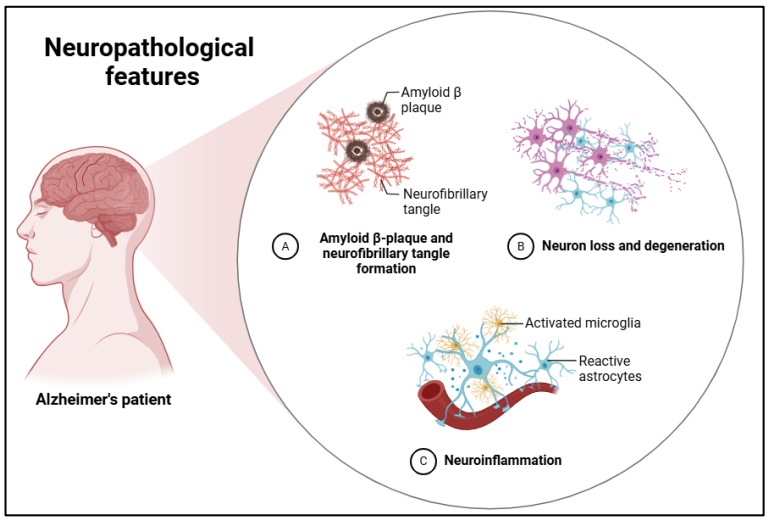


Figure 4. Neuropathological features of AD. In the early stages of AD, abnormal proteins start to aggregate, including β-amyloid plaques and neurofibrillary tangles (A). These deposits have detrimental effects in neurons predominantly cortical and hippocampal neurons, inducing neuronal loss and degeneration (B). During disease progression, neuroinflammation exacerbates, characterized by the overactivation of microglia and astrocytes (C). Collectively, these pathological processes culminate in brain damage, contributing to the cognitive impairment and memory deficits associated with AD [Image was adapted from (from Pais et al., 2023)].

4.1 Amyloid-beta pathology

Amyloid beta (Aβ) pathology refers to the extracellular accumulation and aggregation of Aβ peptides into cerebral amyloid plaques. These peptides, typically 36-43 amino acids long, derive from the cleavage of amyloid precursor protein (APP) by sequential proteolytic processing mediated by three enzymes (α-, β- and γ-secretase), resulting in two distinct pathways: the non-amyloidogenic and the amyloidogenic. The non-amyloidogenic pathway involves the cleavage of full-length APP by α-secretase within the Aβ sequence, precluding Aβ formation. This produces soluble APPα, a neuroprotective fragment, released extracellularly and a C-terminal APP fragment of 83-amino acids (C83) that remains embedded in the plasma membrane. Subsequently, γ-secretase cleavage processes this fragment yields in the non-toxic p3 peptide and the amyloid intracellular domain (AICD). This pathway is considered protective as it does not produce amyloidogenic Aβ peptides and contributes to normal neuronal function (Y. Zhang et al., 2011, 2023). In the amyloidogenic pathway, APP is sequentially cleaved first by β-secretase (beta-site amyloid precursor protein–cleaving enzyme 1, BACE1), releasing soluble APPβ. The retained fragment C99 is digested then by γ-secretase, an enzymatic complex with presenilin 1 (PS1), resulting in the production of Aβ peptides, Aβ40 and Aβ42. Despite Aβ40 being more abundant in the cerebrospinal fluid and brain tissue, Aβ42 is more prone to aggregation and is the predominant form found in the amyloid plaques (Festa et al., 2019; Gu & Guo, 2013). Thus, the balance between these two pathways—amyloidogenic producing Aβ and non-amyloidogenic preventing Aβ formation—is crucial for brain health and is a focus for AD therapeutic strategies aiming to reduce Aβ burden.

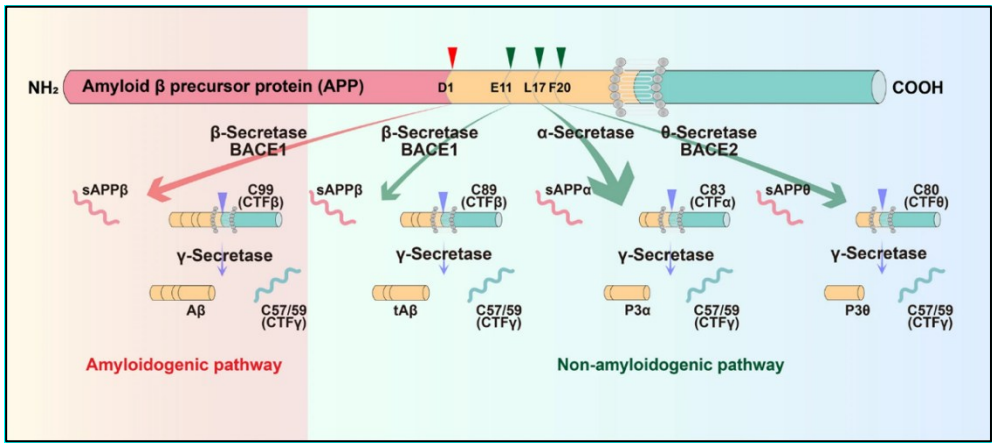


Figure 5. Amyloidogenic and non-amyloidogenic processing pathways of APP in AD. The diagram illustrates the proteolytic cleavage of APP and their implications for AD pathogenesis. In the amyloidogenic pathway (left), APP is initially cleaved by β-secretase (BACE1), resulting in the release of sAPPβ and the formation of the 99-amino-acid C-terminal fragment C99, followed by γ-secretase produces Aβ peptides and the γ-secretase-derived C-terminal fragment (CTFγ), which contributes to the development of amyloid plaques. Conversely, the non-amyloidogenic pathways (right), α-secretase cleaves APP within the Aβ domain, generating neuroprotective sAPPα and C83, thus inhibiting Aβ production (from Y. Zhang et al., 2023).

4.1.1 Evolution of the Amyloid cascade hypothesis

Beginning in the early 1990s, the amyloid cascade hypothesis became the dominant framework for understanding the pathogenesis of AD (Hardy & Higgins, 1992). The "amyloid cascade hypothesis," proposed in 1992, suggests that Aβ deposition is the initial event in the pathological development of AD, leading to tau pathologies, synaptic dysfunction and neuronal loss (Y. Zhang et al., 2023). This hypothesis posits that the accumulation of aberrant extracellular amyloid-β plaques, resulting from the cleavage of amyloid precursor protein, instigates a cascade of detrimental events, including tau protein hyper-phosphorylation and misfolding, ultimately forming intra-neuronal neurofibrillary tangles (Gholami, 2023; Krishaa L. et al., 2023). However, over time, the initial plaque-centric perspective has evolved, with accumulating

evidence indicating that soluble Aβ oligomers, rather than mature plaques are the primary neurotoxic agents responsible for early synaptic dysfunction (Tolar et al., 2021). While widely accepted, particularly due to genetic evidence linking AD-related mutations to Aβ overproduction or increased aggregation-prone Aβ peptides, direct therapeutic interventions targeting Aβ have largely failed to produce significant clinical benefits, prompting a re-evaluation of its singular causal role (Gulisano et al., 2018). Despite these therapeutic setbacks, the amyloid cascade hypothesis continues to serve as a foundational framework for understanding AD pathogenesis, emphasizing the critical role of Aβ dysregulation as a primary driver (Tsering & Prokop, 2024). However, a growing body of evidence suggests that Aβ plaques might also represent a protective adaptation rather than solely a neurotoxic entity, especially when considering its role as an antimicrobial peptide (Y. Zhou et al., 2018). Modern advancements have transformed contemporary interpretations of the theory into a more multifactorial model, wherein Aβ serves as a significant initiator while closely interacting with tau pathology, inflammation, and other cellular stresses (Olejar et al., 2024). This perspective indicates that Aβ peptides, derived from amyloid precursor protein, are a primary instigator in the formation of amyloid plaques and neurofibrillary tangles (Pimplikar, 2009). This process subsequently leads to neurodegeneration and brain atrophy, which are the immediate causes of cognitive decline and clinical progression in Alzheimer's patients (De Ninno et al., 2024).

4.1.2 Amyloid-beta aggregation state

Aβ aggregation is a critical factor in the pathogenesis of AD. After the proteolytic processing of APP in the amyloidogenic pathway, Aβ peptides can spontaneously self-

aggregates into different physical forms. Aβ monomers are soluble structures existing as peptides that tend to assembly with one another leading to the formation of oligomers. The most abundant Aβ monomer in the brain is Aβ40, while the Aβ42 is the most prone to misfolding and aggregation. Aβ42 plays a major role in the seeding of amyloid aggregates and the formation of oligomers. As also mentioned above, the high ratio of Aβ42 to Aβ40 in cerebrospinal fluid (CSF) is used clinically as a biomarker for early detection of AD, indicating higher disease risk and progression. Both peptides differ in their cellular uptake and trafficking pathways, which further influences their neurotoxic effects (Blennow & Zetterberg, 2018; Omtri et al., 2012). Aβ oligomers (Aβo) are the most neurotoxic structures directly can exert their detrimental effects by disrupting synaptic plasticity, which consequently leads to neuronal damage (S. Li & Selkoe, 2020). These include the overstimulation of NMDA/AMPA receptors, intracellular calcium unbalance, impaired neurotransmitter release, and the triggering of tau phosphorylation (Tu et al., 2014). In addition, Maezawa I. et al demonstrated that low nanomolar Aβo can activate microglia activation to release soluble neurotoxic factors, damaging indirectly the integrity of neurons and synapses in primary culture and organotypic hippocampal slices (Maezawa et al., 2011). Protofibrils are intermediate aggregates formed during the transition of oligomeric Aβ peptides into mature amyloid fibrils. Structurally, Aβ protofibrils share features with mature fibrils, such as a core β-sheet organization and flexible N- and C-termini (Williams et al., 2005). As oligomers, protofibrils are considered neurotoxic, contributing to AD pathogenesis. Protofibrils are also metastable, they can dissociate or convert to fibrils under certain conditions. Stabilizing these protofibrils may have therapeutic implications by sequestering Aβ away from toxic pathways (Ono & Tsuji, 2020). Amyloid fibrils are insoluble

aggregates, possessing a β-sheet conformation and they can assemble to form extracellular amyloid plaques, also called senile plaques, in the brain parenchyma during AD progression. The diverse structural forms (polymorphism) of fibrils, dictated by their aggregation conditions, impact their solubility, rate of accumulation, and toxicity (Petkova et al., 2005). Aβ fibrils and plaques are associated to synaptic dysfunction in AD animal models and patients. Fibrillar Aβ deposits are found near disrupted neurites, in regions with reduced spine density, and in areas whit neuronal loss (Cohen et al., 2013). Furthermore, the microinjection of Aβ fibrillar in primate models of AD has shown their assembly in the cerebral cortex leading to neurodegeneration, neurofibrillary pathology, and neuroinflammation (Guela et al., 1998). However, eliminating fibrillar deposits may not fully restore synaptic function if toxic soluble oligomer populations persist. These differences are critical for therapy development, suggesting that interventions targeting oligomer neutralization might provide more immediate functional benefits than strategies focused only on removing fibrillar aggregates (Piers et al., 2018). In addition to the previously described fibrillar deposits, amyloid plaques in AD can be classified into dense-core (neuritic) plaques and diffuse plaques, which vary in morphology, composition, and pathological impact. Dense-core plaques feature a tightly packed fibrillar Aβ core that binds strongly to Thioflavin-S and Congo Red dyes; they are closely linked to dystrophic neurites, activated microglia, and adjacent tau pathology, which makes them highly neurotoxic (Serrano-Pozo et al., 2011). By comparison, diffuse plaques display loosely arranged, non-fibrillar Aβ deposits that fail to bind amyloid-specific dyes, lack a defined core, and show little to no associated neuritic degeneration, indicating they likely reflect earlier or less harmful phases of amyloid accumulation (Röhr et al., 2020; Thal et al., 2002). Apart from parenchymal plaques, Aβ also deposits within

cerebral vessel walls, resulting in cerebral amyloid angiopathy (CAA), a frequent AD comorbidity defined by Aβ1-40-enriched vascular accumulations that compromise vascular integrity, trigger inflammation, and elevate risk of microhaemorrhages and haemorrhagic stroke (Charidimou et al., 2017; Yamada, 2015). The concurrent presence of dense-core plaques, diffuse plaques, and CAA highlight the diverse characteristics of Aβ pathology and emphasizes the need for interventions targeting both parenchymal and cerebrovascular amyloid deposits.

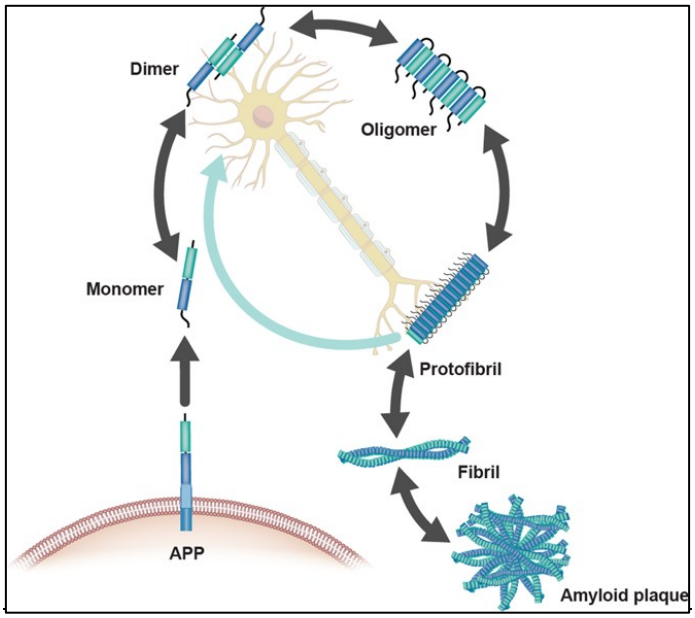


Figure 6: Amyloid-β aggregation states. The image shows the sequential aggregation of Aβ peptides. Initially, Aβ exists as monomers, which can associate to form dimers and then assemble into oligomers. These oligomers undergo further organization into protofibrils, which subsequently elongate and mature into fibrils. Finally, these fibrils aggregate to form dense amyloid plaques (from Hampel et al., 2021).

4.2 Neurofibrillary tangles

Neurofibrillary tangles (NFTs) are intraneuronal filamentous inclusions found in pyramidal neurons, playing a pivotal role in the onset and progression of AD, contributing to neurodegeneration and cognitive impairment (Busche & Hyman,

2020). NFTs are considered pathologic marker of AD development, and their presence in the brain is essential for disease diagnosis. The main component of tangles is the abnormal hyperphosphorylated and aggregated form of tau protein. Normally, tau is an abundant protein found in axons, which interacts with tubulin promoting the microtubules formation and stabilization as well as vesicle transport (X. Hong et al., 2025). Tau is a microtubule-associated protein (MAP) and plays a critical role in maintaining the structural integrity of neurons by stabilizing the microtubular cytoskeleton. The tau protein comprises three structural components: a C-terminal region that interacts with microtubules, a proline-rich region in the middle, and an N-terminal region known as the projection domain, which establishes interactions with other cellular components (Barbier et al., 2019). The classical epitopes bound by the AT8 and AT180 antibodies, commonly used in immunohistochemical studies to detect phosphorylated tau (p-Tau) protein, are located within the proline-rich region of tau (Hanger et al., 2009). In a normal physiological state, tau is regulated such that its phosphorylation by enzymes like mitogen-activated protein kinases (MAPKs), glycogen synthase kinase 3β (GSK3β), and cyclin-dependent kinase 5 (Cdk5) allows it to detach from microtubules (Chakraborty et al., 2024). In AD, this mechanism becomes dysregulated, tau protein undergoes aberrant hyperphosphorylation and becomes insoluble, loses affinity for microtubules and destabilizes the neuronal cytoskeletal network. This disruption impairs intracellular transport and culminates in synaptic dysfunction (Wu et al., 2021). Hyperphosphorylated tau self-assembles into paired helical filaments (PHFs) and leads to the formation of neurofibrillary tangles. In AD, NFTs initially appear in the medial temporal lobe, particularly within the entorhinal cortex. During disease progression, these abnormal misfolded tau protein aggregates then spread to the

limbic regions, notably the hippocampus, before eventually reaching the neocortex. This distinctive pattern of pathogenic propagation has a predictable course, starting in memory and learning-related brain regions and then spreading to additional cortical regions (Macedo et al., 2023). Phosphorylated tau (p-tau) epitopes are found also in CSF. They serve as accurate biomarkers for both the pathological and clinical diagnosis of AD and show increased levels during the preclinical stage of the disease (Suárez-Calvet et al., 2020).

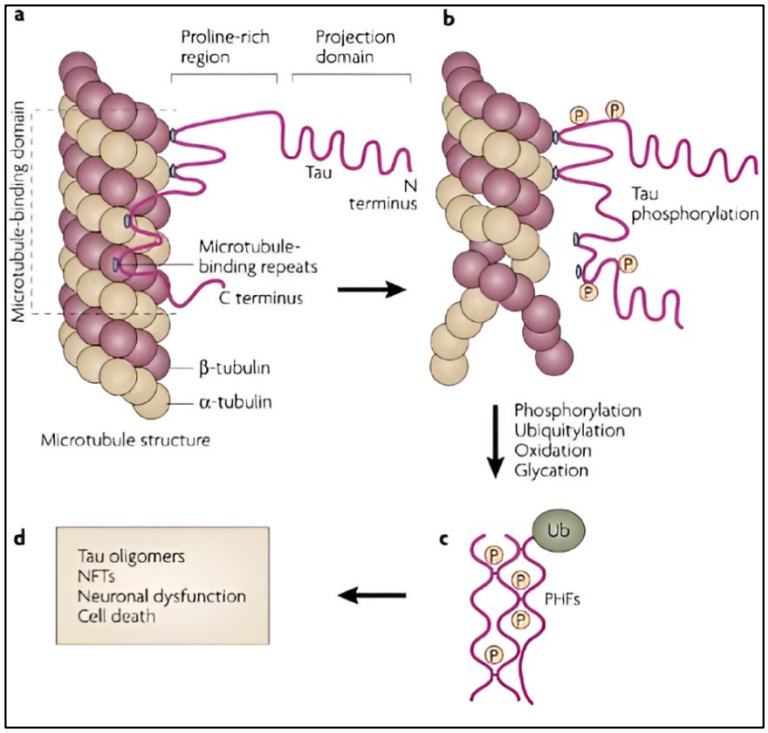


Figure 7. The phosphorylation of Tau protein. a) Microtubules, formed by repeating α- and β-tubulin dimers, constitute the neuronal cytoskeleton. Tau, the predominant microtubule-associated protein, maintains microtubule stability through its repetitive tubulin-binding domains, interlinked by flexible spacers. **b)** Kinases including GSK3β, CDK5, and ERK2 phosphorylate serine/threonine residues diminishing tau's microtubule affinity and inducing microtubule destabilization. **c**) Excessive or aberrant phosphorylation results in tau detaching from microtubules and assuming misfolded conformations that aggregate into paired helical filaments (PHFs). **d**) Subsequent aggregation yield tau oligomers and neurofibrillary tangles comprising heavily phosphorylated, β-sheet-enriched tau reinforced by disulfide bonds. These aggregates disrupt axonal transport, impair synaptic integrity, and promote neuronal death, whereas kinase inhibitors targeting tau can attenuate hyperphosphorylation and mitigate neurofibrillary tangle formation (from Mazanetz & Fischer, 2007).

4.3 Synaptic and neuronal loss

Synaptic loss is one of the earliest pathological features of AD and contributes to the cognitive impairment that characterizes Alzheimer's dementia. In AD, synaptic dysfunction is present at early stages of the disease, preceding amyloid plaque formation. Indeed, alterations in synaptic plasticity appear to be driven by dimers and trimers of amyloid peptides, which are produced during the early stages of the disease (Jeong, 2017). Significant synaptic toxicity is exhibited by Aβ-oligomers, which impair long-term potentiation, a crucial mechanism underlying memory formation, disrupt synaptic plasticity, and reduce neurotransmitter release. Furthermore, synaptic dysfunction is further exacerbated by hyperphosphorylated tau aggregates, which disrupting the neuronal cytoskeleton, alter synaptic structure and transport mechanisms (Luan et al., 2025). Synaptic loss in AD is typically evaluated by immunohistochemical methods using antibodies targeting pre-synaptic or post-synaptic proteins. A commonly analyzed pre-synaptic protein in research on synaptic loss in AD is synaptophysin, which is an integral membrane protein found on small synaptic vesicles in the brain. The immunohistochemical techniques for the study of synaptophysin have demonstrated that the number of synapses decreases as the disease progresses and correlates with cognitive decline (Subramanian et al., 2020). However, it remains unclear whether synaptic loss occurs before, after, or concurrently with neuronal loss. The progression of AD pathology is associated with significant and gradual neuronal loss, resulting in brain atrophy and shrinkage. Neuronal loss begins in the pre-clinical phase of the disease, prior to the formation of amyloid plaques and NFTs, then continues to worsen during the MCI and dementia (Arendt et al., 2015). Initial neuronal loss involves specific brain areas, such as the

entorhinal cortex, basal nucleus of Meynert, and locus coeruleus. Then neuronal loss spreads also in the hippocampus (especially CA1 and subiculum), amygdala, temporal lobe, frontal cortex, and parietal cortex (Goel et al., 2022). Several overlapping processes contribute to neuronal death, rather than a single pathway. Aβ oligomers disrupt synaptic function and alter glutamatergic signalling, leading to calcium dysregulation, mitochondrial dysfunction, and oxidative stress (Granzotto et al., 2020; Tu et al., 2014). Additionally, hyperphosphorylated tau in the dendrites and axons destabilizes microtubules and impairing axonal transport, further compromising vulnerable neural circuits. Neuroinflammatory processes also plays a key role in AD. Activated microglia and astrocytes respond to Aβ and tau pathology by releasing cytokines, reactive oxygen species (ROS), and activate the complement components that can tag synapses for elimination and create a toxic milieu (Jiang & Bhaskar, 2017).

4.4 Neuroinflammation

Neuroinflammation is now recognized as prominent hallmark of AD pathology, contributing to disease progression. Many studies have elucidated the pivotal role of inflammatory processes in the pathogenesis of AD (Heneka et al., 2015). Neuroinflammation, a pathological immune reaction within the central nervous system (CNS) triggered by damage to the CNS or peripheral tissues, results in the generation of inflammatory mediators including cytokines interleukin-1β [IL-1β], IL-6, IL-8, tumor necrosis factor-α [TNF-α]), chemokines, complement factors, and small molecule messengers (prostaglandins, NO, ROS), which collectively intensify the immune response and play a crucial role in the AD pathogenesis (Leng & Edison,

2021; Rubio-Perez & Morillas-Ruiz, 2012). Generally, acute inflammatory processes initially serve as a defensive response against injury to the nervous system, infection, and other noxious stimuli, instead persistent inflammation chronically stimulates the nervous system (C. Wang et al., 2023), releasing numerous pro-inflammatory cytokines and exacerbating the inflammation, and at the end causing neuronal damage and various pathological alterations (Stancu et al., 2019). Evidence indicate that sustained immune response within the brain is not only responsible for neurodegeneration, but also promote and amplify the pathological progression of Aβ and NFTs formation (Aranda-Abreu et al., 2026).

5. Neuronal and astrocyte dysfunction in Alzheimer's disease

In addition to the structural changes observed in AD brains, extensive research has focused on the cellular pathologies and neuronal damage that occur during disease progression (Brion, 2005). Neurons, the fundamental functional units of the nervous system, are among the cell types most severely affected in AD. These cells exhibit synaptic loss, accumulation of tau protein tangles, and deposition of Aβ peptides, leading to impaired communication, functional decline, and eventual neuronal death, particularly in the hippocampus and cerebral cortex (Brion, 2005; Mohandas et al., 2009). Even prior to the onset of clinical dementia, neurons demonstrate significant alterations, including hyperexcitability, elevated action potential firing, and heightened seizure activity. These neuronal changes are associated with increased calcium signaling and elevated glutamate release (Kazim et al., 2021; Targa Dias Anastacio et al., 2022). Further disruption in the endosomal system leads to the accumulation of Aβ and tau tangles, which contribute to neurodegeneration and indicate early critical events in the progression of AD (Nixon et al., 2000; R. Wang &

Reddy, 2017). Glial cells, particularly microglia and astrocytes, have dysregulated functions that potentially contributing to neuronal alterations in AD (Meda, 2001).

Astrocytes play a vital role in preserving the integrity and function of the BBB, regulating neurotransmitter uptake and clearance, modulating synaptic activity, maintaining ion homeostasis, and controlling inflammation (Filous & Silver, 2016). In AD, astrocytes are overactivated, characterized by cellular hypertrophy and increased glial fibrillary acidic protein (GFAP) expression. This reactive state compromises essential homeostatic functions, such as downregulation of astrocytic glutamate transporters, like EAAT2, which reduces glutamate clearance from the synaptic cleft, and directly contributes to neuronal hyperexcitability and excitotoxicity (J. Kim et al., 2024; Price et al., 2021). Reactive astrocytes show impaired clearance of amyloid-β through the glymphatic system and phagocytic mechanisms, subsequently transforming into a source of persistent neuroinflammation by secreting pro-inflammatory cytokines and ROS that further aggravating tau pathology (Escartin et al., 2021). Growing evidence suggests that astrocytes acquire specialized functional states that profoundly influence neurodegeneration during AD pathogenesis. Specifically, reactive astrocytes can polarize into either a neurotoxic A1 phenotype or a neuroprotective A2 phenotype. A1 astrocytes are triggered by pro-inflammatory cytokines from activated microglia, including as IL-1α, TNF-α, and C1q, and are manifesting as diminished homeostatic functions coupled with the release of mediators that actively promote synaptic deletion and neuronal death. In contrast, A2 astrocytes provide neuroprotective effects by elevating neurotrophic factors that support neuronal survival and tissue remodelling (Liddelow et al., 2017). However, transcriptomic and histopathological analyses of the AD brain indicate the prevalence of A1-like astrocytic signatures in regions heavily loaded with amyloid-β plaques and

tau pathology, thereby implicating astrocytes as active participants in driving neurodegeneration (Habib et al., 2020). Importantly, A1 astrocytes are marked by deficient glutamate clearance, altered calcium homeostasis, and diminished metabolic support for neurons, thereby intensifying the neuronal hyperexcitability and synaptic dysfunction evident in early disease stages. The imbalance between neurotoxic A1 and protective A2 astrocyte states makes worse and speeds up neuronal loss, therefore confirming the essential roles of glial-neuronal interactions in the progression and complexity of AD pathology (Dai et al., 2023; Srivastava et al., 2025).

6. Microglia

Microglia, as the principal innate immune cells within the CNS, serve as resident macrophages that monitor neural tissue and defend against infection and CNS injury. While sharing similarities with other tissue-resident macrophages in their functions, brain microglia constitute a distinct and highly specialized population of myeloid-derived cells (Sarlus & Heneka, 2017). Microglial precursors originate from the yolk sac during embryonic development, migrating to colonize the embryonic brain, where specific brain-derived signals guide their unique differentiation. Following their initial colonization of the brain, microglia self-renew to repopulate and maintain their population under homeostatic conditions (Q. Li & Barres, 2018; Tong & Vidyadaran, 2016). As the guardians of the CNS, microglia exhibit a distinctive ramified morphology and perform critical roles in synaptic pruning during neural circuit development, phagocytosing debris and dying cells in response to foreign material or damage, and maintaining overall neural homeostasis (Morrison et al., 2023; Salter & Stevens, 2017). In order to carry out these activities and regulate healthy cerebral activity, microglia are able to monitor the brain parenchyma and respond quickly to

changes in brain homeostasis due to the highly branched cellular projections that extend from the soma (Kofler & Wiley, 2011). However, microglia exhibit a wide and dynamic range of activation states in response to changes in the brain parenchyma, rapidly transforming from a ramified to an amoeboid shape and modifying their intracellular metabolic processes, cytokine and chemokine secretion profiles, and functional capacities to align with the requirements of the surrounding tissue (Colonna & Butovsky, 2017).

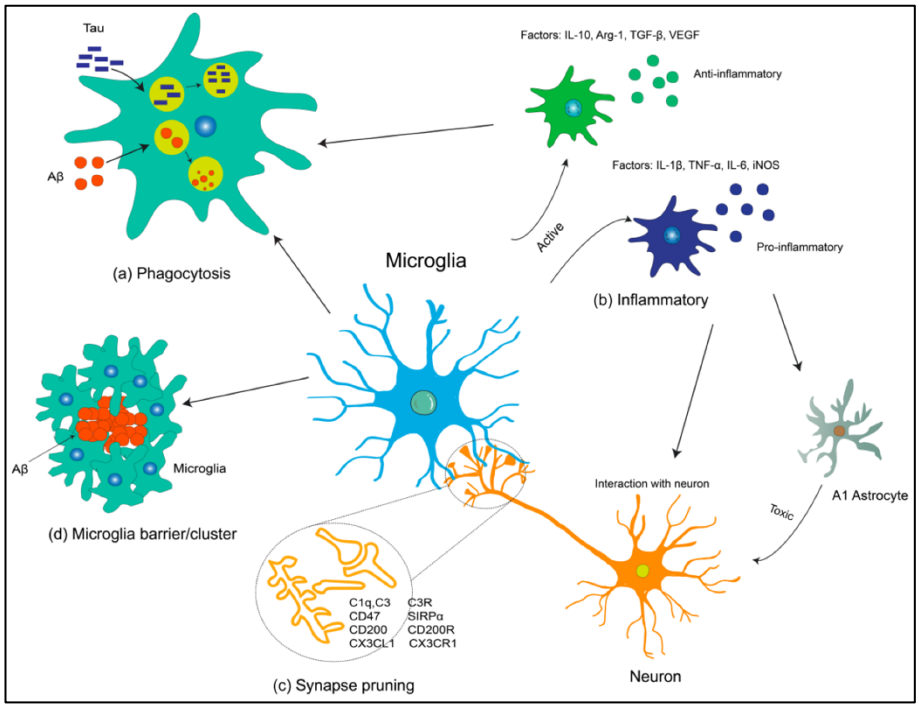


Figure 8. Microglia functions in AD. a) Microglia play a protective role by removing harmful Aβ and tau proteins through phagocytosis. **(b)** Activated microglia can exhibit either pro-inflammatory or anti-inflammatory effects by secreting cytokines. Pro-inflammatory cytokines can activate A1 astrocytes, which are toxic to neurons. Microglia interact with neurons directly or indirectly through physical contact, receptor-ligand binding, and the release of soluble factors like TNF-α, IL-1β, BDNF, and IL-6. **c)** Microglia are involved in the loss of synapses, by synaptic pruning. **d)** Microglia migrate and cluster around amyloid plaques, forming a physical barrier to prevent the spread of Aβ (from Sun et al., 2024).

6.1 The role of microglia in neuroinflammation

Neuroinflammation is primarily driven by glial cells, such as microglia and astrocytes, which play a vital role in maintaining homeostasis within the CNS. Under normal conditions, these cells actively survey the CNS, supporting the functional integrity and viability of neurons (Farooqui, 2017). However, when brain homeostasis is disrupted, both microglia and astrocytes actively participate in the inflammatory response. Through cellular communication, they collaborate to address harmful stimuli and contribute to the resolution of these threats (Y. Wang et al., 2022). Microglia, the highly mobile innate immune cells that constitute approximately 10% of the brain's total cellular population, are pivotal in the process of neuroinflammation. These cells not only initiate the inflammatory response but also recruit peripheral immune cells (Morimoto & Nakajima, 2019). Although neuroinflammation is frequently associated with pathological consequences, particularly in the context of prolonged injury or chronic disorders, it also plays a crucial role in positive physiological processes, such as neurorepair and tissue recovery (W.-W. Chen et al., 2016). In response to pathological stimuli, microglia exhibit dynamic morphological transformations, shifting from a ramified to an amoeboid state (Nimmerjahn et al., 2005). Upon activation, these immune cells release a variety of pro-inflammatory signalling molecules, including cytokines, chemokines, and reactive oxygen species (Kinney et al., 2018). These mediators are crucial for combating pathogens and clearing cellular debris. However, excessive or prolonged microglial activation can lead to detrimental consequences, such as neuronal damage and the progression of CNS disorders (Y. Wang et al., 2022). This chronic activation is a hallmark of many neurodegenerative diseases, including AD, Parkinson's disease, and multiple sclerosis, where persistent neuroinflammation

exacerbates neuronal dysfunction and loss. Historically, microglial activation was described in binary system based on peripheral macrophage nomenclature, where cells were classified into a pro-inflammatory (M1, 'classical activation') or an anti-inflammatory (M2, 'alternative activation') phenotype (Guzman-Martinez et al., 2019; Orihuela et al., 2016). In this historical context, the M1 state was associated with neurotoxic production of cytokines (e.g., TNF-α, IL-1β, IL-6), NO, and ROS induced by stimuli such as Toll-like receptor ligands, interferon-γ (IFN-gamma), LPS, and aggregated Aβ, thereby promoting pathological synaptic pruning and neuronal apoptosis (Colonna & Butovsky, 2017). On the other hand, M2 state was profiled by anti-inflammation signalling (e.g. IL-10 and TGF-β), enhanced phagocytosis, and release of neurotrophic factors aiding tissue repair along with waste clearance (S. Hong et al., 2016; Tang & Le, 2016). However, consensus frameworks in neuroimmunology have shown the irrelevance of such strictly defined M1/M2 dichotomy and ultimately highlighted its fundamentally insufficient description of microglial biology *in-vivo* (Paolicelli et al., 2022). In contrast to peripheral macrophages, microglia undergo ongoing transcriptomic changes that are continuous and plastic across multiple dimensions, not biphasic or polarized. Recent advances in single-cell RNA sequencing and spatial transcriptomics have uncovered an unprecedented heterogeneity of microglial subpopulations and spatial-temporal adaptations (Fumagalli et al., 2025; Ochocka & Kaminska, 2021). This paradigm shift is exemplified by the identification of a particular microglial cell state present in neurodegenerative diseases, alternatively termed Disease-Associated Microglia (DAM) or Microglia Neurodegenerative (MGnD) phenotype (Keren-Shaul et al., 2017; Krasemann et al., 2017). The transition to a DAM/MGnD state is gradual, consisting of two sequential programs: an initial TREM2-independent downregulation

of homeostatic genes (e.g., P2ry12, Cx3cr1, and Tmem119), followed by a TREM2-dependent upregulation of phagocytic, lipid metabolism, and survival-related genes (e.g., Apoe, Cst7 and Spp1) (Keren-Shaul et al., 2017). Instead of being fixed as purely 'detrimental' or 'beneficial, these contemporary complex phenotypes display functional dualities where beneficial control over Aβ plaques in early disease phases changes to chronic, activated but dysfunctional neuroinflammation in advanced stages of neurodegenerative decline.

6.2 Microglia in AD

The traditional M1/M2 framework, previously a useful metaphor for comprehending microglial activation, is significantly altered in AD. Instead of a simple binary transition, single-cell transcriptomics confirms that AD pathology induces a multifaceted development into specialized context-dependent states, particularly DAM (Hansen et al., 2018). Similarly, the emergence of a DAM phenotype occurs in a clearly temporally regulated sequence during amyloid pathology (as previously introduced). Specifically, the first step describes an early TREM2- independent switch involving a loss of homeostatic functions and down-regulation of matching marker genes. Directly after, the 'second step' is entirely TREM2 dependent, whereby microglia actively upregulate lipid metabolism and high-capacity phagocytosis genes to reduce plaque growth. While transient DAM activation helps protect and consolidate plaques in the early stages of the disease, persistent and unresolved DAM activation ultimately contributes to chronic inflammation, which exacerbates tau-induced neurodegeneration (Keren-Shaul et al., 2017; Leyns & Holtzman, 2017). However, dysregulation of microglial plasticity in AD pushes into persistently pro-

inflammatory and maladaptive states which perpetuate cycles of neuroinflammation and tissue damage (DiSabato et al., 2016). Pathogenic microglial responses in AD do not represent discrete activation programs but are due to a lack of resolution of inflammatory signalling. Therapeutic approaches that restore microglial homeostasis, reduce chronic inflammation and enhance reparative function are a promising strategy to counteract neurodegeneration caused by prolonged immune activation.

Microglia play a crucial role in the onset and progression of AD, acting as key regulators of homeostatic balance and responding to pathological changes in the brain (Michelucci et al., 2018). In AD, microglia migrate toward Aβ deposits, where they become activated (Condello et al., 2015; Fang et al., 2018). This activation leads to a shift towards a pro-inflammatory phenotype, triggering both acute and chronic immune responses (Spittau, 2017). Initially, microglia attempt to clear Aβ plaques through phagocytosis, releasing inflammatory cytokines and ROS. While this initial response may be protective, prolonged activation of microglia results in chronic neuroinflammation, which exacerbates neuronal damage and drives disease progression (C. Wang et al., 2023). Genetic studies have further implicated several key microglial genes, such as *TREM2* and *CD33*, in elevating the risk of AD highlighting the crucial role of microglial dysfunction in the disease's pathogenesis (McQuade & Blurton-Jones, 2019). Dysfunctional microglia may fail to effectively clear Aβ plaques, contributing to their accumulation. Furthermore, by promoting tau hyperphosphorylation and the development of neurofibrillary tangles (NFTs) via inflammatory pathways, microglia contribute significantly to tau pathology (Bachiller et al., 2018; Perea et al., 2018).

The recently recognized microglial subtype called DAM has offered invaluable insights into the role of microglia in AD. DAMs, initially observed in the 3xTg-AD

and 5xFAD mouse models, demonstrate a disease-related increase in the inflammatory profile alongside significant alterations in phagocytic, inflammatory, and iron-regulation pathways (Rudan Njavro et al., 2022; Yuan et al., 2020). Microglia exhibit a multifaceted influence in AD, functioning as both neuroprotective and neurodegenerative agents dependent on the disease progression (Franco-Bocanegra et al., 2021; Hemonnot et al., 2019). This inherent complexity emphasizes the importance of microglia as a critical target for therapeutic interventions aimed at modulating their activity to delay or prevent the advancement of AD.

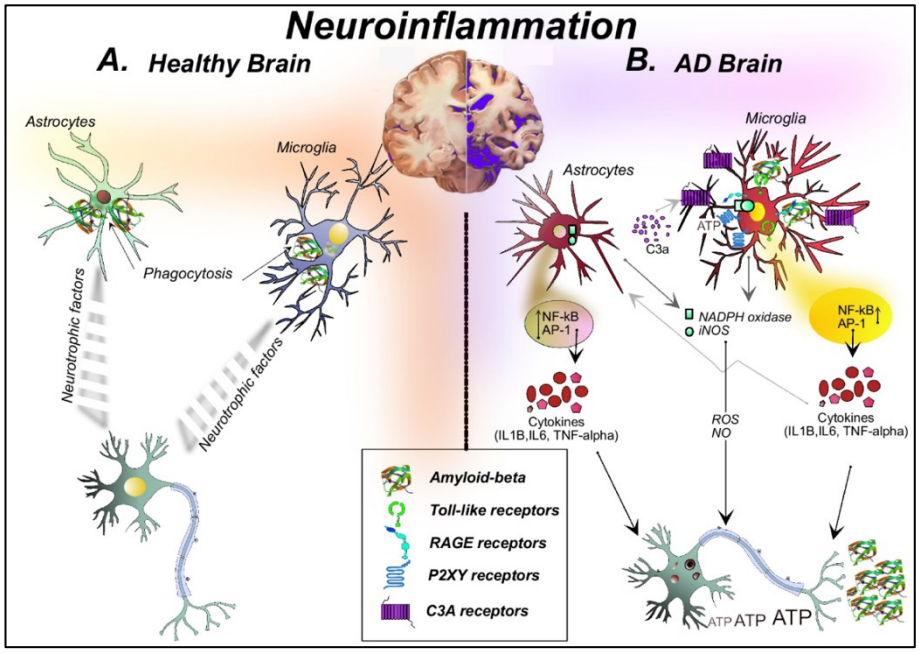


Figure 9. Neuroinflammation in the healthy and AD brain. a) In the healthy brain, microglia and astrocytes maintain neuronal homeostasis by clearing Aβ aggregates and providing neurotrophic support. **b)** The AD brain is characterized by a high burden of Aβ aggregates. The impaired phagocytosis of Aβ and the exaggerated inflammatory response by microglia and astrocytes lead to the accumulation of amyloid plaques. These Aβ aggregates bind to pattern recognition receptors (PRRs) on microglia, triggering the production of cytokines, which in turn contribute to the activation of astrocytes and the induction of neurotoxicity. Additionally, the binding of Aβ aggregates to microglia and astrocytes stimulates the production of reactive oxygen species (ROS) and nitric oxide (NO),

further exacerbating neurotoxicity. The disrupted crosstalk between neurons, astrocytes, and microglia leads to an imbalance in brain homeostasis and promotes neurodegeneration (from Singh, 2022).

7. Natural compounds targeting microglial activation in AD

Recognizing the pivotal role of microglia in exacerbating neuroinflammation and disease progression in AD, current research efforts are increasingly focused on identifying novel therapeutic interventions capable of modulating microglial activation.

7.1 Natural products as a source of therapeutic molecules in AD

Historically, natural products have been integral to traditional medicinal practices for addressing a broad range of diseases and disorders. These remedies typically comprise complex combinations of bioactive constituents, each contributing to their overall pharmacological efficacy, with the foundational concept often centred on the maintenance of vital energy and health (A. Zhang et al., 2011). In contemporary pharmacological research, there has been a notable resurgence of interest in the development of therapeutics derived from natural origins, a trend substantiated by the significant observation that approximately one-third of small-molecule drugs authorized by the U.S. Food and Drug Administration (FDA) between 1981 and 2010 were either directly isolated from or inspired by natural product frameworks (Newman & Cragg, 2012). Natural compounds are highly prized in modern research for their exceptional structural diversity, which enables them to simultaneously affect multiple pharmacological targets, a significant advantage in addressing complex, multifactorial diseases such as AD. A variety of plant-derived phytochemicals,

including flavonoids, alkaloids, and saponins, have demonstrated significant anti-inflammatory and neuroprotective properties relevant to AD pathophysiology (Deng et al., 2023). Specific examples highlight this focused effectiveness against fundamental neurodegenerative processes. Flavonoids, including Quercetin and Kaempferol, have shown the ability to prevent Aβ aggregation, mitigate oxidative stress through the activation of the Nrf2/HO-1 pathway, and attenuate neuroinflammation by promoting an anti-inflammatory phenotype in microglia (Zamanian et al., 2025). For instance, the alkaloid Berberine demonstrates anti-inflammatory effects in primary microglial and BV2 cells by inhibiting NF-κB and MAPK signalling, thereby reducing pro-inflammatory mediators including IL-6, MCP-1, COX-2, and iNOS (Jia et al., 2012). Similarly, Saponin Ginsenoside C-K (Compound K) attenuated Aβ-induced inflammation in BV-2 microglia in vitro, by inhibiting NF-kB activation and cytokine secretion (TNF-α, IL-1β), while protecting HT22 neuronal cells from Aβ toxicity (C. Xie et al., 2025). Collectively, these findings indicate the capacity of natural compounds to modulate key pathological mechanisms associated with AD, such as oxidative stress, neuroinflammation, and neuronal injury. A range of natural compounds have demonstrated efficacy in attenuating LPS-induced microglial activation, a key contributing element to neuroinflammation linked to neurodegenerative pathologies. For instance, curcumin has been shown to inhibit LPS-induced inflammatory cytokine production and oxidative stress within BV2 microglial cells through the miR-137-3p/NeuroD1 signalling pathway (Gao et al., 2019). Isoflavone formononetin attenuated LPS-induced pro-inflammatory cascades while elevating estrogen receptor β expression in BV2 microglia (El-Bakoush & Olajide, 2018). Triptolide exhibited substantial suppression of TNF-α, IL-1β, and nitric oxide production even in primary microglial

cultures, thereby indicating potent anti-inflammatory properties (H.-F. Zhou et al., 2003). Another compound, panduratin A substantially reduced LPS-induced activation of BV2 microglia, encompassing both NO and inflammatory cytokine release (Jamornwan et al., 2022). Several natural products demonstrate anti-inflammatory and neuroprotective attributes within Aβ-activated models. A critical analysis of secondary plant metabolites revealed that compounds such as epigallocatechin-3-gallate, luteolin, myricetin, and silibinin acted as potent inhibitors of Aβ fibril formation in Thioflavin-T assays and enhanced behavioural and histopathological outcomes in transgenic mouse models of AD (Stefanescu et al., 2020). Furthermore, insights from medical plant science highlight the utility of phytochemicals possessing acetylcholinesterase inhibitory activity, underscoring their significance in counteracting Aβ-induced pathology (Tan & An, 2020). Aromatic Turmerone, isolated from Curcuma longa, effectively suppressed Aβ-induced expression of iNOS, cyclooxygenase-2, matrix metalloproteinase-9, and inflammatory cytokines in microglia by inhibiting the activation of NF-kB, JNK, and p38 MAPK pathways, thereby safeguarding HT22 neurons from microglial toxicity (S. Y. Park et al., 2012). These observations are further supported by *in vivo* evidence, which highlights the capacity of natural products to ameliorate neuropathological alterations and cognitive deficits in animal models of AD. Some evidence demonstrate that plant extracts enriched with polyphenols, flavonoids, terpenoids, and other bioactive compounds, such as Ginkgo biloba extract, curcumin formulations, and various herbal blends are effective in suppressing Aβ plaque formation, preventing the degradation of synaptic markers, and enhancing memory performance in AD animal models, including APPSwe/PS1dOE, 5xFAD, and 3xTg-AD mice (Andrade et al., 2019; X. Chen et al., 2021; Da Rosa et al., 2022). In fact, Sabogal-Guáqueta et al. demonstrated

that the oral administration of quercetin (25 mg/kg) in aged (18-21 months old) 3xTg-AD mice significantly diminished β-amyloid levels, reduced tau protein phosphorylation, and suppressed glial cell activation within the hippocampus and amygdala, concurrently improving learning ability and emotional function (Sabogal-Guáqueta et al., 2015). Taken together, these results highlight the therapeutic promise of natural products for AD and emphasize their increasing importance in modern pharmaceutical development.

7.2 Screening Italian plants for anti-inflammatory and neuroprotective effects

This study is conducted in the context of the National Biodiversity Future Center (NBFC), a major national research initiative supported by the PNRR (Piano Nazionale di Ripresa e Resilienza). This is a national project that focuses on exploring and integrating Italian biodiversity for applications in human health (Cena & Labra, 2024). This interdisciplinary study focuses on bioprospecting specific Italian plant species to uncover natural compounds with possible anti-inflammatory and neuroprotective properties relevant to microglial regulation in AD. The objective of this work is to assess the capacity of plant-derived extracts to modulate microglial activation under inflammatory conditions. Focus will be targeted on identifying non-toxic natural compounds and evaluating their effects on cytokine secretion and NO release in microglial cells, alongside their potential neuroprotective activity in a neuroblastoma cell line. The current study wants to evaluate these natural extracts from Italian flora to modulate microglial responses to inflammatory stimuli, thereby identifying compounds with novel neuroinflammatory effects. These extracts were

obtained using a methanolic extraction procedure (as detailed in the Materials and Methods section), a standard technique chosen to ensure the efficient recovery of a broad spectrum of bioactive constituents. The plant panel included: Althaea officinalis, Salvia pratensis, Petasites paradoxus, Adenophora lilifolia, Allium lusitanicum, Dianthus superbus, Succisa pratensis, Typha laxmannii, Acalypha virginica, Actinidia deliciosa, Allium angulosum, Cistus monspeliensis, Diospyros kaki, Gratiola officinalis, Heuchera sanguinea, Lythrum salicaria, Sanguisorba officinalis, Verbascum thapsus, Artemisia abrotanum, Glandularia tenera, Malva alcea, Pulsatilla montana, and Rosa canina.

To evaluate biological activity of natural extracts in microglia, we utilized: (1) SIM-A9 cells, an immortalized microglial line derived from postnatal C57BL/6J mouse cortex, for initial screening of viability and inflammatory responses; and (2) primary microglia isolated from neonatal 3xTg-AD mice. Initially, cell viability studies were conducted to establish non-cytotoxic concentrations of the extracts. Then, we evaluate the capability of the natural extracts to modulate the release of pro-inflammatory cytokines. Instead, the N2A neuroblatoma cell line was used to assess the neuroprotective effects of natural extracts against glutamate-induced neurotoxicity. This research expands our comprehension of plant-derived compounds as modulators of microglial activation and neuroinflammation. Based on this screening, four extracts: Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis were selected as the best candidates with anti-inflammatory properties.

7.3 The properties of the four selected plants

7.3.1 Althaea officinalis

Althaea officinalis, commonly known as marshmallow, denotes a perennial herbaceous species of the Malvaceae family, with a native range encompassing regions of Europe, North Africa, and Western Asia (Khalighi et al., 2021). Traditionally, various part of the plant including roots, leaves, and flowers has been utilized in the treatment of respiratory ailments, gastritis, dermatological conditions, and inflammatory diseases. Phytochemical analyses have identified that Althaea officinalis contains a diverse range of bioactive constituents, including mucilage, flavonoids, triterpenes, phenolic acids, essential oils, tannins, and amino acids, which all contribute to its recognized therapeutic properties (Banaee et al., 2017; Gudej, 1991). Most pharmacological study has concentrated on extracts derived from Althaea officinalis roots and flowers. Root extracts exhibit significant anti-inflammatory and antioxidant properties, notably by inhibiting the generation of TNF-α, IL-6, and ROS in LPS-stimulated monocytic and endothelial cell lines (Bonaterra et al., 2020, 2022). Flower extracts have demonstrated anti-carcinogenic properties in a rat model of hepatocellular carcinoma, presumably through the attenuation of oxidative and inflammatory damage (Z. Wang et al., 2023). Further research indicates that Althaea officinalis formulations exhibit protective and immunomodulatory effects in models of respiratory infections, stomach ulcers, and nephrotoxicity, underscoring the medicinal flexibility of the species (Hage-Sleiman et al., 2011; Šutovská et al., 2009; Talebi et al., 2014). Conversely, Althaea officinalis leaf extracts remain minimally studied, especially concerning its impact on the central nervous system. A key exception is the research conducted by Rezaei and Alirezaei, which illustrated that Althaea officinalis leaf extract provided neuroprotective effects in a 6-hydroxydopamine rat model of Parkinson’s disease by mitigating lipid peroxidation,

enhancing behavioural outcomes, and preserving substantia nigra neurons from dopaminergic degeneration (Rezaei & Alirezaei, 2014). Despite these results, no prior research has assessed Althaea officinalis leaf extract in microglia or in animal models of AD. Consequently, our research constitutes the first-ever systematic assessment of Althaea officinalis leaf extract on microglial activation (SIM-A9 and 3xTg-AD primary microglia) and neuronal resilience to glutamate toxicity, thereby enhancing the existing comprehension of Althaea officinalis beyond its conventional applications and previously documented Parkinson's-related neuroprotection.

7.3.2 Adenophora lilifolia

Adenophora lilifolia, often known as lilyleaf ladybell, is a perennial herb from the Campanulaceae family that grows throughout Europe and parts of Asia (Farkas, 2021; Kovács et al., 2024). Although no studies have examined the biological activity of Adenophora lilifolia, closely related species within the Adenophora genus or chemically analogous genera have exhibited significant anti-inflammatory and antioxidant properties, indicating a substantial potential for the development of novel therapeutic agents. For instance, extracts from Eupatorium adenophorum leaves (EASL-AE) were demonstrated to protect human intestinal Caco-2 cells from tert-butyl hydroperoxide (t-BHP)–induced oxidative and inflammatory damage by diminishing intracellular ROS, downregulating NF-κB signaling, and inhibiting pro-inflammatory cytokines such as TNF-α and IL-1β (Zheng-qiang et al., 2024). Similarly, the compound cryptochlorogenic acid (CCGA), derived from Ageratina adenophora, exhibited potent anti-inflammatory effects in LPS-stimulated RAW264.7 macrophages via blockade of the IKK/IκB/NF-κB pathway, alongside reductions in NO, iNOS, cyclooxygenase-2, and pro-inflammatory cytokines (TNF-α, IL-1β, IL-6,

IL-8) (Ma et al., 2022). These results are relevant to our research, since they illustrate how analogous plant derivates can influence inflammatory pathways in microglial activation. Additional evidence is provided by Adenophorae radix powder (roots of Adenophora triphylla), which demonstrated anti-inflammatory properties in mouse models by suppressing allergic acute inflammation and symptoms of xylene-induced contact dermatitis (Hu et al., 2019). These findings underscore the broader anti-inflammatory potential across the Adenophora lineage. Adenophora species have been extensively utilized in East Asian medicine for the treatment of chronic cough, bronchitis, and respiratory inflammation (M.P, 2010; W. S. Park et al., 2019). Chemical analyses of the genus indicate the presence of saponins, flavonoids, triterpenes, alkaloids, and polysaccharides (M. Zhang et al., 2021), which correspond to the bioactive components found in numerous anti-inflammatory natural products. No studies examine the use of Adenophora species in the mitigation of inflammatory state associated to neurodegenerative disorders, thereby emphasizing the novelty of the present study with respect to its effects on microglial function and AD-associated neuroinflammation.

7.3.3 Dianthus superbus

Dianthus superbus, also known as fringed pink, belonging to the Caryophyl-laceae family, is native to Europe, Asia, and parts of Russia (D. H. Kim et al., 2019). This plant is valued not only for its delicate and fragrant flowers but also for its medicinal properties. Historically, this plant, known as 'Qumai' in traditional Chinese medicine, has been extensively utilized in herbal healing practices across China, Korea, Iran, and Mongolia (López-Expósito et al., 2011; J.-Q. Yu et al., 2012). It is particularly esteemed for its diuretic, anti-inflammatory, and detoxifying properties, which make it

an effective treatment for urinary tract disorders, such as infections, kidney stones, and edema (Y.-C. Wang et al., 1998; J.-Q. Yu et al., 2012). The plant is composed of diverse pharmacologically active compounds, such as triterpenes, saponins, flavonoids, glycosides, antho-cyanins, and peptides (Shimizu et al., 1982; Yun et al., 2016b). Despite its historical significance in traditional medicine, modern science is now actively investigating its mechanisms. Among these, Maltol glycoside within Dianthus superbus has been shown to suppress NO production and inducible nitric oxide synthase (iNOS) expression in LPS-stimulated RAW264.7 murine macrophages (Yuan et al., 2022).

More significantly for the neuroimmunology field, newly discovered phytoecdysteroids from Dianthus superbus potently suppressed LPS-induced NO production in BV2 microglia, and molecular docking analyses indicating potential direct interactions with neuroinflammatory signaling pathways (Hou et al., 2023). This discovery is significantly important to our research as it presents the initial proof that Dianthus superbus comprises chemicals capable of influencing microglial activation, the primary trigger of neuroinflammation in AD. However, despite this encouraging report, Dianthus superbus has yet to be examined in AD-associated microglial models or assessed for anti-neuroinflammatory activity in primary microglia. Beyond microglial modulation, Dianthus superbus exhibits promising neuroprotective effects. Compounds derived from Dianthus superbus effectively protected HT22 hippocampal neurons against glutamate-induced oxidative stress, a validated model of excitotoxicity pertinent to early AD pathology, especially hippocampal atrophy. As the hippocampus is one of the earliest regions impacted in AD (Rao et al., 2022), the capacity of Dianthus superbus-derived molecules to inhibit glutamate-mediated neuronal death underscores their therapeutic promise and

indicates that Dianthus superbus may regulate key pathways underpinning neuronal viability. Despite promising findings, Dianthus superbus is largely unexplored in neurodegenerative diseases, especially AD. No research has investigated Dianthus superbus extracts in microglial systems related to amyloid-induced inflammation, nor evaluated its combination anti-inflammatory and neuroprotective effects.

7.3.4 Succisa pratensis

Succisa pratensis or devil's-bit scabious, is a perennial plant initially categorized in the Dipsacaceae family and now part of Caprifoliaceae, distributed across Europe, Asia, and Africa (Scabious, 2024). Phytochemical investigations have revealed the presence of diverse bioactive compounds, including flavonoids, phenolic acids, triterpenes, essential oils, and saponins (Kowalczyk, 1999; Witkowska-Banaszczak & Długaszewska, 2017). Of these, essential oils and hydrophilic compounds from the leaves and flowers extracts of SP exhibit *in vitro* antimicrobial activity against Gram-positive and Gram-negative bacteria (Witkowska-Banaszczak & Długaszewska, 2017), while flavonoids derived from the plant have demonstrated anti-inflammatory effects through modulation of NF-κB signaling pathways in HepG2 human hepatocellular carcinoma cells (Witkowska-Banaszczak et al., 2020), indicating anti-inflammatory potential in vitro study. Many studies on Succisa pratensis have focused on its ecological adaptability, conservation, and general phytochemistry, rather than biomedical applications. No studies examined Succisa pratensis effects in contexts related to neuroinflammation. Therefore, our research reveals for the first time the study of Succisa pratensis in microglial and neuronal models. Succisa pratensis has been recently studied in relation to brain-related disorders. Recently, Giammona et al., showed that Succisa pratensis leaf extracts have strong anti-cancer effects on

glioblastoma (GBM) cells by inhibition of proliferation, restriction of migration, alteration of oxidative stress responses, and facilitation of metabolic shifts (Giammona et al., 2024). Although this study was conducted in a neoplastic setting instead of a neurodegenerative condition, the results still show that Succisa pratensis has bioactive molecules that can change cellular pathways that are important for keeping neural tissue stable, like oxidative stress, cytoskeletal organization, and signalling related to inflammation. Contrarily to Succisa pratensis, related species within the Caprifoliaceae family, such as Lonicera japonica and Lonicera caerulea, have exhibited *in vitro* anti-inflammatory effects by reducing TNF-α and IL-6 production in LPS-activated RAW264.7 macrophages (C. Li et al., 2024; Lin et al., 2021).

a) Althaea officinalis **b) Adenophora lilifolia**

c) Dianthus superbus **d) Succisa pratensis**

Figure 10. The four selected plants in our project (NBFC): **a)** Althaea officinalis, **b)** Adenophora lilifolia, **c)** Dianthus superbus, and **d)** Succisa pratensis.

AIM OF THE STUDY

The purpose of this study was to identify new plant-derived extracts with anti-inflammatory and neuroprotective potential relevant to Alzheimer's disease, using *in vitro* cellular models. This objective was achieved through a systematic and stepwise approach as follows:

1. Screening and Safety Assessment

- Systematically screen various plant extracts to identify and eliminate cytotoxic candidates.
- Select non-toxic extracts for further investigation.

2. *In Vitro* Evaluation of Anti-Inflammatory Properties

- Evaluate the anti-inflammatory activity of selected extracts on Lipopolysaccharide (LPS) and Amyloid-beta (Aβ 1-42, Aβ 25-35) stimulated microglia to mimic the inflammatory conditions.
- Determine the inhibition of inflammatory mediators, such as NO and pro-inflammatory cytokines (TNF-α and IL-6).

3. *In Vitro* Evaluation of Neuroprotective Effects

- Determine the neuroprotective potential of the selected extracts against glutamate-induced neurotoxicity in neuronal cell culture.

Overall Goal

The main goal of this research was to identify safe plant-derived compounds that exhibit both anti-inflammatory and neuroprotective effects in cell-based model systems. This work aimed to emphasize their potential as candidates for future therapeutic strategies to affect neuroinflammation, which is commonly associated with neurodegenerative disorders, such as AD.

MATERIALS AND METHODS

1. Reagents

The SIM-A9 murine microglial cell line was acquired from the American Type Culture Collection (ATCC® CRL-3265TM, Manassas, VA, USA). The Dulbecco's Modified Eagle Medium F12 (DMEM-F12; CAT. N-L0090) culture medium was obtained from Biowest, France. L-glutamine (Catalog No. 25-015-CI_24123012) and Penicillin-Streptomycin (P/S; Catalog No. 30-002-CI) were purchased from CORNING, United States. Additional reagents, including Dimethyl sulfoxide (DMSO; Catalog No. D8418), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder (Catalog No. M5655-1G), Fetal Bovine Serum (FBS; Catalog No. F7524), and Horse Serum (H/S; Catalog No. H1270), Trypsin-EDTA, Sodium Nitrite ($NaNO_2$; Catalog No. 237213-BULK) were obtained from Sigma-Aldrich.

2. Plant material and extract preparation

A total of twenty-three methanolic extracts from naturally derived plant leaves, including Althaea officinalis, Salvia pratensis, Petasites paradoxus, Adenophora lilifolia, Allium lusitanicum, Dianthus superbus, Succisa pratensis, Typha laxmannii, Acalypha virginica, Actinidia deliciosa, Allium angulosum, Cistus monspeliensis, Diospyros kaki, Gratiola officinalis, Heuchera sanguinea, Lythrum salicaria, Sanguisorba officinalis, Verbascum thapsus, Artemisia abrotanum, Glandularia tenera, Malva alcea, Pulsatilla montana, and Rosa canina, originating from Italian flora, were prepared by Prof. Flavia Guzzo’s laboratory (Department of Biotechnology,

University of Verona) within the framework of the PNRR _ National Biodiversity Future Center (NBFC) project. The plant species were purchased from the institutional nursery "Veneto Agricoltura" (Montecchio Precalcino, Vicenza, Italy) in collaboration with Prof. Guzzo's laboratory and cultivated in the greenhouse facility of the University of Verona until flowering arrived. Vegetative aerial organs (leaves and young stems) were collected from two individual plants per species and combined, and the same extraction procedure was applied to all twenty-three plant samples. Fresh material was immediately frozen in liquid nitrogen, ground to powder with an A11 basic analytical mill (IKA-Werke, Staufen, Germany) and stored at −80 °C. Approximately 1 g of frozen powder underwent extraction using 10 volumes (w/v) of 100% LC-MS grade methanol (Honeywell, Seelze, Germany). The sample was vortexed for 30 s, sonicated on ice for 10 min in a 40 kHz ultrasonic bath (SOLTEC, Milano, Italy), then centrifuged at 14,000× g for 10 min at 4 °C. The supernatant was concentrated via speed-vac system (Heto-Holten; Frederiksborg, Denmark). The dried extract was re-solubilized in an equivalent volume of ethanol and characterized by UPLC-ESI-HRMS analysis.

3. Phytochemical profiling of plant extracts

Each extract was diluted 1:20 in LC-MS-grade water (Honeywell) and filtered through 0.22 µm Minisart filters (Sartorius-Stedim Biotech, Göttingen, Germany). Phytochemical profiling was conducted by untargeted metabolomics by injecting 1.2 and 10 µL samples into the UPLC-ESI-HRMS system, operated in FAST-DDA negative and positive ionization modes using the previously described instruments and methods (Bianchini et al., 2025). Three orthogonal parameters, i.e., the accurate mass (deduced from the m/z ratio and isotopic pattern), retention time, and

fragmentation pattern (from the FAST-DDA analysis), were utilized for the tentative identification of phytochemicals for each extract and compared against a proprietary library of authentic standard compounds, an in silico proprietary library of plant compounds, and existing scientific literature and public databases (e.g., Pubchem, MoNA, MassBank, Human Metabolome Database). In cases where specific information was unavailable, metabolites were putatively identified using Metfrag for in silico analysis, querying the PubChem database with experimental MS data. The positive ionization mode analysis was exclusively employed to validate molecular ions initially detected in the negative ionization mode.

4. Preparation of plant extracts for cellular assays

The dried plant extracts (10 mg each) were resuspended in dimethyl sulfoxide (DMSO) to achieve a final concentration of 50 mg/mL for subsequent cellular tests. Each extract was reconstituted in 50 µL of DMSO and vortexed for 10 seconds under sterile conditions. This process was repeated with another 50 µl of DMSO, and the mixture was vortexed for an additional 10 seconds. The samples were then incubated in a water bath at 37 °C for 5 minutes, followed by vortexing for 6 to 10 seconds; this process was done three times. The procedure was reiterated with the addition of 50 µL of DMSO at each stage, resulting in a final volume of 200 µL DMSO per sample (50 mg/mL). The resuspended extracts were divided into 20 µL aliquots in 0.5 mL Eppendorf tubes and preserved at −20 °C for future use.

5. SIM-A9 cell culture

SIM-A9 is an immortalized microglial cell line, derived from mouse cerebral cortex tissues, acquired from the American Type Culture Collection (ATCC® CRL-3265TM, Manassas, VA, USA). SIM-A9 microglial cell lines were cultured and maintained in complete growth media comprising Dulbecco's Modified Eagle Medium F-12 (DMEM/F12) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% horse serum (H/S), 1% penicillin-streptomycin (P/S), and 1% L-glutamine. The cells were incubated at 37°C in a humidified atmosphere with 5% CO_2. Prior to passaging, the cells were rinsed with phosphate-buffered saline (PBS) and detached with Trypsin-EDTA solution (Sigma-Aldrich, Darmstadt, Germany) for 2 minutes at 37°C with 5% CO_2. The cells were seeded into 96-well plates at a density of 5,000 cell/well and allowed to adhere for about 24 hours before experimental treatment. This consistent cell density was used in all assays to make the experiment reproducible.

6. 3xTg-AD mouse

The triple transgenic mouse, 3xTg-AD, generated by the group of LaFerla is the best AD model reproducing the main features of human AD pathology. 3xTg-AD model develops an age-related progressive neuropathological phenotype that includes Aβ plaques and tau pathology due to the expression of three human mutant genes, observed in AD familial patients: PS1 (M146V), βAPP (Swedish), and tau (P301L). PS1 gene encodes for presenilin transmembrane protein involved in the activity of γ-secretase. APP gene encodes for transmembrane APP that is cleaved by β-secretase to form different peptides. Mutations of PS1 and APP genes correlate with pathological accumulation of Aβ plaques. (Oddo et al., 2003). In this study, 3xTg-AD pups from

postnatal days 3 to 7 (P3–P7) were used to isolate primary microglial cells. No evidence of changes in microglia isolated from neonatal 3xTg-AD mice are documented, while alterations in Ca^{2+} homeostasis in embryonic 3xTg-AD neurons have been demonstrated in primary cultures, suggesting an early molecular defect that precedes Aβ and tau pathology and may involve glial cells via dysregulated signalling (Smith et al., 2005).

For this study, the 3xTg-AD mice (MMRRC stock no. 34830-JAX), were purchased from the Jackson Laboratory. The research conducted complies with all relevant ethical guidelines. Research involving animals was authorized by the Ethical Committee from the University of Verona and by the Italian Ministry of Health, Department of Veterinary Public Health, Nutrition and Food Safety, Directorate General of Animal Health and Veterinary Medicine (authorization no. 164/2016-PR and 876/2021-PR), as required by Italian legislation (D. Lgs 26/2014) as per the application of European Directive (2010/63/UE).

7. Primary microglia culture

Hippocampal and cortical brain regions were rapidly dissected from new-born 3xTg-AD mice (3 to 7 days old) and dissociated using the Adult Brain Dissociation Kit (catalog no. 130-107-677, Miltenyi Biotec) and gentleMACS Dissociator (Miltenyi Biotec), according to the manufacturer's instructions. After removing debris and red blood cells, samples were labelled with a non-neuronal biotin antibody cocktail (Miltenyi Biotec) to collect mixed glial cells. These mixed glial cells were cultured in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine in T-75 flasks at 37°C with 5% CO_2 for 11–14 days. The culture medium was completely replaced with a fresh medium every three days. Then, the

microglial cells were isolated by shaking the flask at 200 rpm at 37°C for 2 hours, followed by centrifugation of the cell supernatant at 1200 rpm for 6 minutes at 25°C. The isolated primary microglial cells were resuspended and seeded onto 96-well plates at a density of 30,000 cells/well for further experiments.

8. Neuroblastoma N2A cell culture

Mouse neuroblastoma cells (Neuro-2a or N2A) were purchased from the American Type Culture Collection and maintained in DMEM/F12 medium supplemented with 10% FBS, 5% H/S, 1% P/S, and 1% L-glutamine. The cells were incubated at 37˚C in a humidified environment with 5% CO_2 and normally grew to 80-90% confluency within each 48–72 h. For passaging and experimental seeding, the culture medium was aspirated off, cells were washed twice with PBS at pH 7.4 and detached with a Trypsin-EDTA solution (both from Sigma-Aldrich Darmstadt, Germany) for 2 min at 37°C. The detached cells were resuspended in complete medium and seeded into 96-well plate at a density of 20,000 cell/well. Cells were then allowed to adhere for 24 h before the experimental treatments.

9. Aβ1-42 oligomer preparation

Oligomeric Aβ1-42 was prepared as reported previously (Costantini et al., 2005; Klein, 2002). The lyophilized Aβ1-42 peptide (Bachem AG, Cat#4014447) was reconstituted in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; Sigma, Cat# 105228) to a final concentration of 1 mM and incubated at room temperature for 60 minutes in a sealed vial. The solution was then aliquoted into sterile microcentrifuge tubes (10 µL per tube, 1 mM), and the HFIP was evaporated overnight under sterile hood at room

temperature. Subsequently, the tubes were centrifuged in a SpeedVac for 10 minutes to ensure complete drying. The resulting dried peptide films (10 µL/vial, 1 mM) were stored over a desiccant at −80 °C until required for further use. To obtain Aβ1-42 oligomers, the dried peptide film was reconstituted in dimethyl sulfoxide (DMSO) to a concentration of 5 mM. This concentrated solution was subsequently diluted with ice-cold, phenol red-free Ham's F-12 medium (BioSource) to achieve a final concentration of 100 µM. The diluted solution was agitated by vortexing for 30 seconds and subsequently incubated at 4 °C for 24 hours. Then, the solution was centrifuged at 15,000 × g for 10 minutes at 4 °C to eliminate insoluble aggregates. The supernatant, containing soluble Aβ oligomeric species, was collected and its concentration determined using the MicroBCA protein assay (Pierce, Rockford, IL).

10. Characterization of Aβ1-42 Oligomers by Western Blotting

To determine the oligomeric state of soluble Aβ1-42 preparation, Western Blot analysis were conducted under non-reducing and non-denaturing conditions as described by Costantini et al., 2005. Aβ1-42 peptides were diluted in lithium dodecyl sulfate sample buffer and loaded, without heating, onto precast 12% Bis-Tris NuPAGE gel (Invitrogen). Electrophoresis was carried out in MES running buffer (Invitrogen), and the proteins were transferred on polyvinylidene difluoride (PVDF) membrane using NuPAGE transfer buffer (Invitrogen). Subsequently, the membranes were blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated overnight at 4 °C with mouse monoclonal antibody (6E10; 1:3000; Thermo Fisher Scientific, Cat. No. MA5-51794), recognizing residues 1-17 of Aβ. Following washing with TBS-T,

the membranes were then incubated with HRP-conjugated anti-mouse secondary antibody (1:5000; Thermo Fisher Scientific, Cat. No. 31430) for 1 h. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) system.

11. Aβ25-35 preparation

Aβ25-35 (A4559, Sigma–Aldrich, St. Louis, MI, USA) was diluted in sterilized double-distilled water to achieve a final concentration of 1 mM. This solution was then incubated at 37°C for 7 days to facilitate the fibrillary aggregation of Aβ25-35. Subsequently, the fibrillar Aβ25-35 was maintained at -20°C (Ji et al., 2019; Pike et al., 1995).

12. Immunofluorescence staining

The purity of primary microglial cell was assessed by an immunofluorescence staining, using a microglia marker Iba-1 (Ionized calcium-binding adapter molecule 1), an astrocyte marker GFAP (Glial Fibrillary Acidic Protein), and DAPI (4′,6-diamidino-2-phenylindole), a blue-fluorescent DNA-marker. Primary microglia cells were seeded (10,000 cells/well) in DMEM/F12 medium supplemented with 10% FBS, 1% penicillin-streptomycin, and 1% L-glutamine on a sterilized Falcon® 8-well chamber slide (Product Number: 354118, CORNING USA), and incubated at 37 °C for 48 h. Following incubation, the cells were fixed with 4% paraformaldehyde for 10 minutes and washed twice with PBS to remove residual fixative. The cells were then blocked in a blocking solution (0.2% Triton X-100, 2% Bovine serum albumin, and 2% Normal goat serum in PBS) for 1 hour at room temperature. The cells were

incubated with primary antibodies, including rabbit anti-Iba-1 polyclonal antibody (1:1000, Wako) and rat anti-GFAP monoclonal antibody (1:1000, DAKO), for 2 hours at room temperature. After primary antibody incubation, the cells were washed three times with PBS containing 0.05% Tween-20, followed by a 1-hour incubation at room temperature with secondary antibodies Alexa 488-conjugated goat anti-rabbit (1:1000, Invitrogen) and Alexa 647-conjugated chicken anti-rat (1:1000, Invitrogen). Subsequently, the cells were washed three times with PBS containing 0.05% Tween-20 and stained with 4',6-diamidino-2-phenylindole (DAPI) solution for 7 minutes at room temperature. Finally, the cells were washed once with PBS containing 0.05% Tween-20 and mounted using DABCO Mounting Medium (Sigma, P8136). Image acquisition was performed using a Zeiss Axio Imager.Z2 microscope (20x magnification). Culture purity was quantified with images using a manual cell count made by counting with the multi-point Tool within ImageJ (Fiji). In each preparation, the number of Iba-1 + microglia or GFAP+ astrocytes is expressed as a fraction of the total DAPI-stained nuclei in numerous random fields. Quantitative evaluations verified significant enrichment of the cultures, with Iba-1+ cells marked at 98.8% in the total population and thus indicating high purity of microglial cultures.

13. Cell viability

Cell viability was assessed utilizing the 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to evaluate the cytotoxic effects of naturally derived plant extracts on SIM-A9 cells, primary microglia, and N2A neuroblastoma cells. The cells were seeded in flat-bottom 96-well plates at a density of 5,000 cells/well for SIM-A9, 30,000 cells/well for primary microglia, and 20,000 cells/well for N2A cells. They were then incubated under standard conditions (37°C, 5% CO_2)

for 24 hours. The following day, the cells were treated with varying concentrations of the compounds (10, 25, 50, 100, and 200 µg/mL) and incubated for an additional 24 hours under the same conditions. 0.5% DMSO (vehicle) was used as negative control and staurosporine (1 µM) as a positive control, respectively. Staurosporine is a potent non-selective protein kinase inhibitor and was used as a positive control for apoptosis to ensure sensitivity of the assay and to verify cell death detection. After treatment, the cells were exposed to an MTT solution at a final concentration of 0.5 mg/mL and incubated for 1 hour at 37°C in the dark. Following incubation, the supernatant was carefully removed, and the formazan crystals were dissolved using 100 µL/well of DMSO. The absorbance intensity was quantified using a microplate reader (VICTOR, PerkinElmer) at 570 nm wavelength. The data were calculated as follows: [$(Test\ Sample\ count - Blank\ count)$ / $(Vehicle\ Control\ count - Blank\ count)$] ×100, expressed as % of live control.

14. Nitrite quantification

The production of NO was quantified using a colorimetric Griess assay. The microglia cells were seeded into flat 96-well plates at densities of 5,000 cells/well for SIM-A9 microglia and 30,000 cells/well for primary microglia. The cells were incubated under standard conditions (37°C, 5% CO_2) for 24 hours. Subsequently, the cells were pre-treated with plant extracts at 10, 25, 50, 100, and 200 µg/mL doses for 24 hours and then co-treated with LPS at a concentration of 1 µg/ml for an additional 24 hours. LPS, a major constituent of the outer membrane of Gram-negative bacteria, was employed as a strong inducer of pro-inflammatory response by activated microglial cells, promoting the expression of inducible nitric oxide synthase (iNOS), thereby

causing measurable NO generation (Z. Xie et al., 2002). The NO concentration was determined by analyzing the nitrite released from the LPS-treated microglia using Griess reagent, which consisted of 1% sulfanilamide dissolved in 5% phosphoric acid (H_3PO_4) and 0.1% N-(L-naphthyl)-ethylene diamine dihydrochloride. Equal volumes of the cell culture medium and Griess reagent solution were mixed, and the absorbance was measured at 550 nm using a microplate reader (VICTOR, PerkinElmer). A sodium nitrite ($NaNO_2$, Sigma-Aldrich, USA) standard curve was utilized as a reference to quantify the NO levels under each experimental condition.

15. ELISA assay

Production of pro-inflammatory cytokines, such as TNF-α and IL-6, was measured by enzyme-linked immunosorbent assay (ELISA). SIM-A9 and primary microglia cells were plated in 96-well flat-bottom plates at the concentration of 5,000 and 30,000 cells/well respectively and incubated at 37 °C with 5% CO_2 for 24 h. The cells were pre-treated with plant extracts for 24 h in doses ranging from 10, 25, 50, 100, and 200 µg/mL for SIM-A9 and 10, 50, and 100 µg/mL for primary microglia. Then, the cells were co-stimulated with LPS, 1 µg/mL. Co-treatment was maintained for 24 hours. In separate experiments, primary microglial cells were also co-treated with Aβ peptides (Aβ1-42 at 5 µM or Aβ25-35 at 10 µM) for 24h. Positive control was represented by the cells treated with LPS or Aβ plus 0.5% DMSO (vehicle). Following treatment, supernatants were collected and centrifuged at 2,000 rpm for 5 min at 4°C. After centrifugation, supernatants were collected and assessed for pro-inflammatory cytokines, such as TNF-a and IL-6 by ELISA kits (Mabtech AB, Sweden) according to the manufacturer's guidelines. ELISA assays were performed starting from a

coating step of 96-well clear polystyrene ELISA microplate (Merck, Germany, Cat. #CLS3896) with 50 µL/well of capture antibody (at a concentration of 2 µg/mL for TNF-α and at 1 µg/mL for IL-6) overnight at 4 °C. The day after, the plates were blocked with 100 µL/well of blocking solution (PBS + 0.05% Tween-20 + 0.1% BSA) at RT for 1 hour and washed five times. For TNF-α and IL-6 measurements, samples were diluted 10-fold in cell culture medium (11 µL sample + 99 µL medium; final volume of 110 µL). Standards and diluted samples (50 µL/well) were placed for 2 h at room temperature. The standard curve for TNF-α ranged from 3.9 to 1000 pg/mL, and the IL-6 standard curve ranged from 15.6 to 4000 pg/mL. After five washes, 50 µL/well of biotinylated detection antibodies (0.5 µg/mL for TNF-α, and 1 µg/mL for IL-6) was added for one hour and followed by subsequent incubation with 50 µL/well of Streptavidin-HRP (1:1000) for an additional hour. Finally, 50 µL/well of TMB substrate was added, and incubated at RT for 10 minutes, protected from direct light. The reaction was then stopped by adding 50 µl/well of 1 M sulfuric acid (H_2SO_4), and absorbance was measured at 450 nm using a microplate reader (VICTOR, PerkinElmer) within 15 minutes.

16. Glutamate-induced N2A neurotoxicity

N2A cells were seeded at a density of 2×10^4 cells per well in flat-bottom 96-well plates and incubated overnight. Glutamate was freshly prepared in DMEM/F-12 culture medium containing 10% FBS at the final concentration of 50 mM. Glutamate is commonly used as excitotoxic reagent to bring about neuronal cell damage. The cells were pretreated with the plant extracts at concentrations of 10, 25, 50, 100 and 200 µg/mL for 24 h. To induce neurotoxicity, the cells were co-treated with 50 mM

glutamate for a further 4 h. Cells treated with 50 mM glutamate alone (plus 0.5% DMSO) were used as toxicity positive control, whereas cells treated only with the culture medium containing 0.5% DMSO (vehicle) was used as the negative control (indicating 100% viability). Following treatment, cell viability was determined by MTT assay. Briefly, 10 μL of MTT solution (5 mg/mL, in PBS) was added to each well to reach a final concentration of 0.5 mg/mL, followed by incubation of the plates for 1 hour at 37°C in dark. The supernatant was removed after incubation and the purple formazan crystals were dissolved by adding 100 μL/well of DMSO. The absorbance was read at 570 nm on a VICTOR (PerkinElmer) microplate reader. The data were calculated as a percentage of cell viability compared with the negative control group.

17. Statistical Analysis

The data were presented as the mean ± SEM from (at least) three independent experiments. Statistical differences between groups were assessed using a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test, performed with GraphPad Prism 8.0.2 software (GraphPad Software Inc., San Diego, CA, USA). Differences with *P*-values < 0.05 were considered statistically significant.

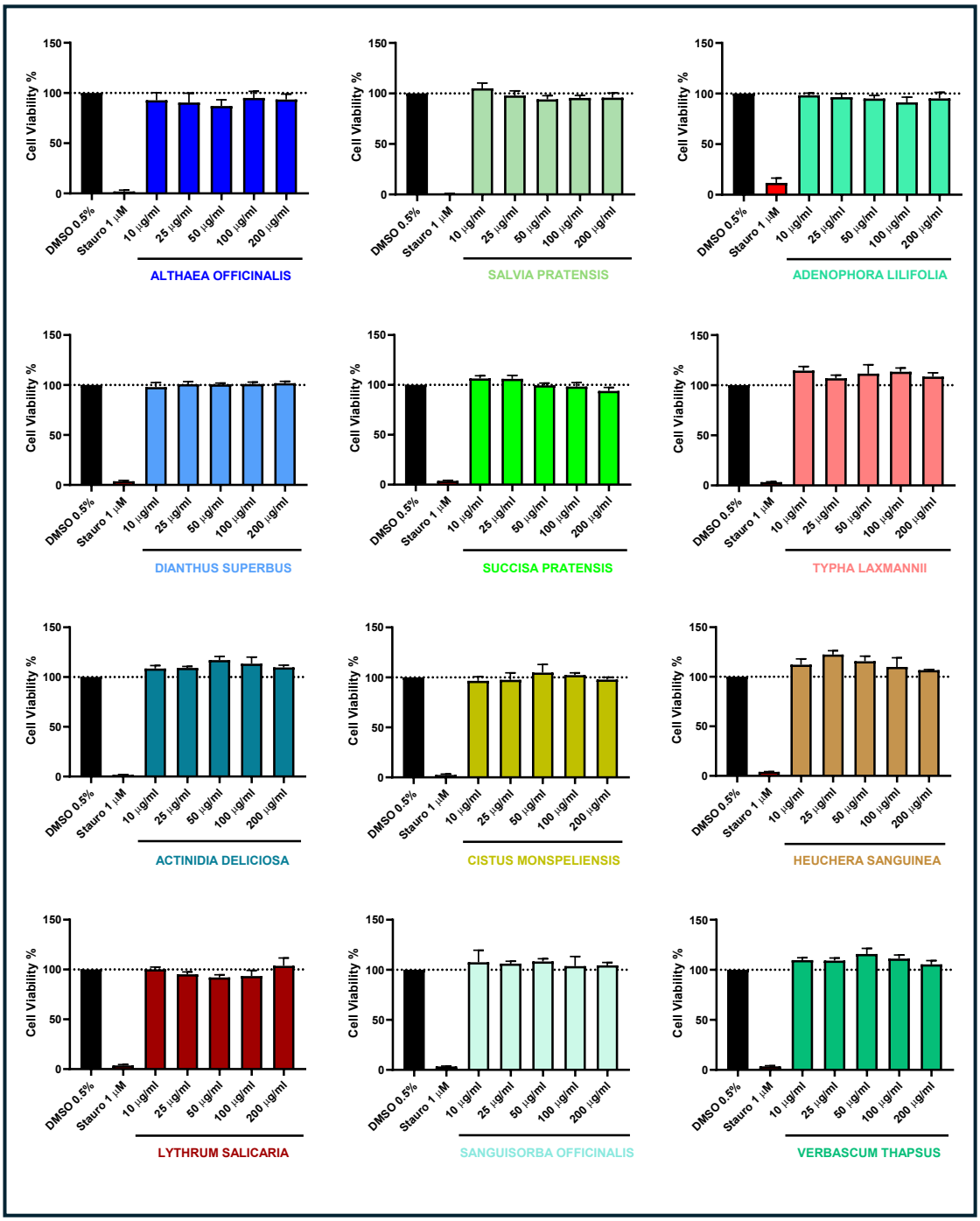
RESULTS

Firstly, 23 plant-derived extracts prepared by Prof. Guzzo's lab underwent screening for cytotoxicity within SIM-A9 microglial cells at different concentrations. Extracts identified as non-toxic were subsequently evaluated in primary microglial cultures. These safe extracts were then assessed for their anti-inflammatory activity by measuring NO production and the secretion of pro-inflammatory cytokines, including TNF-α and IL-6, induced by LPS in both cell types. The four most potential anti-inflammatory extracts, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis, were next tested for their capacity to attenuate Aβ -induced neuroinflammation, specifically the TNF-α and IL-6 production in primary microglia upon exposure to Aβ peptides. Concurrently, their neuroprotective effects were evaluated also in N2A neuroblastoma cells stimulated by glutamate.

1. The impact of plant extracts on SIM-A9 microglial cell viability

To determine the non-toxic concentrations of 23 plant extracts on SIM-A9 microglial cells, cell viability was evaluated using the MTT assay. Cells were cultured at 5,000 cells/well, allowed to adhere overnight, and subsequently treated with extract concentrations of 10, 25, 50, 100, and 200 μg/ml for 24 hours. The results revealed that 17 out of the 23 extracts, namely Althaea officinalis, Salvia pratensis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Cistus monspeliensis, Heuchera sanguinea, Lythrum salicaria, Sanguisorba officinalis, Verbascum thapsus, Rosa canina, Allium lusitanicum,

Acalypha virginica, Diospyros kaki, and Malva alcea, were non-toxic at all tested concentrations **(Figure 11)**. In contrast, six extracts displayed varying degrees of cytotoxicity. Specifically, Glandularia tenera induced toxicity at the highest tested dose (200 µg/ml); Artemisia abrotanum showed toxicity at concentrations ≥100 µg/ml; and Petasites paradoxus, Gratiola officinalis, and Allium angulosum were toxic at concentrations ≥50 µg/ml; while Pulsatilla montana was toxic at all tested concentrations **(Figure 12)**. 0.5% DMSO (vehicle) was used as negative control and staurosporine (1 µM) as a positive control.


Cell Viability %
150
100
50
0
DMSO 0.5%
Stauro 1 µM
10 µg/ml
25 µg/ml
50 µg/ml
100 µg/ml
200 µg/ml
ALTHAEA OFFICINALIS
SALVIA PRATENSIS
ADENOPHORA LILIFOLIA
DIANTHUS SUPERBUS
SUCCISA PRATENSIS
TYPHA LAXMANNII
ACTINIDIA DELICIOSA
CISTUS MONSPELIENSIS
HEUCHERA SANGUINEA
LYTHRUM SALICARIA
SANGUISORBA OFFICINALIS
VERBASCUM THAPSUS

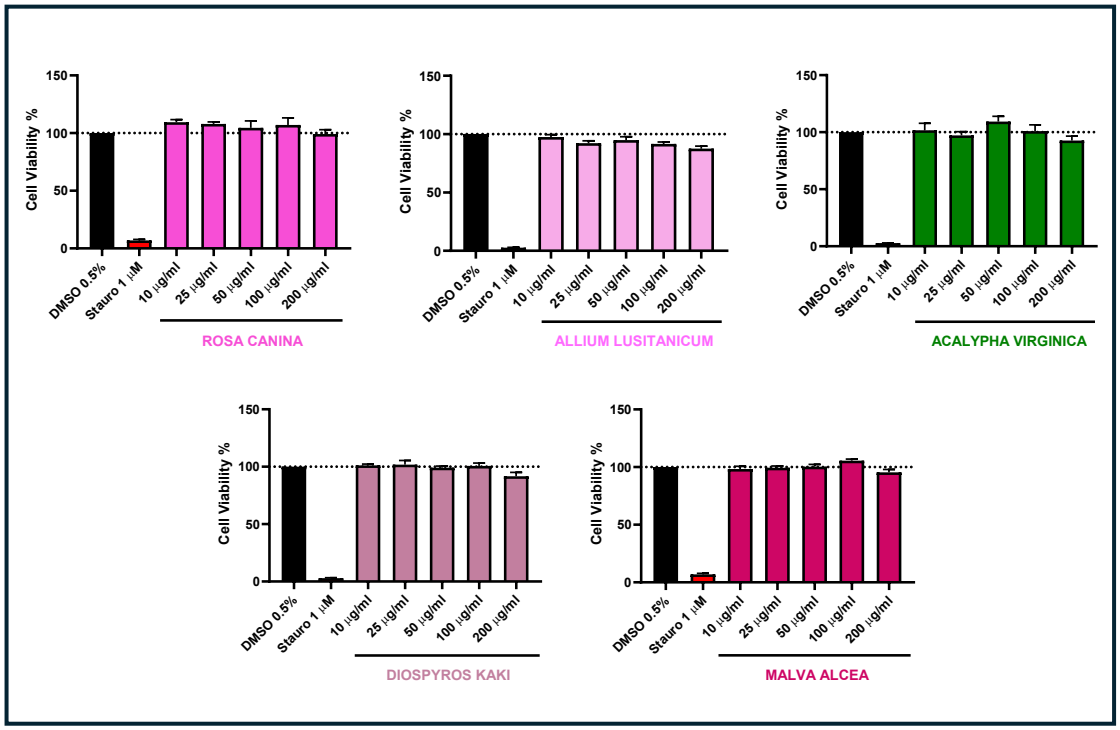


Figure 11. Lack of toxic effects of plant extracts on SIM-A9 cells. None of the 17 plant extracts at the tested concentrations (10, 25, 50, 100, and 200 µg/ml) exhibited toxicity in murine microglial SIM-A9 cells after 24 hours of treatment, as assessed by the MTT assay. Experimental controls included 0.5% DMSO as the negative control and staurosporine (1 µM) as the positive control. The values were represented the mean ± SEM.

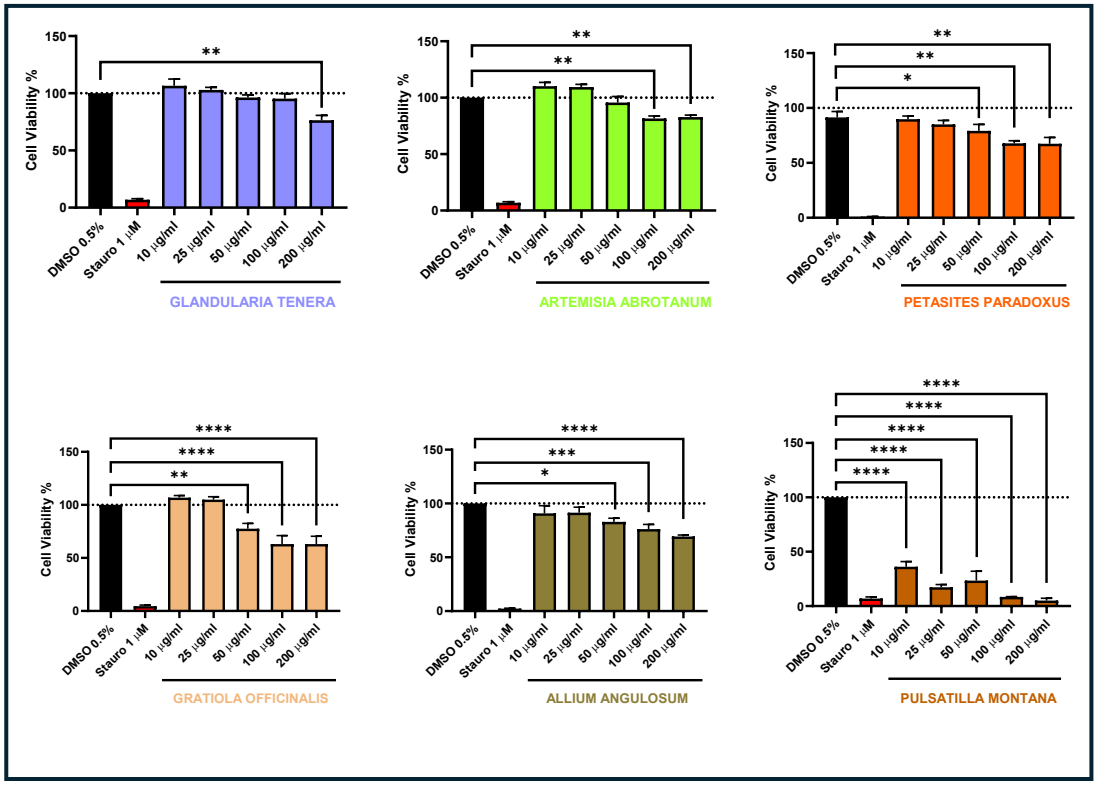
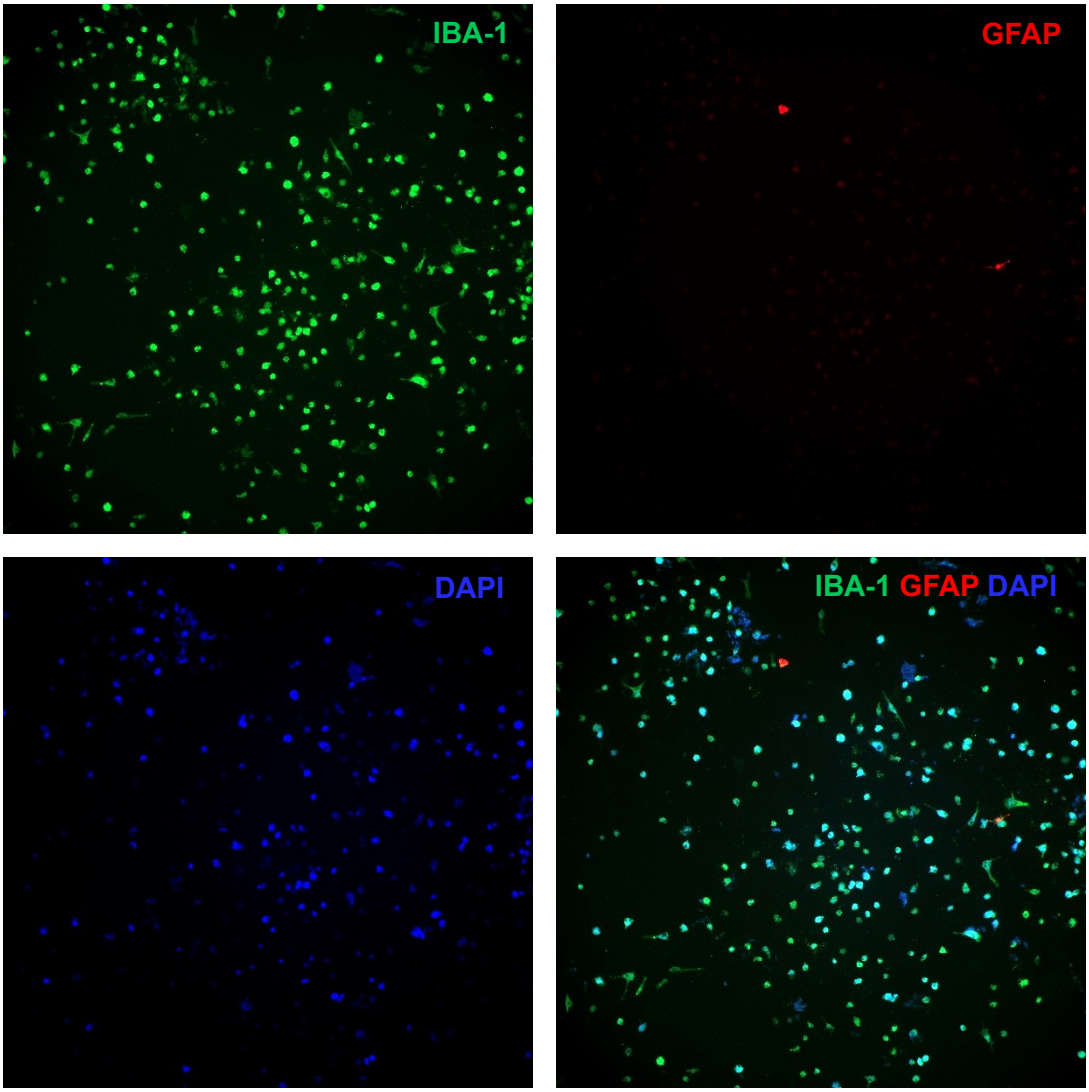


Figure 12. The toxic effects of plant extracts on SIM-A9 cells. MTT assay demonstrated cytotoxic effects of these extracts on SIM-A9 cell viability after 24 hours of stimulation. Glandularia tenera (toxic at 200 µg/ml), Artemisia abrotanum (≥100 µg/ml), Petasites paradoxus, Gratiola officinalis, and Allium angulosum (≥50 µg/ml), while Pulsatilla montana at ≥10. Staurosporine (1 µM) was used as a positive control. The values were represented the mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, compared with the 0.5% DMSO control group.

2. The purity of isolated primary microglia

Before testing the effects of extracts on primary cells, the purity of the isolated primary microglia was confirmed by immunofluorescence staining. Iba-1 (Ionized calcium-binding adapter molecule 1) positive cells were predominant throughout the cell culture, whereas a limited number of GFAP (Glial Fibrillary Acidic Protein) positive astrocytes were detected. The nuclei were labeled with DAPI. The cellular culture was acquired by AxioImager Z2 microscope (Zeiss, Germany) at 20X magnification. The images were quantified by counting the number of cells using the

Multi-point tool in ImageJ (Fiji). As shown in **Figure 13**, the Iba-1+ microglia comprised 98.8% of total cells and GFAP+ astrocytes were only 1.2%. The high Iba-1: GFAP ratio confirms the isolation of pure microglial population.



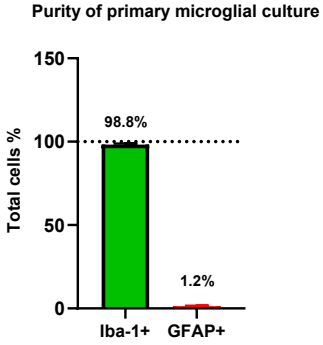
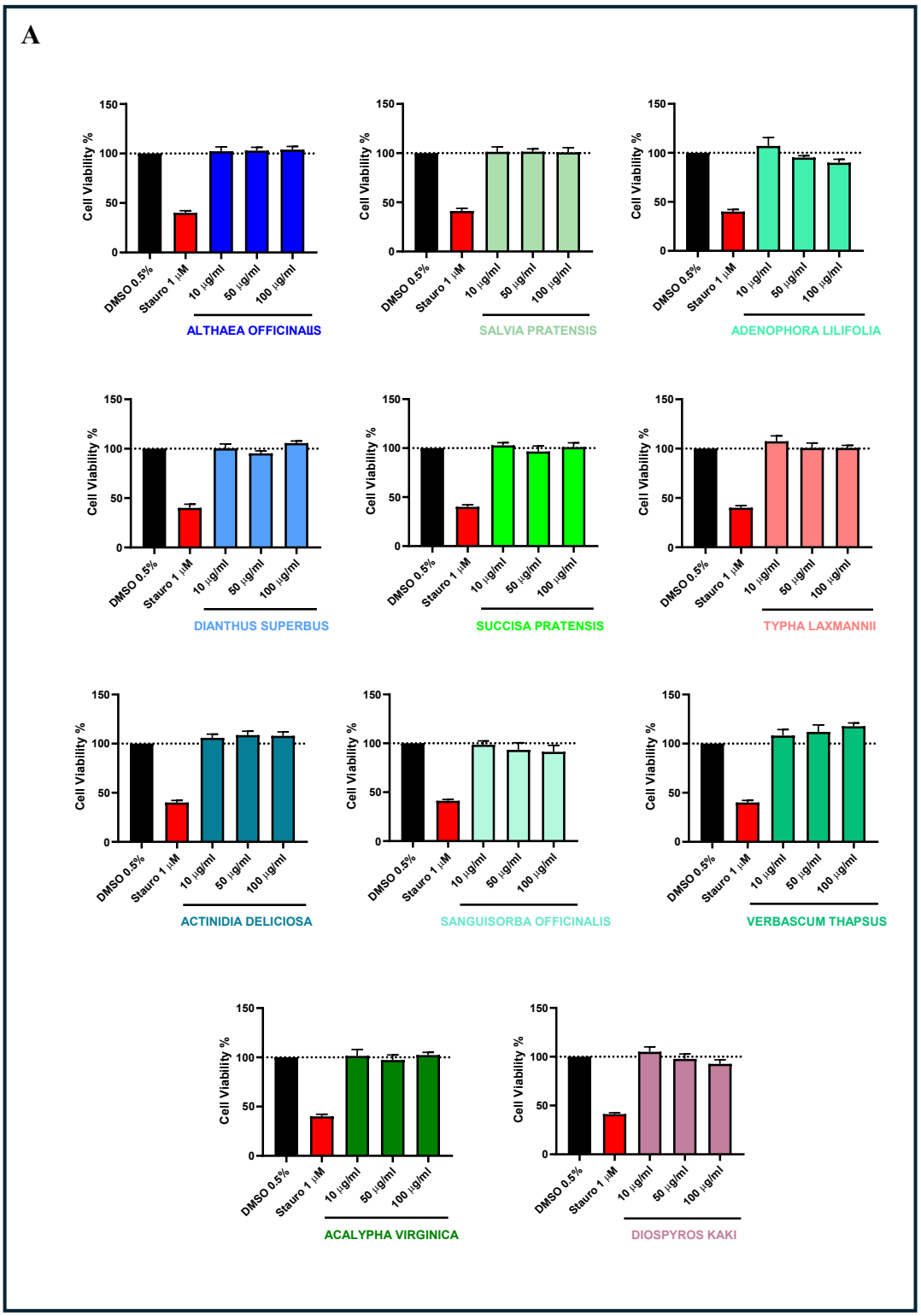


Figure 13. The high purity of isolated primary microglia. Representative immunofluorescence images of isolated primary microglia from neonatal 3xTg-AD mice. A high number of microglia is labelled with Iba-1 (in green), whereas GFAP (in red) reveals few astrocytes. All nuclei were stained with DAPI (in blue). The graph below shows the percentage of Iba-1+ microglia and GFAP+ astrocytes.

3. The effect of plant extracts on primary microglial cells viability

The plant extracts previously identified safe in SIM-A9 microglial cell lines were evaluated then on primary microglia viability. Primary microglial cells were seeded at a density of 30,000 cells per well, incubated overnight, and subsequently treated with selected plant extracts at concentrations of 10, 50, and 100 µg/ml for a 24-hour period. As illustrated in **Figure 14a**, eleven extracts, including Althaea officinalis, Salvia pratensis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Sanguisorba officinalis, Verbascum thapsus, Acalypha virginica, and Diospyros kaki, did not impact primary microglia viability at all the tested concentrations. Whereas six extracts exhibited varying degrees of cytotoxicity (**Figure 14b**). Particularly, Malva alcea and Cistus monspeliensis demonstrated toxicity at concentrations ≥100 µg/ml; Rosa canina displayed toxicity at concentrations ≥50 µg/ml; while Allium lusitanicum, Lythrum salicaria, and Heuchera sanguinea significantly diminished microglial cells viability across all tested doses (10, 50, and 100 µg/ml). 0.5% DMSO (vehicle) was used as negative control, and staurosporine (1 µM) as the positive control. Collectively, these findings indicate that eleven natural extracts were non-toxic to both SIM-A9 cells and primary microglia at the tested concentrations.


A
Cell Viability %
150
100
50
0
DMSO 0.5%
Stauro 1 µM
10 µg/ml
50 µg/ml
100 µg/ml
ALTHAEA OFFICINALIS
SALVIA PRATENSIS
ADENOPHORA LILIFOLIA
DIANTHUS SUPERBUS
SUCCISA PRATENSIS
TYPHA LAXMANNII
ACTINIDIA DELICIOSA
SANGUISORBA OFFICINALIS
VERBASCUM THAPSUS
ACALYPHA VIRGINICA
DIOSPYROS KAKI

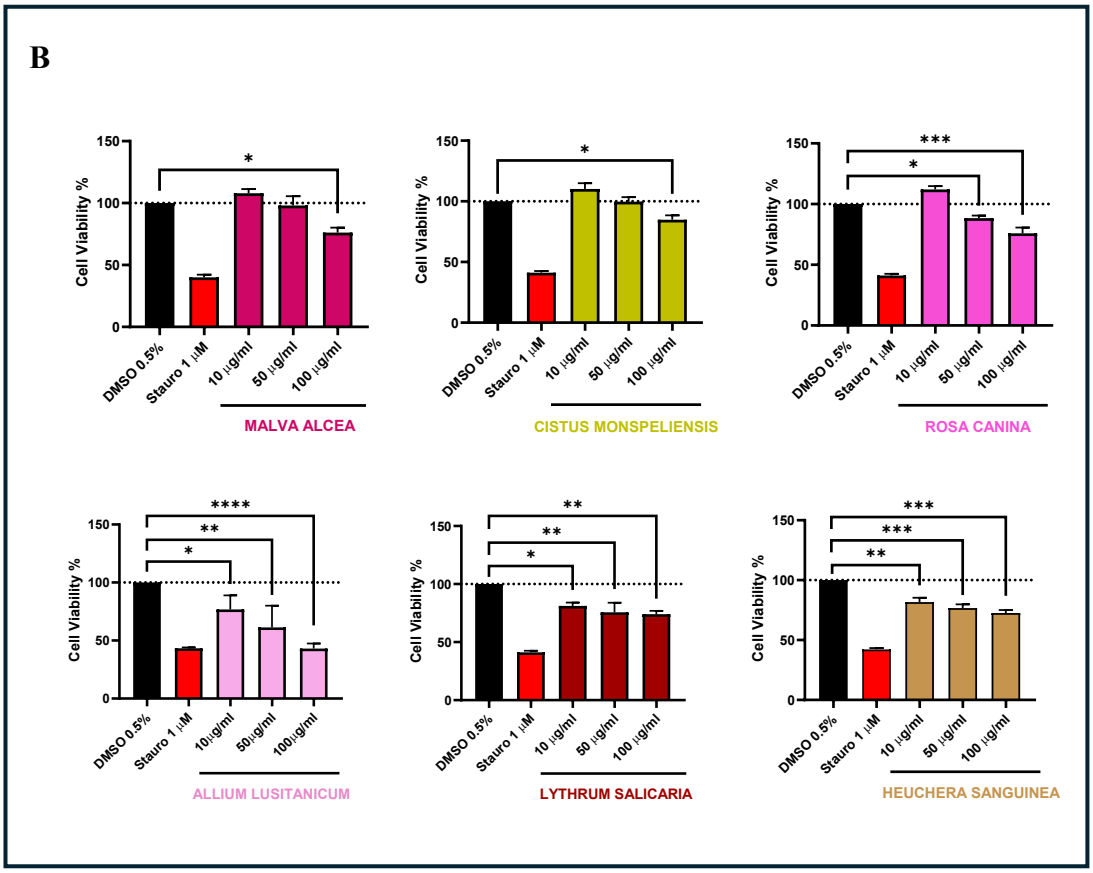


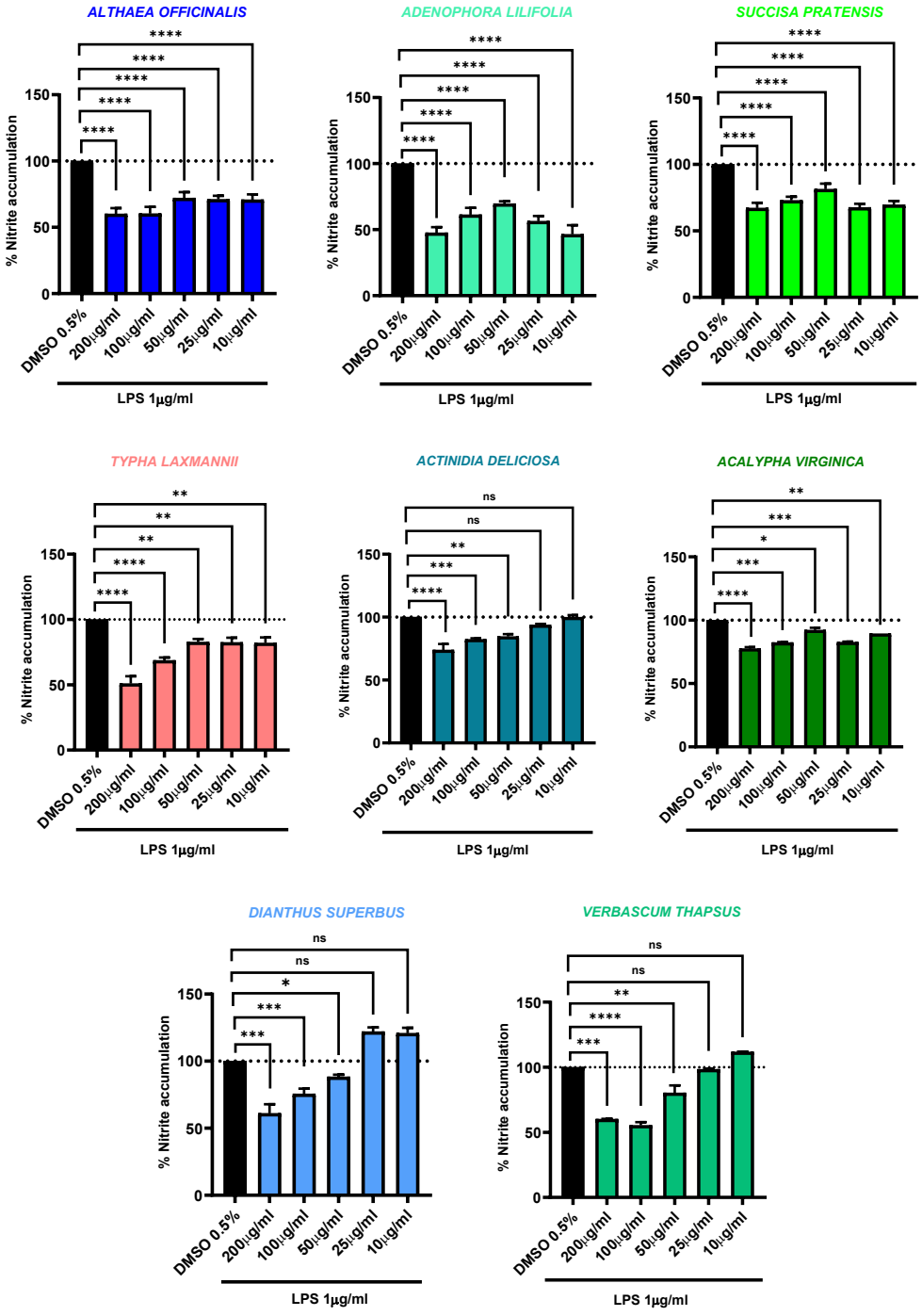
Figure 14. Impact of plant extracts on primary microglial cells viability. a) Althaea officinalis, Salvia pratensis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Sanguisorba officinalis, Verbascum thapsus, Acalypha virginica, and Diospyros kaki show no toxic effect at all tested concentrations (10, 50, and 100 µg/ml) after 24h. **b)** Contrarily, Malva alcea, Cistus monspeliensis, Rosa canina, Allium lusitanicum, Lythrum salicaria, and Heuchera sanguinea show cell toxicity. Staurosporine (1 µM) was used as a positive control. The values were expressed as the mean ± SEM, with statistical significance indicated as **P*<0.05, ***P*<0.01, ****P*<0.001, and *****P*<0.0001, compared to the 0.5% DMSO control group.

4. Effect of natural extracts on the NO production in the LPS-stimulated SIM-A9 cells

Chronic microglia activation leads to excessive NO production, forming reactive nitrogen species (RNS) that can contribute to neuronal cell death (Lull & Block,

2010). Since microglia activation is a source of pro-inflammatory mediators, we investigated the anti-inflammatory potential of eleven plant-derived extracts, Althaea officinalis, Salvia pratensis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Sanguisorba officinalis, Verbascum thapsus, Acalypha virginica, and Diospyros kaki, by evaluating their effects on LPS-induced NO production in SIM-A9 microglial cells. All eleven extracts had been previously confirmed to be non-toxic to both SIM-A9 cells and primary microglia across all tested concentrations. NO levels were quantified using the colorimetric Griess assay, and its reduction was calculated as percentage. Eight of the plant extracts (Althaea officinalis, Adenophora lilifolia, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Acalypha virginica, Dianthus superbus, and Verbascum thapsus) effectively reduced LPS-induced NO production at one or more tested concentrations (10–200 µg/ml) after 24 hours of treatment, although a clear dose-dependent response was observed only with some extracts (**Figure 15a**). However, Actinidia deliciosa, Dianthus superbus, and Verbascum thapsus did not exhibit NO reduction at concentrations of 25 µg/ml (or lower). Instead, Salvia pratensis, Sanguisorba officinalis, and Diospyros kaki (**Figure 15b**) showed no significant effect on NO production at any tested concentration. To further validate these *in vitro* findings, the eight extracts were then tested on primary microglia to assess their impact on LPS-induced NO production.

A


ALTHAEA OFFICINALIS
ADENOPHORA LILIFOLIA
SUCCISA PRATENSIS
TYPHA LAXMANNII
ACTINIDIA DELICIOSA
ACALYPHA VIRGINICA
DIANTHUS SUPERBUS
VERBASCUM THAPSUS
% Nitrite accumulation
DMSO 0.5%
200µg/ml
100µg/ml
50µg/ml
25µg/ml
10µg/ml
LPS 1µg/ml

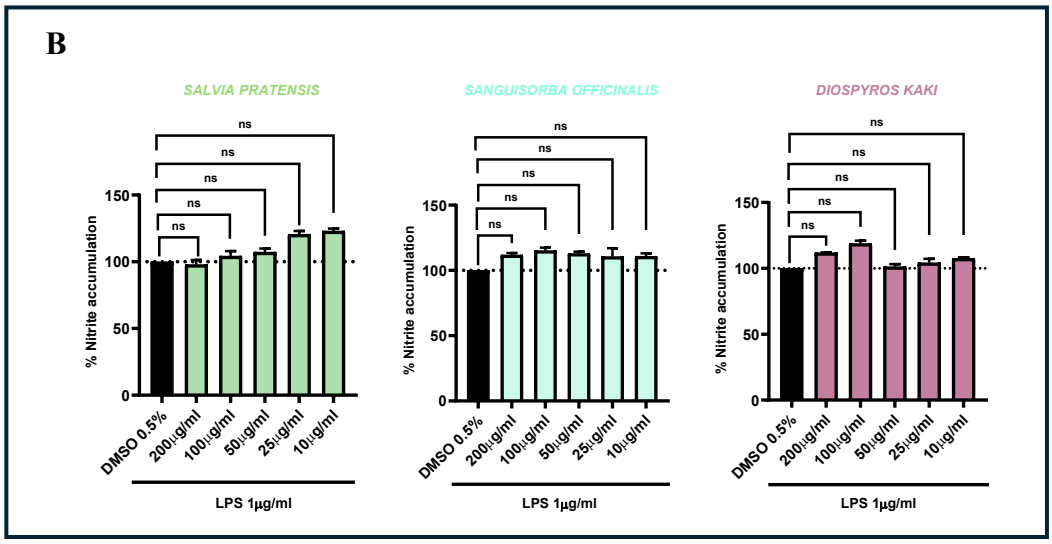


Figure 15. The effect of plant extracts on the NO production in LPS-treated SIM-A9 cell line. SIM-A9 cells were pre-treated with plant extracts (10, 25, 50, 100, and 200 µg/mL) for 24 hours, followed by co-treatment with LPS (1 µg/mL) for an additional 24 hours. The release of NO was measured using the Griess assay. **a)** Althaea officinalis, Adenophora lilifolia, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Acalypha virginica, Dianthus superbus, and Verbascum thapsus significantly reduced the NO production in a dose-dependent manner. **b)** Salvia pratensis, Sanguisorba officinalis, and Diospyros kaki did not show any significant effect on NO production at any of the tested concentrations. Values were expressed as the mean ± SEM. Statistical significance is indicated as follows: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, compared to the 0.5% DMSO + LPS (1 µg/mL).

5. Effect of natural extracts on the NO production in LPS-activated primary microglia

The eight plant extracts that have demonstrated a reduction in the NO production in SIM-A9 cells (Althaea officinalis, Adenophora lilifolia, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Acalypha virginica, Dianthus superbus, and Verbascum thapsus) were further investigated for their anti-inflammatory effects against LPS-induced NO production in primary microglia. Primary microglial cells were cultured

at a density of 3×10^4 cells per well in a 96-well plate. Cells were pre-treated with the selected plant extracts at concentrations of 10, 50, and 100 µg/ml for 24 hours, followed by co-treatment with 1 µg/ml of LPS for an additional 24 hours. NO production was subsequently quantified using the colorimetric Griess assay, with 0.5% DMSO + LPS (1 µg/ml) as the positive control. The results indicated that six of the extracts, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, and Actinidia deliciosa, significantly reduced the LPS-induced NO production compared to the positive control (0.5% DMSO + LPS-treated cells) **(Figure 16a)**. Conversely, Verbascum thapsus and Acalypha virginica did not exhibit any significant reduction in NO levels in LPS-treated primary microglia **(Figure 16b)**. Based on these findings from both SIM-A9 and primary microglia cells, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, and Actinidia deliciosa were identified as effective in attenuating LPS-stimulated NO production. These findings underscore the anti-inflammatory potential of these six extracts and warrant further investigation into their broader impact on other inflammatory mediators, including pro-inflammatory cytokines.

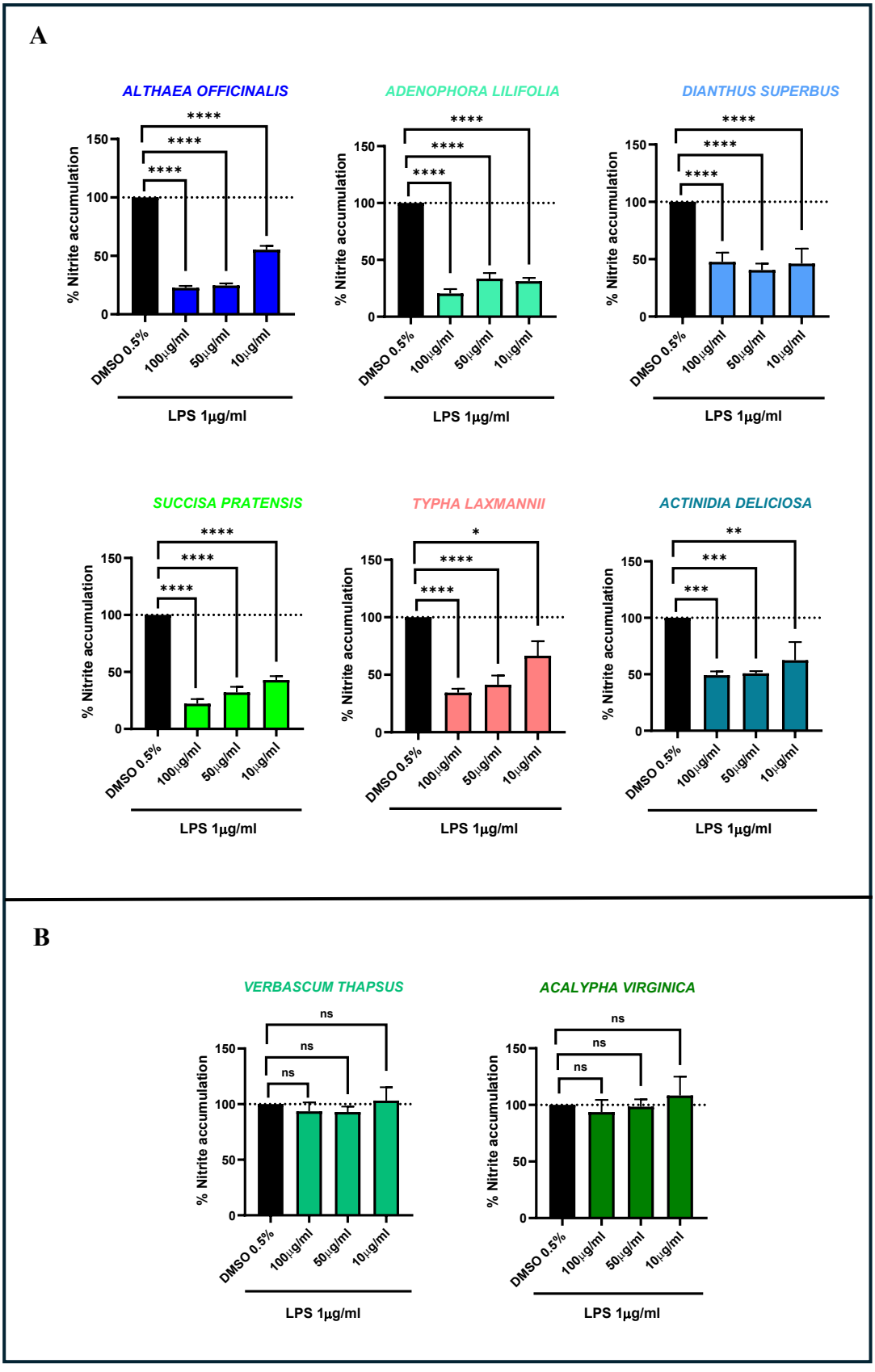


Figure 16. The impact of plant extracts on NO generation in LPS-treated primary microglia. Primary microglia were pretreated with plant extracts (10, 50, and 100 µg/mL) for 24 hours, followed by co-treatment with LPS (1 µg/mL) for additional 24 hours. Nitrite concentration (µM) in culture

supernatants was measured using the Griess assay. **a)** Althaea officinalis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, and Actinidia deliciosa significantly reduced NO production in a dose-dependent manner compared to LPS-treated cultures. **b)** Verbascum thapsus and Acalypha virginica showed no significant reduction in NO levels. Values were expressed as mean ± SEM. Statistical significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ vs. LPS control.

6. The plant extracts modulate the release of pro-inflammatory cytokine in LPS-induced SIM-A9 microglial cells

This study investigated whether six bioactive plant extracts (Althaea officinalis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, and Actinidia deliciosa), previously identified for their NO-modulating effects, can mitigate LPS-induced pro-inflammatory cytokine release in SIM-A9 microglial cells. SIM-A9 cells were pre-treated with these extracts at concentrations of 10, 25, 50, 100, and 200 µg/ml for 24 hours, followed by co-treatment with LPS (1 µg/ml) for additional 24 hours. Cell culture supernatants were subsequently collected, and the levels of pro-inflammatory cytokines, TNF-α and IL-6, were quantitatively determined by ELISA according to the manufacturer's instructions. 0.5% DMSO + LPS (1 µg/ml) was used as the positive control. Our findings demonstrated that LPS alone (1 µg/ml) significantly induced the production of both TNF-α and IL-6. However, the pre-treatment of cells with Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis significantly attenuated LPS-induced TNF-α production compared to the positive control (0.5% DMSO + LPS-treated cells) (**Figure 17a**). In contrast, Actinidia deliciosa and Typha laxmannii did not significantly reduce TNF-α levels (**Figure 17b**). Regarding IL-6, ELISA have revealed that extracts from Althaea officinalis, Adenophora lilifolia, Dianthus

superbus, and Succisa pratensis dose-dependently reduced IL-6 production in response to LPS stimulation (**Figure 18a**). Among these, only Dianthus superbus did not achieve statistical significance in IL-6 reduction at its lowest tested concentration (10 µg/ml). Conversely, Typha laxmannii and Actinidia deliciosa failed to exhibit any significant reduction in IL-6 levels at any tested concentrations in LPS-treated SIM-A9 cells (**Figure 18b**). Collectively, these results demonstrate that Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis possess anti-inflammatory properties by modulating LPS-induced pro-inflammatory cytokine release (TNF-α and IL-6) in SIM-A9 microglial cell lines.

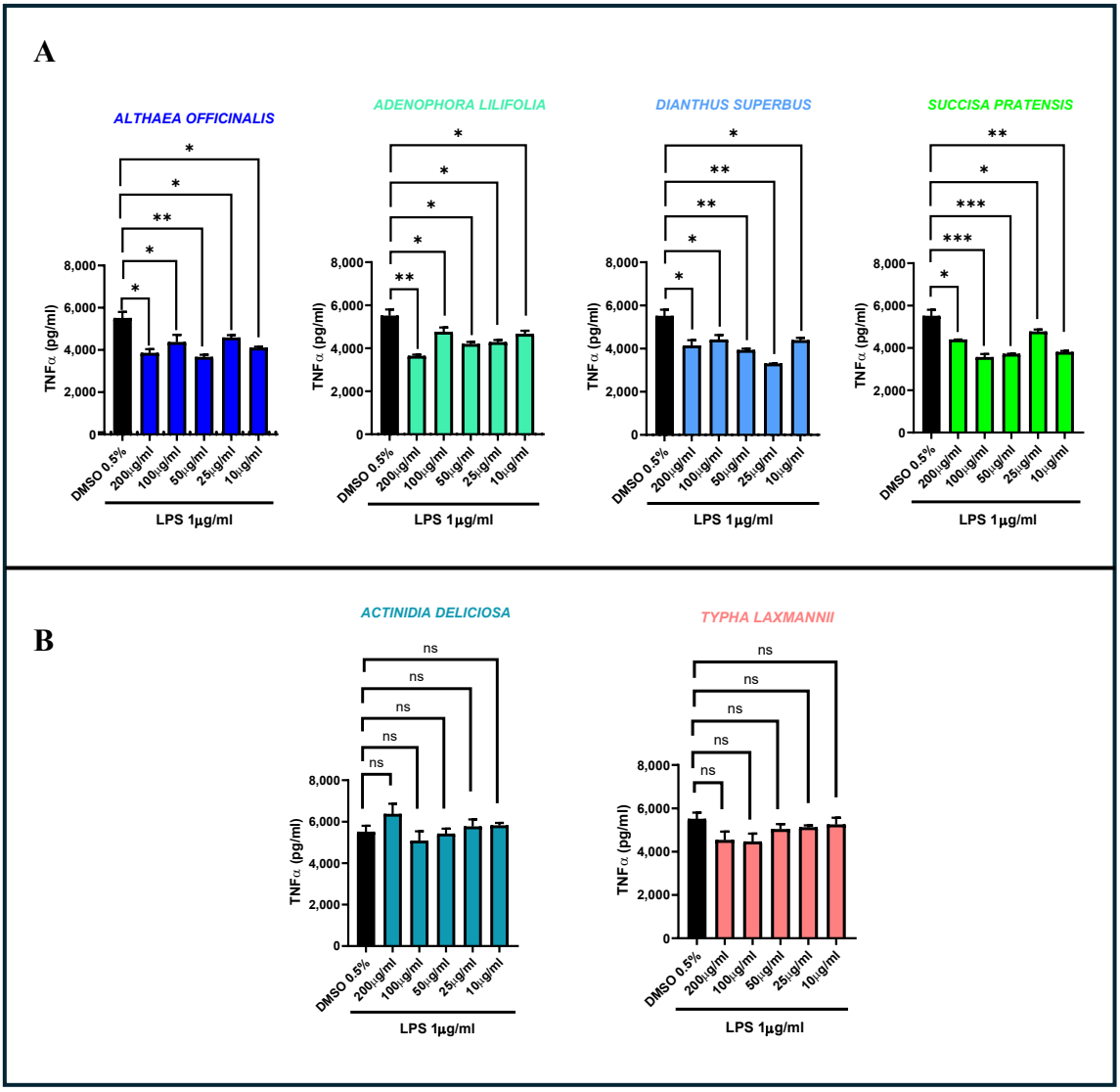


Figure 17. Plant extracts modulate in TNF-α release in LPS-treated SIM-A9 cells. SIM-A9 were pre-treated with the extracts at indicated concentrations for 24h, and next co-incubated with LPS (1 µg/mL) for additional 24h. The levels of TNF-α (measured in pg/mL) were assessed using an ELISA assay. **a)** Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis significantly reduced TNF-α secretion. **b)** Actinidia deliciosa and Typha laxmannii did not show any reduction in TNF-α levels. Data were presented as mean ± SEM, with statistical significance indicated as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, when compared to the 0.5% DMSO + LPS 1 µg/mL control.

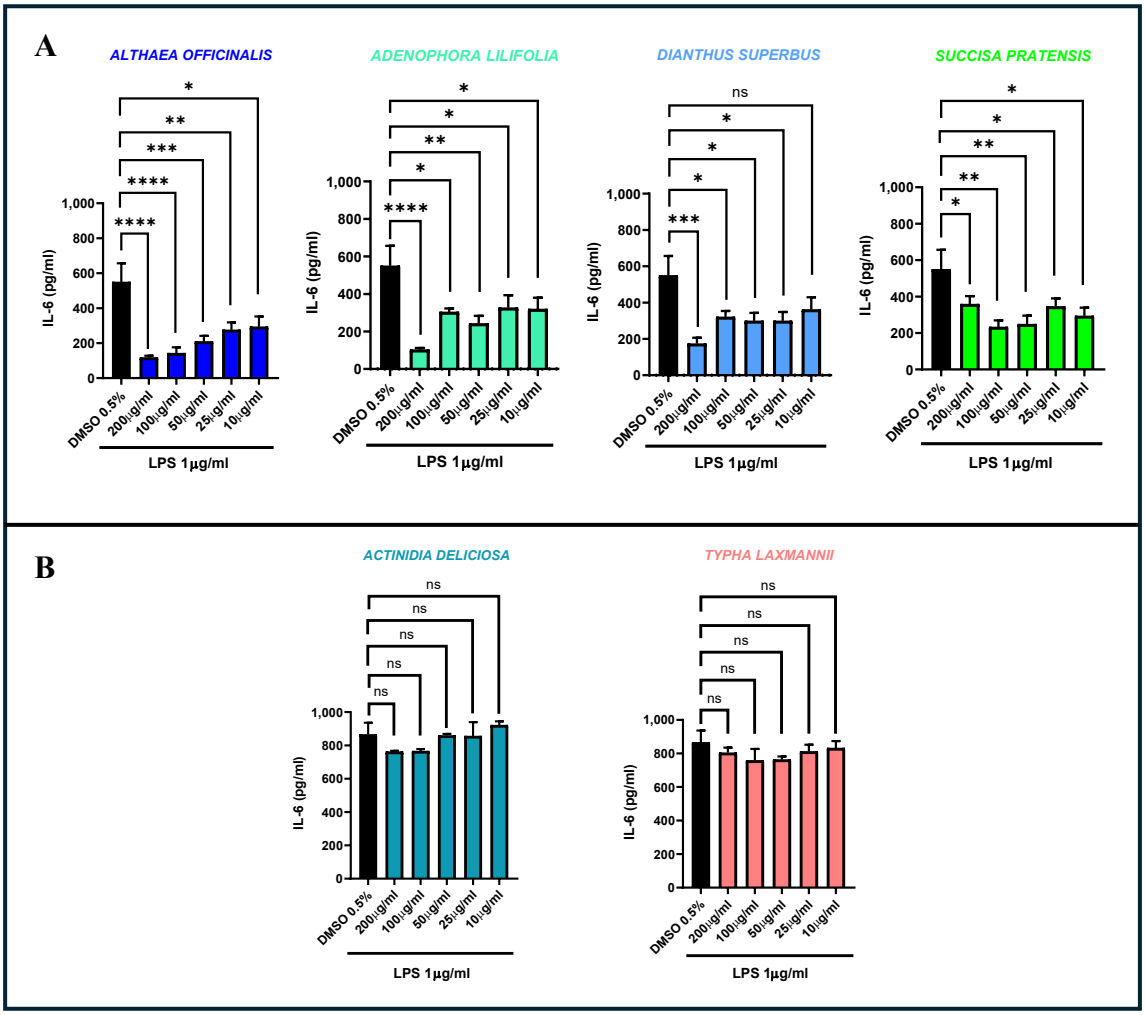


Figure 18. Plant extracts modulate IL-6 release in LPS-treated SIM-A9 microglial cells. a) The pro-inflammatory cytokine IL-6 was significantly decreased by extracts derived from Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis, indicating strong anti-inflammatory potential. **b)** Actinidia deliciosa and Typha laxmannii showed no significant changes in IL-6 levels. Values are presented as mean ± SEM. Statistical differences compared to the 0.5% DMSO + LPS control were noted as follows: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

7. Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis inhibit the release of TNF-α and IL-6 in LPS-treated primary microglia

The anti-inflammatory properties of the selected plant extracts, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis were further assessed in primary microglial cultures. Primary microglial cells were seeded at a density of 3×10^4 cells/well in 96-well plates. Cells underwent a 24-hour pre-treatment with each extract at concentrations of 10, 50, and 100 μg/mL, followed by co-treatment with LPS (1 μg/mL) for additional 24 hours. Subsequently, culture supernatants were collected for the quantification of TNF-α and IL-6 levels by ELISA. 0.5% DMSO + LPS was used as positive control. Following LPS (1μg/ml) stimulation, primary microglia exhibited a robust increase in both TNF-α and IL-6 secretion. Pre-treatment with all four plant extracts resulted in a significant reduction in TNF-α levels in LPS-activated primary microglia (**Figure 19**). Similarly, IL-6 secretion showed a notable decrease across all tested concentrations of Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis (**Figure 20**). Treatment with each plant extract alone for 24 or 48 h without LPS, had no effect on basal TNF-α or IL-6 secretion in SIM-A9 and primary microglia (**Figure 21**).

These findings validate the results obtained from SIM-A9 cells, reinforcing the conclusion that these plant-derived extracts possess significant anti-inflammatory properties. Specifically, their capacity to inhibit LPS-induced TNF-α and IL-6 production highlights their therapeutic potential for modulating neuroinflammatory responses in both immortalized and primary microglial models. Based on their

consistent anti-inflammatory effects in both SIM-A9 cells and primary microglia, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis were selected for further characterization. To gain insight into the phytochemical composition potentially underlying their biological activities, an untargeted UPLC–HRMS metabolomic analysis was performed.

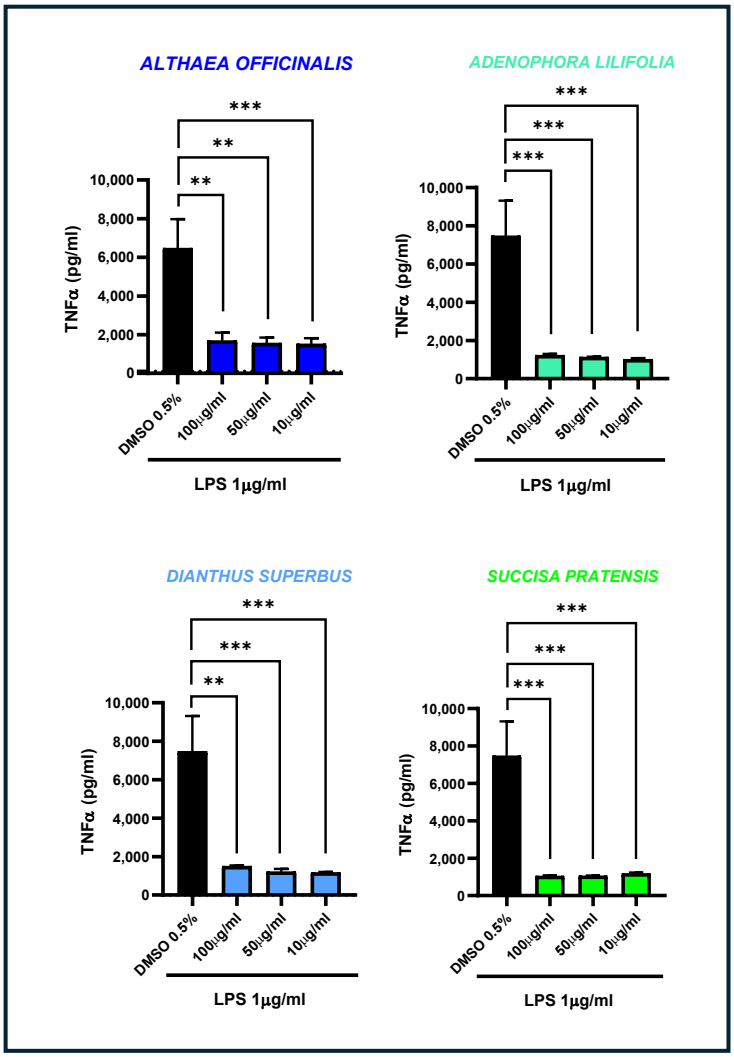


Figure 19. The inhibitory effects of plant extracts on TNF-α production in LPS-stimulated primary microglia. Primary microglial cells were pre-treated with plant extracts at the indicated concentrations (10, 50, and 100 µg/mL) for 24 hours, followed by a 24-hour co-treatment with 1 µg/mL LPS. TNF-α levels (pg/mL) in the culture supernatants were quantified using an ELISA assay. Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis significantly reduced LPS-induced TNF-α release compared to the control group (0.5% DMSO + LPS), demonstrating their anti-

inflammatory properties. Data are represented as mean ± SEM. Statistical analysis was conducted using one-way ANOVA followed by Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

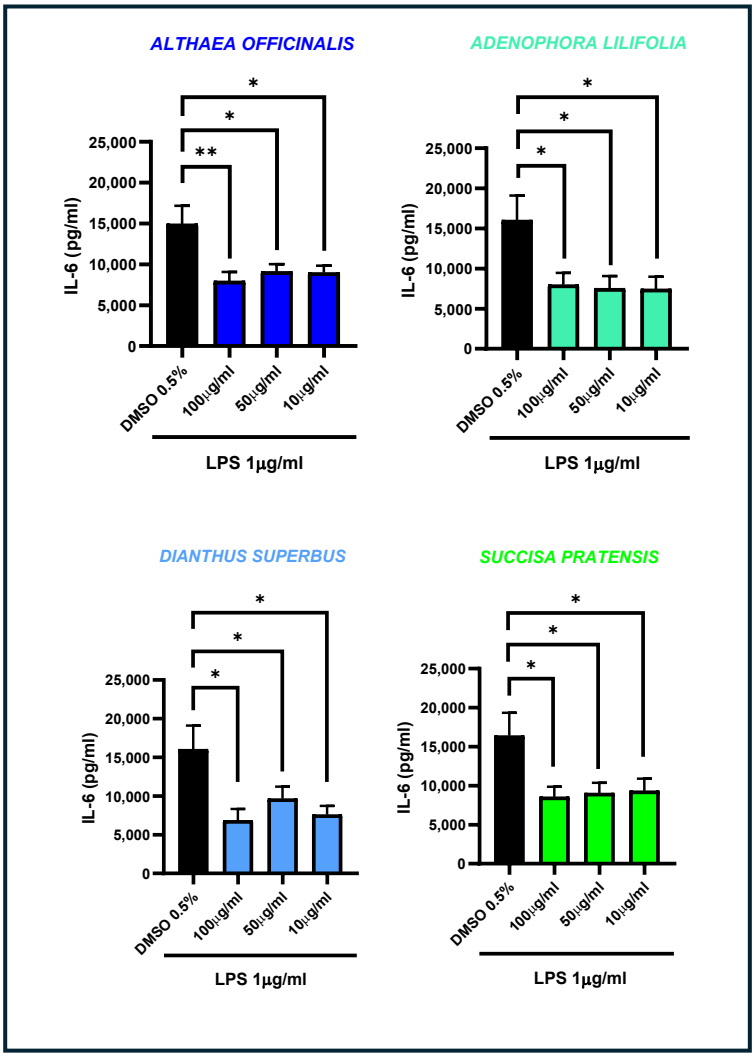


Figure 20. Plant extracts reduce the IL-6 levels in LPS-activated primary microglia. Treatment of primary microglial cells with Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis (10–100 µg/mL) before to LPS (1 µg/mL) stimulation resulted in significant reduction of IL-6 levels. Quantitative analysis by ELISA confirmed a significant reduction in IL-6 production across the tested dose range, underscoring the potent anti-inflammatory properties of these extracts. Data are shown as mean ± SEM. Significance: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$ versus LPS control (one-way ANOVA, Tukey's test).

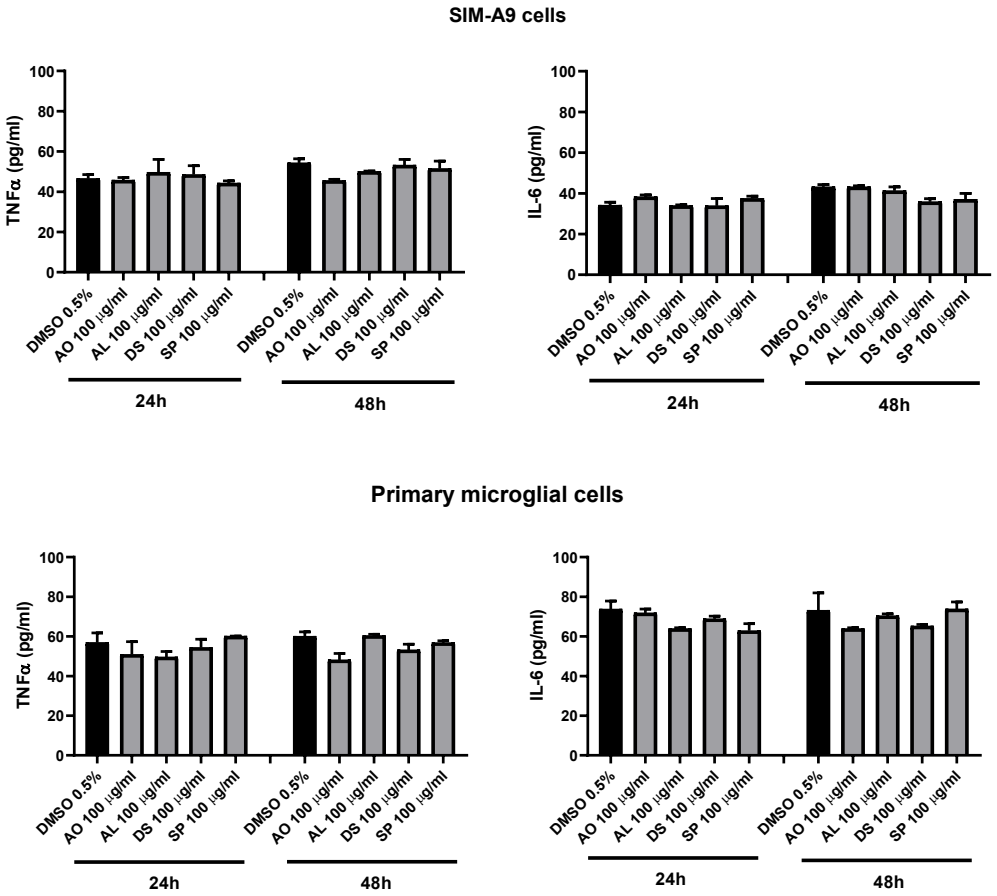


Figure 21. The plant extracts alone have no effect on basal pro-inflammatory cytokine release in microglial cells. SIM-A9 and primary microglia cells were treated with 100 µg/mL of Althaea officinalis (AO), Adenophora lilifolia (AL), Dianthus superbus (DS), and Succisa pratensis (SP) for 24 h and 48 h. DMSO 0.5% was used as a control. The basal levels of TNF-α and IL-6 were measured by ELISA. Data are presented as mean ± SEM (n=3), (One-way ANOVA, Tukey's test).

8. Metabolite profile of the 4 extracts

Leaf methanolic extracts of Althaea officinalis, Adenophora lilifolia, Succisa pratensis, and Dianthus superbus underwent untargeted metabolomic analysis utilizing Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry (UPLC–HRMS). **Figure 22** displays the base peak chromatograms (BPCs) of the samples, obtained in negative ion mode. The chromatographic study identified many bioactive compounds specific to each species, indicating their varied phytochemical composition.

In the Althaea officinalis extract, the chromatogram was predominantly characterized by flavonoids and phenolic derivatives, such as hypolaetin-8-O-glucuronide, kaempferol, luteolin, quercetin, and a sulfate-conjugated molecule designated as YL (sulfate). These derivates are recognized for their antioxidant and anti-inflammatory capabilities, aligning with the traditional therapeutic attributes of Althaea officinalis (Banaee et al., 2017; Bonaterra et al., 2020). The extract of Adenophora lilifolia exhibited many flavones and hydroxycinnamate derivatives, particularly methyl luteolin, tetrahydroxyflavone-C-rutinoside, and coumaroyl quinic acid, along with additional minor constituents. The metabolites, primarily from the flavonoid and phenolic acid groups, enhance the plant's documented antioxidant and anti-inflammatory properties (Kovács et al., 2024; Yoon et al., 2024). For Dianthus superbus, the chromatographic profile was defined by gypsogenic acid, madecassic acid, and tetrahydroxyflavone-C-diglucoside, in addition to many unidentified secondary metabolites. Gypsogenic acid and madecassic acid are triterpenoid saponins known for their antibacterial, wound-healing, and cytoprotective properties (Hou et al., 2023; Yuan et al., 2022; Yun et al., 2016a). The existence of these bioactive saponins and flavones underscores the phytochemical intricacy of Dianthus

superbus. The chromatogram of Succisa pratensis exhibited a diverse profile predominantly characterized by chlorogenic acid, luteolin, apigenin, quinic acid, dicaffeoylquinic acid, and akebia saponin D. The existence of phenylpropanoids (including chlorogenic and dicaffeoylquinic acids) and flavones (luteolin, apigenin) signifies substantial antioxidant potential, whilst akebia saponin D, a triterpenoid saponin, may enhance the plant's anti-inflammatory characteristics (Witkowska-Banaszczak et al., 2020; Witkowska-Banaszczak & Długaszewska, 2017).

Collectively, the UPLC–HRMS study demonstrated that each extract has a distinct molecular fingerprint, primarily including flavonoids, phenolic acids, and triterpenoid saponins. To evaluate their potential under disease-relevant conditions, the four selected extracts were evaluated for their ability to mitigate Aβ-induced neuroinflammatory responses in primary microglia by assessing TNF-α and IL-6 release.

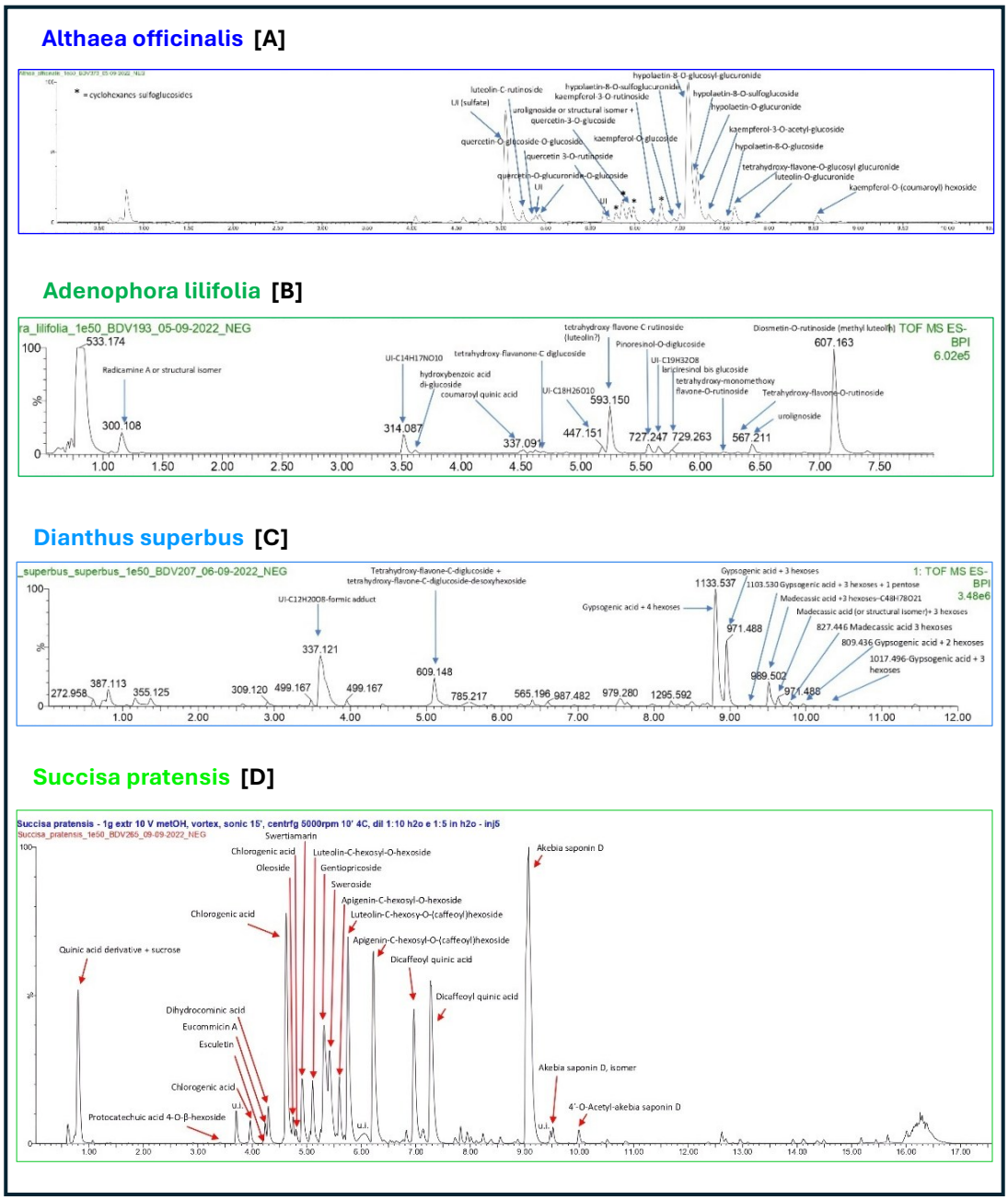


Figure 22. UPLC–HRMS chromatograms of plant extracts. BPCs of leaf methanol extracts from Althaea officinalis (A), Adenophora lilifolia (B), Dianthus superbus (C), and Succisa pratensis (D) were evaluated using UPLC–HRMS in negative ion mode. The chromatograms show different extraction profiles associated with major types of metabolites, such as flavonoids, phenolic acids, and triterpenoid saponins (Data provided by Prof. Guzzo's lab).

9. Detection of oligomeric forms of Amyloid beta 1-42 by Western Blot

Western blot analysis showed the presence of oligomeric Aβ1-42 assemblies under non-denaturing and non-reducing conditions. Soluble oligomeric Aβ1-42 samples were separated by electrophoresis on a 12% Bis-Tris NuPAGE gel using MES running buffer and transferred to PVDF membrane. Subsequently, membranes were incubated with the monoclonal antibody 6E10 (1:3000), which targets residues 1–16 of Aβ. The resulting immunoblot clearly displayed bands corresponding to monomeric (~4 kDa), trimeric (~12 kDa), and tetrameric (~16 kDa) species of Aβ1-42 (**Figure 23**). The figure shows in Lane 1 the molecular weights, in lanes 2-4 the increasing concentration of the oligomeric Aβ1-42 preparations (lane 2 1µg, lane 3 2µg and lane 4 4µg). This specific oligomeric pattern aligns consistently with previously reported profiles of low-molecular-weight Aβ1-42 assemblies identified under similar electrophoretic conditions (Costantini et al., 2005). These findings confirm that the Aβ1-42 preparation predominantly consisted of monomeric, trimeric, and tetrameric forms, with only trace formation of higher molecular weights (~40–60 kDa) and no detectable fibrillar structures after a 24h incubation at 4 °C.

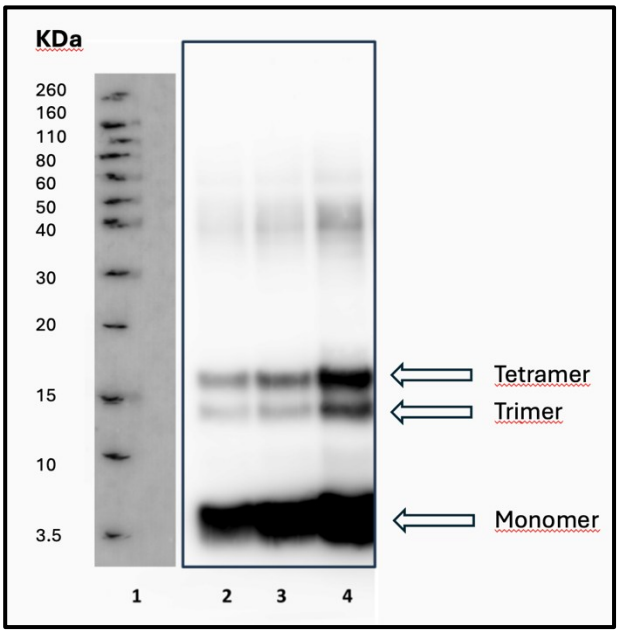


Figure 23. Western blot of oligomeric preparations of Aβ1-42. Representative immunoblot showing monomeric and oligomeric (trimeric and tetrameric) forms of soluble Aβ1-42 detected using the anti-Aβ monoclonal antibody 6E10 (recognizing residues 1–17). Samples were separated on precast 12% Bis-Tris NuPAGE gel and transferred on PVDF membranes under not reducing and not denaturing conditions. Detection was achieved using a horseradish peroxidase–conjugated secondary antibody (1:5000) and enhanced chemiluminescence system. Lane 1: molecular weight markers; lanes 2–4: increasing concentrations of oligomeric Aβ1-42 preparation, 1-2-4 µg respectively. Bands corresponding to Aβ monomer (about 4 kDa), trimer (about 12 kDa), and tetramer (about 16 kDa) are indicated by arrows.

10. Plant extracts attenuate Aβ-induced pro-inflammatory cytokine production in primary microglia

Previous studies have established that Aβ peptides trigger the release of pro-inflammatory mediators from microglia, thereby contributing to the neuroinflammation (Dheen et al., 2005; Moreira et al., 2024; Shukla & Sharma, 2011). The four plant extracts, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis, which demonstrated significant anti-inflammatory activity against LPS-induced responses, were selected to assess their capacity to modulate the Aβ-induced pro-inflammatory cytokines release in primary microglia.

Primary microglial cells were stimulated with non-toxic concentrations of oligomeric Aβ1-42 (5 μM) and fibrillar Aβ25-35 (10 μM). To assess optimal non-toxic concentrations, an MTT assay was conducted to evaluate the dose- response of oligomeric Aβ 1-42 (0.5, 1, 2.5, 5 and 10 μM) and fibrillar Aβ 25-35 (2, 5, 10 and 20 μM) on primary microglial viability. These concentrations, including Aβ1-42 (5 μM) and Aβ25-35 (10 μM) that we selected for subsequent experiments, did not compromise primary microglial cell viability as demonstrated by cytotoxicity assays shown in **Figure 24**. Microglia were cultured in 96-well plates at a density of 3×10^4 cells/well in DMEM/F-12 supplemented with 10% FBS for 24 hours. Subsequently, cells were then pre-treated with Althaea officinalis, Adenophora lilifolia, Dianthus superbus or Succisa pratensis, (50 μg/mL) for 24 hours in the same medium. Following pre-treatment, cells were co-stimulated with the plant extracts in combination with either Aβ1-42 (5 μM) or Aβ25-35 (10 μM) in DMEM/F-12 containing 1% FBS for an additional 24 hours. 0.5% DMSO + Aβ was used as positive control. Then, TNF-α and IL-6 levels were quantified in the culture supernatants by ELISA. The cell stimulation with oligomers of Aβ1-42 (5 μM) and DMSO resulted in a significant increase in TNF-α and IL-6 secretion by primary microglia; however, pre-treatment with Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis (each at 50 μg/mL) markedly reduced the levels of both cytokines in Aβ1-42 oligomers –stimulated cells (**Figure 25**). This indicates a strong inhibitory effect of all four extracts on Aβ1-42–induced pro-inflammatory responses. Similarly, when microglia were stimulated with fibrillar Aβ25-35, the cells exhibited a significant increase in TNF-α and IL-6 production. Pretreatment with Althaea officinalis, Adenophora lilifolia, Dianthus superbus, or Succisa pratensis (50 μg/mL) significantly attenuated this response, leading to

markedly lower cytokines levels compared to the positive control (**Figure 26**). Collectively, these findings demonstrate that all four plant extracts effectively suppressed the release of Aβ-induced pro-inflammatory cytokines in primary microglia under both experimental conditions, highlighting their potential therapeutic relevance in mitigating neuroinflammation.

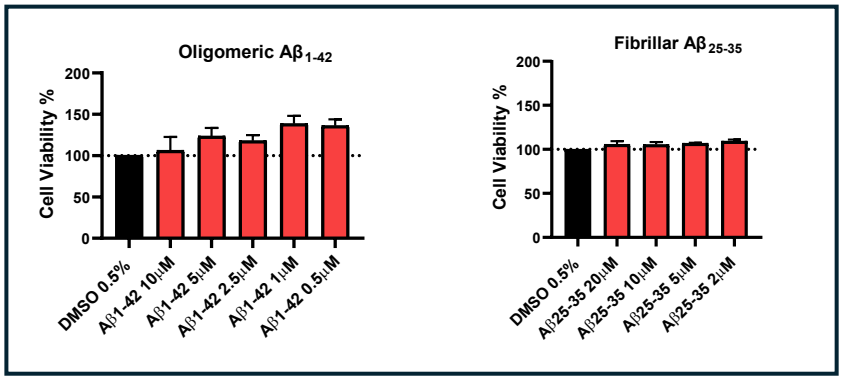


Figure 24. Effect of Aβ peptides on primary microglial viability. Primary microglial cells were stimulated for 24h with different concentrations of oligomeric Aβ1-42 (0.5, 1, 2.5, 5, and 10 μM) and fibrillar Aβ25-35 (2, 5, 10 and 20 μM). MTT assay was performed to evaluate cell viability and expressed as a percentage relative to 0.5% DMSO control. Data are shown as mean ± SEM.

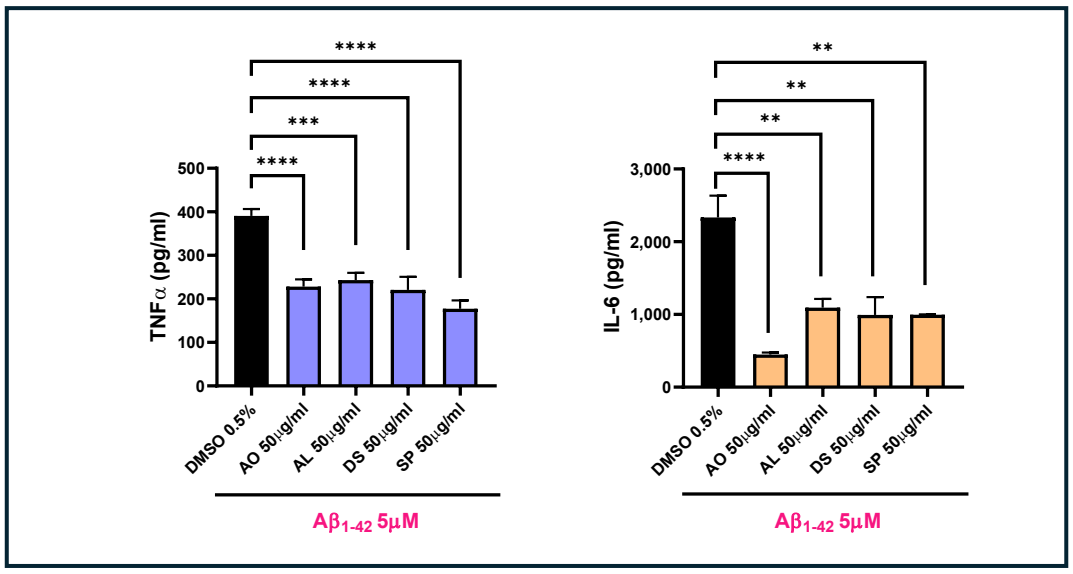


Figure 25. Plant extracts reduce the release of TNF-α and IL-6 in Aβ1–42-treated primary microglia. Primary microglia were pretreated with 50 µg/mL of Althaea officinalis (AO), Adenophora lilifolia (AL), Dianthus superbus (DS), or Succisa pratensis (SP) extracts prior to stimulation with oligomeric Aβ1-42 (5 µM). The concentrations of TNF-α and IL-6 (pg/mL) were quantified by ELISA. All four extracts significantly reduced cytokine levels compared to the positive control group (0.5% DMSO + Aβ1-42). Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

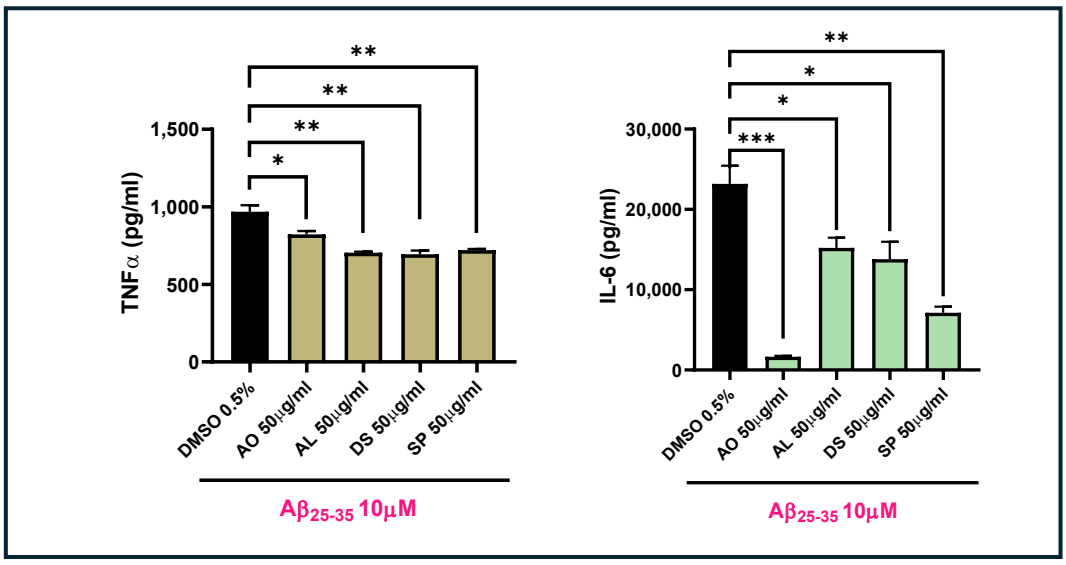


Figure 26. The inhibition of Aβ25-35-triggered pro-inflammatory cytokine production in primary microglia by plant extracts. Fibrillar Aβ25-35 (10µM) stimulation was carried out following pre-incubation of primary microglial cells with Althaea officinalis (AO), Adenophora lilifolia (AL), Dianthus superbus (DS), or Succisa pratensis (SP) extracts at a concentration of 50 µg/mL. The levels of TNF-α and IL-6 (expressed in pg/mL) in the culture supernatants were quantified by ELISA. The analysis demonstrated that all four plant extracts significantly reduced the release of both cytokines compared to the positive control group (0.5% DMSO + Aβ25-35). Data are presented as mean ± SEM. Statistical significance was assessed by one-way ANOVA with Tukey's multiple comparisons, where: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$).

11. Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis exhibit no cytotoxic effects on N2A cell line

Once establishing the anti-inflammatory properties of natural extracts on microglial cells, we assessed their toxicity and their neuroprotective effects on neuroblastoma cell line N2A. We assessed the cell viability of N2A cells following treatment with Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis using the colorimetric MTT assay. N2A cells were plated at a density of 2×10^4 cells per well and incubated overnight. Then, the cells were treated with different concentrations (10, 25, 50, 100, and 200 µg/mL) of the plant extracts for 24 hours, after which cell viability was measured. 0.5% DMSO as the control and Staurosporine (1 µM) as a positive control, respectively. The results indicated that all four extracts did not exert cytotoxic effects across the tested concentrations following 24 hours of exposure (**Figure 27**). The extracts were subsequently selected for the evaluation of their neuroprotective effects against glutamate-induced neurotoxicity.

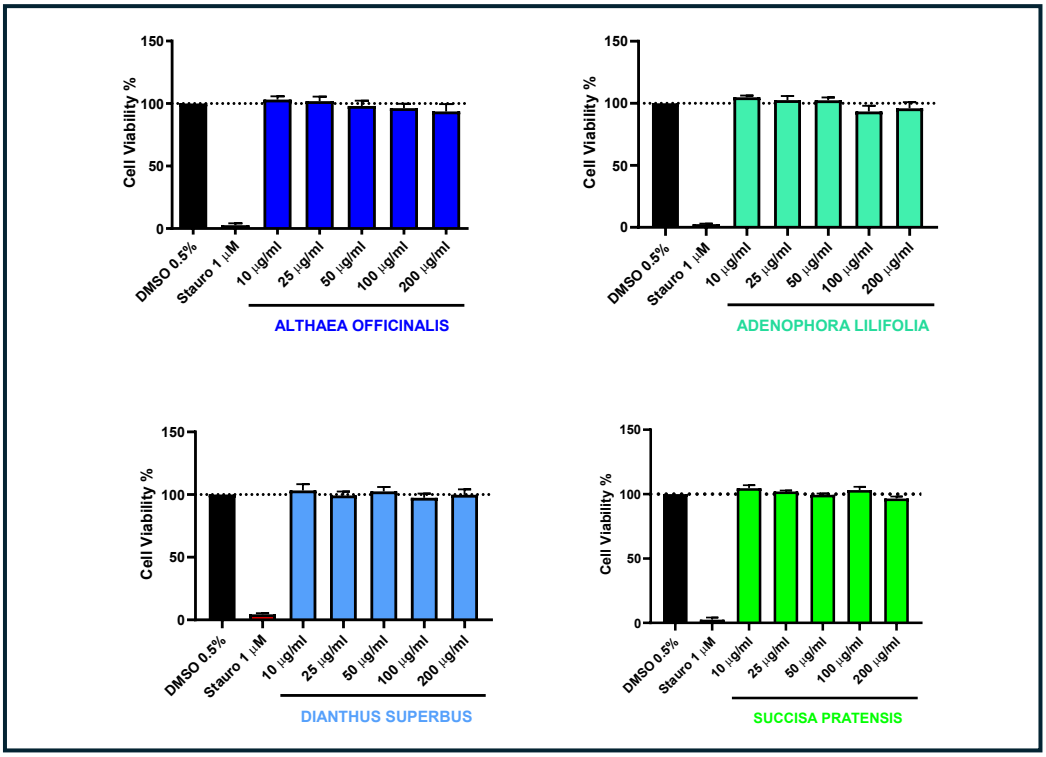


Figure 27. Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis exhibit no toxic effects on N2A cell viability. N2A cells were exposed to Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis at different concentrations ranging from 10, 25, 50, 100 to 200 μg/mL for 24 h. Cell viability was subsequently assessed utilizing the MTT assay. No cytotoxic effects were observed for any of the tested extracts across the entire concentration range. 0.5% DMSO was used as control, while 1 μM staurosporine was used as the positive control. Data are presented as mean ± SEM.

12. The effect of plant extracts on glutamate-induced neurotoxicity in N2A cells

The neuroprotective potential of Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis was evaluated in glutamate-induced N2A cells. Cells were prepared by seeding at a density of 2×10^4 cells per well. Subsequently, cells received a 24-hour pretreatment with the plant extracts across a concentration range of 10, 25, 50, 100, and 200 μg/mL, followed by a 4-hour co-exposure to 50 mM glutamate to induce excitotoxic cell damage. A positive control group, treated only with glutamate, consistently exhibited approximately ≥50% cell death, while cells maintained in culture medium with 0.5% DMSO served as the negative control. Cell viability was quantitatively assessed using the MTT assay. The experimental findings indicated that Althaea officinalis significantly mitigated glutamate-induced cytotoxicity at all concentrations except 10 μg/mL, with maximal neuroprotection was evident at 200 μg/mL (**Figure 28a**). Similarly, Dianthus superbus displayed dose-dependent protective activity at 25, 100, and 200 μg/mL, leading to an enhancement of N2A cell viability when compared to the glutamate-treated cells (**Figure 28b**). In contrast, neither Adenophora lilifolia nor Succisa pratensis demonstrated neuroprotective effects at any concentrations tested (**Figure 28c, d**). Overall, these

results collectively suggest that Althaea officinalis and Dianthus superbus exert neuroprotection against glutamate-induced toxicity in N2A cells at higher concentrations.

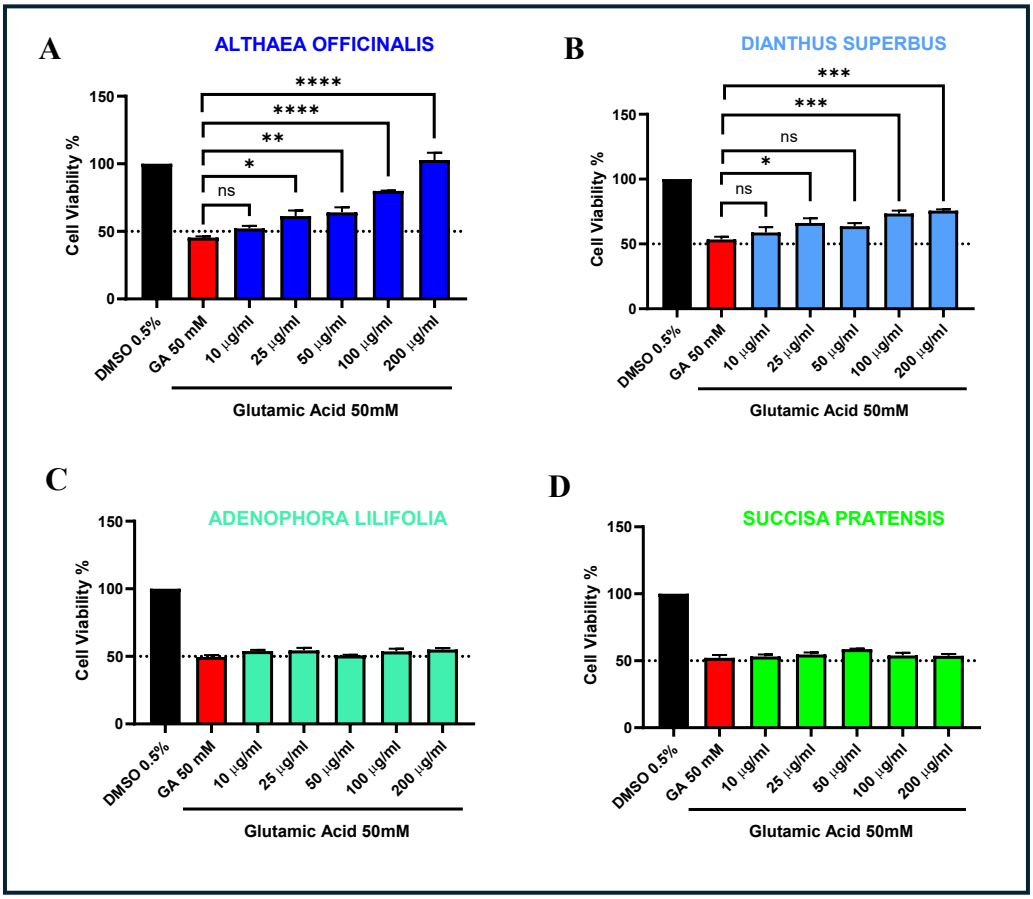


Figure 28. Neuroprotective effects of plant extracts against glutamate-induced toxicity in N2A Cells. N2A cells were pretreated with Althaea officinalis, Adenophora lilifolia, Dianthus superbus, or Succisa pratensis extracts (10–200 µg/mL) for 24 h, then exposed to 50 mM glutamate (GA) for 4 h. Cell viability was measured using the MTT assay. Althaea officinalis **(a)** and Dianthus superbus **(b)** significantly protected the cells in a dose-dependent manner, especially at higher doses, while Adenophora lilifolia **(c)** and Succisa pratensis **(d)** had no significant effects. 0.5% DMSO was used as negative control, while glutamate as positive control. Data are presented as mean ± SEM. Statistical significance was compared to the GA 50mM control with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$.

Collectively, a comprehensive evaluation identified Althaea officinalis, Adenophora lilifolia, Dianthus superbus, and Succisa pratensis demonstrated safety and effectively reduced the release of NO and pro-inflammatory cytokines induced by LPS and Aβ. Furthermore, Althaea officinalis and Dianthus superbus provided protection against glutamate-mediated neurotoxicity. Therefore, Althaea officinalis and Dianthus superbus can be considered as promising best candidates with significant anti-inflammatory and neuroprotective properties, highlighting their potential for addressing neuroinflammation and neuronal degeneration, commonly associated with neurodegenerative disorders, such as AD.

DISCUSSION

The present study is part of the National Biodiversity Future Center (NBFC)'s project, the first National Research and Innovation Center dedicated to biodiversity and funded by the MUR through European Union funds (NextGenerationEU). The objective of this project was to develop strategies for monitoring, conserving, restoring and valorising the biodiversity of species across Italian regions. This effort contributes to European Union target of protecting 30% of Italy's territory by 2030 (Cena & Labra, 2024; National Biodiversity Future Center, n.d.). Within this framework, the present study, supported by the PNRR (Piano Nazionale di Ripresa e Resilienza, CN5) focused on exploring Italian biodiversity for applications in human health. The main goal was to identify promising natural compounds with anti-inflammatory and neuroprotective properties that could potentially be developed into new drugs for the treatment of neurodegenerative disorders, such as AD. To achieve this objective, twenty-three methanolic leaf extracts prepared in Prof. Guzzo's laboratory (Dept. of Biotechnology, University of Verona), and then evaluated the cytotoxicity of these extracts (Althaea officinalis, Salvia pratensis, Petasites paradoxus, Adenophora lilifolia, Allium lusitanicum, Dianthus superbus, Succisa pratensis, Typha laxmannii, Acalypha virginica, Actinidia deliciosa, Allium angulosum, Cistus monspeliensis, Diospyros kaki, Gratiola officinalis, Heuchera sanguinea, Lythrum salicaria, Sanguisorba officinalis, Verbascum thapsus, Artemisia abrotanum, Glandularia tenera, Malva alcea, Pulsatilla montana, and Rosa canina) in the SIM-A9 mouse microglia cell line. Since microglia play a crucial role in neuroinflammation and AD progression, they represent the most promising therapeutic targets for development of novel interventions. Seventeen out of the

twenty-three methanolic plant leaf extracts (Althaea officinalis, Salvia pratensis, Adenophora lilifolia, Allium lusitanicum, Dianthus superbus, Succisa pratensis, Typha laxmannii, Acalypha virginica, Actinidia deliciosa, Cistus monspeliensis, Diospyros kaki, Heuchera sanguinea, Lythrum salicaria, Sanguisorba officinalis, Verbascum thapsus, Malva alcea, and Rosa canina) displayed a non-toxic profile across a range of concentrations (10, 25, 50, 100, and 200 µg/mL). Subsequently, extracts derived from Althaea officinalis, Salvia pratensis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, Actinidia deliciosa, Sanguisorba officinalis, Verbascum thapsus, Acalypha virginica, and Diospyros kaki also demonstrated no significant cytotoxic effects in primary microglial cultures. Testing these natural extracts in primary cells provide an *in vitro* model much more faithful to what occurs *in vivo*, unlike immortalized cell lines. Primary microglia were isolated from neonatal 3xTg-AD mice, one of most widely used transgenic models of AD. This primary culture method allowed us to assess the preliminary evaluation of immunomodulatory potential of the extracts within a genetically relevant AD context. Then, to study the anti-inflammatory properties of natural extracts, we assessed their effect on the modulation of NO production and the release of pro-inflammatory cytokines in activated microglia cells, simulating the inflammatory condition present in AD. Consequently, strategies aimed at reducing microglial activation and proinflammatory mediators may represent a promising therapeutic approach for targeting the pathological mechanisms underlying AD (Block & Hong, 2005; Crescitelli et al., 2023; Heneka & O'Banion, 2007).

NO has a dual role in AD: on one side it can serve as a neuroprotective factor, which reduce associated risk factors, and on another side it can act as a harmful agent in AD, potentially worsening the pathology (Azargoonjahromi, 2023). The production of NO

by chronic activation of microglia is a feature of neuroinflammation and plays a significant role in the progression of AD and other neurodegenerative disorders, such as Parkinson's disease (Heneka et al., 2015; Hickman et al., 2018; Lull & Block, 2010). In the current investigation to mimic the inflammatory condition, we stimulated microglial cells with LPS for 24 hours, triggering their activation with the release of pro-inflammatory mediators, such as TNF-α and IL-6, and the production of NO. Notably, six natural extracts, Althaea officinalis, Adenophora lilifolia, Dianthus superbus, Succisa pratensis, Typha laxmannii, and Actinidia deliciosa, significantly affect the NO production, causing a reduction in LPS-stimulated microglia cell line and primary cells cultures. These results suggest that these natural extracts may have potential anti-inflammatory effects, acting on nitric oxide, which is known to have various modulating effects on inflammation and plays a role in regulating immune responses. Several other plant extracts, such as Curcuma Comosa, Ficus Religiosa and Mucuna Pruriens have been reported to diminish NO production in LPS-activated microglial cells (Jantaratnotai et al., 2006; Jung et al., 2008; Rachsee et al., 2021). While these plants do not belong to the same families as our extracts, they do contain similar bioactive metabolites — namely flavonoids, phenolic acids and saponins, which are well known for their strong anti-inflammatory effects. Our study makes an original contribution by identifying these extracts as novel candidates for the modulation of NO-mediated neuroinflammation, as the anti-inflammatory activity of our specific extracts have not been previously investigated.

The activated microglia also release pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, which are key contributors to neuroinflammation and neurodegeneration in AD (Glass et al., 2010; Heneka et al., 2015; Miao et al., 2023). These inflammatory mediators exacerbate neuronal damage and synaptic dysfunction, while also

promoting Aβ aggregation and tau hyperphosphorylation through the activation of pathways including NF-κB, MAPK, and JAK/STAT (Kacimi et al., 2011; Rather et al., 2021; Yadav et al., 2024). Moreover, abnormal cytokine release by microglia can promote the infiltration of peripheral immune cells into the central nervous system, thereby exacerbating inflammatory responses (X. Chen et al., 2023; Mundt et al., 2019). Specifically, TNF-α has been implicated in neuronal apoptosis, impairing synaptic function, and increasing Aβ production (Liao et al., 2004; K. M. Park & Bowers, 2010), whereas IL-6 exacerbates tau hyperphosphorylation, impairs synaptic function, and promotes further Aβ accumulation (Kerkis et al., 2024; Mrak, 2001; W.-Y. Wang, 2015). As a result, the continuous release of these cytokines by overactive microglia contributes to exacerbate neuroinflammatory processes, which in turn enhance neurodegeneration in AD. Thus, the regulation of cytokine production represents a crucial therapeutic strategy for mitigating microglia-mediated pathology. Our investigations revealed that extracts derived from Althaea officinalis, Adenophora lilifolia, Dianthus superbus and Succisa pratensis markedly reduced the secretion of TNF-α and IL-6 induced by LPS in both SIM-A9 cells and primary microglia. These results suggest that these extracts may modulate key inflammatory signalling pathways, potentially including TLR4-mediated NF-κB and MAPK activation. Based on phytochemical profiling of Althaea officinalis, Adenophora lilifolia, Dianthus superbus and Succisa pratensis, we noted the presence of molecules that are recognized to inhibit the NO production, interfering with the inducible iNOS, and the production of pro-inflammatory cytokines via NF-κB and MAPkinases pathway. UPLC–HRMS chromatographic analysis indicated that the extracts are abundant in flavonoids, phenolic acids, and triterpenoid saponins. Althaea officinalis presents high concentration of a glycosylated flavonoid, hypolaetin-8-O-glucosyl-glucuronide, and

other flavonoids derivates, such as luteolin, quercetin, kaempferol. Adenophora lilifolia exhibits several derivates of flavone-O-rutinoside, Dianthus superbus contain Gypsogenic acid and madecassic acid derivates, while in Succisa pratensis we found high concentration of triterpenoid, such as Akebia saponin, and different flavonoid derivates. The effects of flavonoids to suppress NO and pro-inflammatory cytokines production in activated macrophages are well documented (Bonaterra et al., 2020; Cho et al., 2003; Hämäläinen et al., 2007; Kao et al., 2011). The triterpenoids, such as madecassic acid and Akebia saponin, show the same anti-inflammatory effects as flavonoids. Madecassic acid isolated from Centella asiatica inhibits the production of NO, prostaglandin E_2 (PGE_2), TNF-α, interleukin-1β (IL-1β), and IL-6 via the downregulation of NF-κB activation in LPS-stimulated RAW 264.7 murine macrophage cells (Won et al., 2010). Akebia saponin is also an abundant constituent of the rhizome of Dipsacus asper Wall (DAW), a well-known traditional Chinese medicine for its analgesic and anti-inflammatory properties. This saponin has been shown to attenuate neuroinflammation *in vivo* by suppressing the activation of microglia and the expression of TNF-α, IL-1β, cyclooxygenase-2 (COX-2). Additionally, it has been found to improve the memory impairments in the Aβ1–42-induced dementia model, suggesting that Akebia saponin may be a potential agent for AD treatment (X. Yu et al., 2012). As previously shown, the anti-inflammatory effects of the single isolated molecular components from natural extracts are well-documented, but in this study, we can't exclude their synergistic effects with other constituents, thus, further investigations are needed to explore them.

To simulate the neuroinflammatory and pathological conditions of AD *in vitro*, microglial cells were treated with Aβ1-42 and Aβ25-35 peptides to induce significant pro-inflammatory responses. In this experimental context with relevance to AD, the

treatment with Althaea officinalis, Adenophora lilifolia, Dianthus superbus and Succisa pratensis significantly inhibited the release of TNF-α and IL-6 in Aβ-stimulated microglia. Notably, the effects of these four natural extracts on microglial cells stimulated with Aβ has not been previously studied. This suggests their potential to reduce the release of inflammatory mediators by Aβ and positions them as possible modulators of microglia–mediated neuroinflammatory processes in AD treatment.

Further underscoring their therapeutic potential, the systemic anti-inflammatory efficacy of these plant genera is well-established across diverse *in vivo* models of acute and chronic inflammation. For instance, Althaea officinalis formulations have been demonstrated significant reductions in paw edema, gastric mucosal injuries, and localized vascular swelling in rodent models, which correlated with decreased leukocyte infiltration and down-regulation of systemic inflammatory indices (Hage-Sleiman et al., 2011; Zaghlool et al., 2019). Similarly, Dianthus superbus extracts have strong *in vivo* anti-inflammatory activity by significantly reducing airway inflammation, cellular infiltration and vascular permeability in mouse models of allergic asthma and vascular leaking (Shin et al., 2012). In addition, oral administration of Adenophora root extracts have been reported to protect against tissue injury and reduce inflammatory cell recruitment in mice with induced organ inflammation, significantly suppressing the expression of pro-inflammatory cytokines such as TNF-α and IL-6 (S.-M. Park et al., 2022). Likewise, triterpenoid saponins and flavonoids discovered in Succisa pratensis are also compounds well-known for anti-inflammatory and immunomodulation actions with hydrophilic extracts shown to inhibit pathways impacting cell damage (Anwar et al., 2026; Witkowska-Banaszczak & Długaszewska, 2017). Collectively, these findings further support the rationale for

studying these extracts as possible regulators of microglial neuroinflammatory processes in AD model.

In addition to their established anti-inflammatory properties, we evaluated the neuroprotective properties of the natural extracts. Two of the natural extracts, Althaea officinalis and Dianthus superbus, exhibited neuroprotective effects against glutamate excitotoxicity. Althaea officinalis exhibited the most potent neuroprotective impact by maintaining neuronal survival after glutamate exposure, while Dianthus superbus had modest protective efficacy.

The neuroprotective efficacy of Althaea officinalis was previously demonstrated by Rezaei & Alirezaei in 2014. In this study, the authors used an aqueous extract of Althaea officinalis leaves, which exerts neuroprotective effects in the rat model of 6-OHDA-induced hemi-Parkinsonism. The treatment improved behavioural deficits, reduced oxidative stress and protected neurons of the substantia nigra pars compacta. The authors hypothesized that the neuroprotective effect of Althaea officinalis is attributed to its content of polyphenols, flavonoids, and antioxidant compounds, which can scavenge ROS and reduce the oxidative stress implicated in 6-OHDA neurotoxicity (Rezaei & Alirezaei, 2014; Yun et al., 2016a). Whereas Yun et al. demonstrated that two metabolites isolated from Dianthus superbus (4-hydroxy-benzeneacetic acid and 4-methoxybenzeneacetic acid) significantly exerted a neuroprotective effect against glutamate-induced cell death in hippocampal neuronal HT22 cells, supporting a potential use of Dianthus superbus or its metabolites in neurodegenerative diseases associated with excitotoxicity and oxidative stress (Rezaei & Alirezaei, 2014; Yun et al., 2016a).

A key limitation of this study is the translational gap between the experimental models utilized and the complex pathology of human AD. The *in vitro* and primary cell models focused on acute inflammatory stimulation (e.g., LPS or Aβ exposure) to assess transient cellular responses; however, AD is defined by a chronic, progressive, and multifactorial neuroinflammatory process that develops over decades. Accordingly, although these findings provide valuable insight into the molecular pathways and cellular responses modulated by the investigated plant extracts, caution is warranted when translating results obtained from acute *in vitro* models to the complex and progressive pathophysiology of human AD (Heneka et al., 2015; Leng & Edison, 2021).

Overall, our *in vitro* studies have demonstrated the anti-inflammatory effectiveness of natural extracts, in both immortalized and primary microglial cells, as well as their neuroprotective ability in the neuroblastoma cell line. Further studies are needed to validate these neuroprotective properties in primary neuronal culture. Based on our findings, Althaea officinalis emerged as the most promising candidate, showcasing strong anti-inflammatory and neuroprotective efficacy. Future investigations should focus on isolating the bioactive compounds responsible for these effects, together with a detailed investigation of the underlying molecular mechanisms, including signalling pathways involved in neuroinflammation and neuroprotection. Importantly, additional *in vivo* studies using experimental models of AD will be essential in the future to validate the therapeutic efficacy of Althaea officinalis in regulating neuroinflammatory responses by reducing microglial activation and improving cognitive functions, therefore confirming the effectiveness of Althaea officinalis as a plant-based therapeutic approach for AD.

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