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INVESTIGATION ON *BACOPA MONNIERI* (L.) WETTST. AND *CAMELLIA SINENSIS* (L.) KUNTZE, ALONE AND IN COMBINATION, AS CANDIDATES FOR POTENTIAL NEUROTHERAPEUTIC AGENTS

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Abstract

Bacopa monnieri (BM) and *Camellia sinensis* (CS) are natural plants used as nutraceutical products with neuroprotective properties. BM is a memory and learning enhancer (Brimson et al., 2021), while CS is a supplement with high antioxidant activity. Several previous studies have investigated the neurotherapeutic effect on a single extract, but there is currently no evidence regarding the combination of BM and CS. This study was a preliminary investigation to explore the effects of BM and CS, both alone and in combination, on microglial activation, and to explore their antioxidant potential in neurons. Using lipopolysaccharide (LPS)-stimulated BV2 microglia as an *in vitro* model of neuroinflammation, we report that both extracts can significantly attenuate the expression of pro-inflammatory cytokines TNF-α and Interleukin-6, and the chemokine MCP-1, in a concentration-dependent manner as well as the combination of BM and CS. Further, nitrite concentration (Griess assay) and iNOS expression (Western immunoblot analysis) reveal that both test agents, alone and in combination, can significantly attenuate microglial nitric oxide (NO) production in response to LPS. Interestingly, the combination of BM and CS reduced H_2O_2-induced cytotoxicity in N2a cells and ameliorated neuroinflammatory reactions in LPS-stimulated microglia BV2 cells via inhibitions of NF-κB and MAPK with an additive effect.

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List of abbreviations

Abbreviation	Definition
5-HT2A	Serotonin 2A
5-HT6	Serotonin 6
6-OHDA	6-hydroxy dopamine
ABTS+	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
Ach	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ADHD	Attention deficit hyperactivity disorder
AF64A	Ethylcholine aziridinium ion
Akt	Protein kinase B
ALS	Amyotrophic Lateral Sclerosis
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	One-way analysis of variance
Arg-1	Arginase-1
ASK1	Apoptosis signal-regulating kinase 1
ATP	Adenosine 5′-triphosphate
Aβ	Amyloid β-peptide
BAN	*Bacopa monnieri* encapsulated in lactoferrin conjugated PEG-PLA-PCL-OH based polymersomes
BBB	Blood-brain barrier
BM	*Bacopa monnieri* (L.) Wettst.
BMECs	Brain microvascular endothelial cells
BuChE	Butyrylcholinesterase
C	Catechin
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
CAMs	Cell adhesion molecules
CCK8	Cell Counting Kit 8
CCL2	Chemokine (C-C motif) ligand 2

Abbreviation	Definition
CCL5	Chemokine (C-C motif) ligand 5
CCR2	C-C chemokine receptor type 2
CD16 Fc (Fcγ receptor III)	Cluster of differentiation molecule 16
CD206	Cluster of differentiation molecule 206, mannose receptor
CD32	Cluster of differentiation molecule 32
CD64	Cluster of differentiation molecule 64
CD86	Cluster of differentiation molecule 86
CE	Chloroform extract
CNS	Central nervous system
COMT	Catechol-O-methyl transferase
COVID-19	coronavirus disease 2019
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CREB	cAMP response element-binding protein
CS	*Camellia sinensis* (L.) Kuntze
CSF	Cerebrospinal fluid
DAMPs	Damage-associated molecular patterns
DAT	Dopamine transporters
D-GAL	D-galactose
DM2	Diabetes mellitus type 2
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DP	Diazepam
DR5	Death receptor 5
EC	Epicatechin
ECG	Epicatechin gallate
EGC	Epigallocatechin
EGCG	Epigallocatechin gallate
ERK	Extracellular signal-regulated kinase
FIZZ1	Found in Inflammatory Zone 1
GABA	Gamma-aminobutyric acid

Abbreviation	Definition
GAE	Gallic acid equivalent
GC	Gallocatechin
GCG	Gallocatechin gallate
GFP	Green fluorescent protein
GluR1	Glutamate receptor 1
GluR2	Glutamate receptor 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HD	Huntington's disease
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
IDO	Indoleamine 2,3-dioxygenase
IFN-ɣ	Interferon-ɣ
IFNs	Interferons
IGF-1	Insulin-like growth factor-1
IKK	The IκB kinase
IL-1	Interleukin-1
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-1β	Interleukin-1β
IL-23	Interleukin-23
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IκB	Inhibitor of nuclear factor kappa B
JNK	The c-Jun N-terminal kinase
LD	Levodopa
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein

Abbreviation	Definition
L-DOPA	L-dihydroxyphenylalanine
l-NNA	Nw-Nitro-l-arginine
LPS	Lipopolysaccharide
LRRK2	Leucine-rich repeat kinase 2
MAGL	Monoacylglycerol lipase
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MCP-1	Macrophage chemoattractant protein-1
MEKK1	Mitogen-activated protein kinase kinase kinase 1
MEKK4	Mitogen-activated protein kinase kinase kinase 4
mGluR8	Metabotropic glutamate receptor 8
MK2/3	Mapkap Kinase 2/3
MK-801	Dizocilpine
MLK2	Mixed-lineage kinase 2
MLK3	Mixed-lineage kinase 3
MPP+	1-methyl-4-phenylpyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
MSK1	Mitogen- and stress-activated kinases-1
MSK2	Mitogen- and stress-activated kinases-2
Na_2-EDTA	Ethylenedinitrilotetraacetic acid disodium salt dihydrate
$NaNO_2$	Sodium Nitrite
n-BE	n-Butanol extract
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
nHE	n-Hexane
NLRP3	Leucine-rich repeat and pyrin domain-containing protein 3

Abbreviation	Definition
NLRs	Nucleotide-binding and oligomerization domain-like receptors
NMDA	N-methyl-D-aspartate
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
OGD	Oxygen-glucose deprivation
OHSCs	Organotypic hippocampal slice cultures
PAMPs	Pathogen-associated molecular patterns
PARK7	Parkinson's disease protein 7
PD	Parkinson's disease
PEP	Prolyl endopeptidase
PGE2	Prostaglandin E2
PGHS	Prostaglandin H synthases
PI	Phosphatase inhibitor
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
PKB/PKA	Protein kinase A/protein kinase B
PKC	Protein kinase C
PKC-α	Protein kinase C-α
PNS	Peripheral nervous system
PRAK	p38 regulated/activated kinase
PRRs	Pattern Recognition Receptors
PTZ	Pentylenetetrazole
PVDF	Polyvinylidene difluoride
RhoGAP	RHO GTPase-activating proteins
RIG-I	Retinoic acid-inducible gene I-like receptors
RIPA	Radioimmunoprecipitation assay
ROS	Reactive oxygen species
SAPKs	Stress-activated protein kinases
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
SBME	Standardised BM extract

Abbreviation	Definition
SCI	Spinal cord injury
SDAT	Senile dementia of Alzheimer's type
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SNAP	S-nitroso-N-acetyl-penicillamine
SNpc	Substantia Nigra pars compacta
SOD	Superoxide dismutase
STZ	Streptozotocin
T3	Triiodothyronine
TAB2	TAK1 kinase binding protein 2
TAIK-1	Transforming growth factor-activated kinase 1
TAK1	TGF-beta-activated kinase 1
TAO1	Thousand-and-one amino acids 1
TAO2	Thousand-and-one amino acids 2
TBS	Tris-buffered saline
TBS-T	1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent
TDP-43	Transactive response DNA-binding protein-43
TEAC	Trolox equivalent antioxidant capacity
TGF-β1	Tumour growth factor-β1
TGY motif	Thr-Gly-Tyr motif
Th2	T helper 2 cells
TJs	Tight junction ultrastructure
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNF-α	Tumour necrosis factor-α
TPC	Total phenolic content
Tpl2	Tumor progression locus 2
TPY motif	Thr–Pro–Tyr motif
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
TREM2	Triggering receptor expressed on myeloid cells 2
TrkA	Tyrosine protein kinase receptors A

Abbreviation	Definition
TxY motif	Threonine-x-tyrosine motif
VPA	Valproic acid
WHO	World Health Organization
YM-1	Chitinase 3-like 3

Chapter 1

Introduction

Neurological disorders are described medically as conditions that affect the brain as well as the nerves and spinal cord found throughout the human body. A variety of symptoms may result from structural, biochemical, or electrical abnormalities in the brain, spinal cord, or other nerves (Organization, 2006). There are numerous recognised neurological disorders, some of which are common but the majority of which are rare, such as Alzheimer's disease (AD), Parkinson's disease (PD), Multiple sclerosis (MS), and epilepsy. Mental disorders are psychiatric illnesses or diseases that manifest primarily as abnormalities of thought, emotion, or behaviour, causing either distress or functional impairment; they are also considered neurological disorders (Patel et al., 2016). Congenital neurological disabilities are neurological disorders that are present at birth. Included are epilepsy, learning disabilities, neuromuscular disorders, autism, attention deficit hyperactivity disorder (ADHD), brain tumours, and cerebral palsy (Bennett & Karnes, 1998). According to a new study based on WHO data, mortality rates due to neurological diseases in the developed world increased from 1989–1991 to 2012–2014. The reported increase in neurological disease mortality, which was most significant in the United States, demonstrates the emergence of neurological diseases as a global health risk (Pakpoor & Goldacre, 2017). In both developed and developing countries, 25% of individuals develop one or more mental or behavioural disorders at some point in their lives. These disorders are typically diagnosed with the same reliability and precision as the majority of common physical disorders. Some disorders can be eliminated, and all can be successfully managed and treated (Joy, 2021). Recent research indicates that inflammation/neuroinflammation plays a crucial role in neurological disorders. In various neurodegenerative diseases, such as Huntington's disease (HD), Parkinson's disease (PD), and Alzheimer's disease (AD), the role of inflammatory mediators in the central nervous system (CNS) has been studied. Brain immune cells, known as microglia, initiate the earliest and most significant responses to injury or stress. Positive and negative peripheral and CNS symptoms are produced by inflammation. Neuroinflammation refers to inflammation within the CNS. Inflammation of the periphery may affect the central nervous system and result in neurological issues (Muthuraju, Zakaria, Karuppan, & Al-Rahbi, 2020).

Neuroinflammation is a condition which is the theoretical cause of various types of neurological disorders, including Alzheimer's disease (AD), Parkinson's disease (PD), Multiple Sclerosis (MS), epilepsy, Amyotrophic Lateral Sclerosis (ALS), Huntington's disease (HD), ischemic/traumatic brain injury (TBI), spinal cord injury (SCI), migraine and depression (Gilhus & Deuschl, 2019). The characteristics of neuroinflammation are associated with the release of cytokines. Microglia, which are effector cells of the innate immune system that reside in the central nervous system (CNS), play crucial roles in brain homeostasis. In a healthy brain, microglia exert neuroprotective functions by providing innate immunity, eliminating apoptotic cells, and modifying synaptic connectivity (Leng & Edison, 2021). Activation of microglia can result in substantial pathogenic alterations and neurobehavioral consequences, such as depression and cognitive impairments (Norden & Godbout, 2013). Microglia can be biologically polarised to either establish a classical (pro-inflammatory; M1) or alternative (anti-inflammatory and pro-healing; M2) phenotype (David & Kroner, 2011). In pathological conditions, microglial cells replicate the activation of monocytes in peripheral tissues by activating in a distinctive manner. Thus, lipopolysaccharide (LPS), Aβ peptide related to Alzheimer's disease, interferon-ɣ (IFN-ɣ), HIV-related molecules, prion protein, ATP, and cytokines are well-documented microglial cell activators (Nakamura, 2002). Lipopolysaccharide (LPS) is the most intriguing cytokine inducer in microglia (Z. Chen et al., 2012). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a strain of coronavirus that causes coronavirus disease 2019 (COVID-19), which is a prime illustration of neuropsychiatric complications associated with acute neuroinflammation. Extensive inflammation with cytokine storm was observed in the olfactory bulbs and medulla oblongata of the brain, reflecting the loss of smell and many CNS symptoms (Banerjee & Viswanath, 2020). There are more than 300 authentic cytokines, including chemokines, lymphokines, interferons (IFNs), and growth factors. The pleiotropic nature of cytokines is a characteristic shared by all cytokines; a given cytokine may induce proliferation in one cell type but growth inhibition in another (Becher, Spath, & Goverman, 2017). The most prominent characteristics of microglial phenotypes are the expression of distinct cell surface receptors and the release of soluble factors with distinct functions. A new schema was required to describe the phenotype of microglia. The M1 phase, which can be induced *in vivo* and *in vitro* by microbe-associated molecular patterns (MAMPs) such as LPS, granulocyte-macrophage colony-stimulating factor (GM-CSF) and inflammatory cytokine such as IFN-ɣ, has been established as a descriptor for classically activated microglial

cells. Microglia cannot return to a resting state unless anti-inflammatory triggers released by other microglia are first received. These additional stages have been categorised as alternative activation and have a number of healing responses (Xu et al., 2003). The M2a phenotype is stimulated by IL-4 or IL-13 and participates in Type 2 T helper cell (Th2) responses to allergens or parasite killing and encapsulation. Agonists of the IL-1 receptor and Toll-like receptor (TLR) induce the M2b phenotype, which promotes Th2 differentiation and antibody production. IL-10 and glucocorticoid hormones induce the M2c phenotype, which plays a role in immunoregulation, matrix deposition, and tissue remodelling (Franco & Fernández-Suárez, 2015). Microglia with the M1 phenotype exhibits upregulation of CD16 Fc receptors, CD32, CD64, CD86, interleukin (IL)-1β, IL-6, IL-12, IL-23, tumour necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and chemokine (C-C motif) ligand 5 (CCL5). Microglia with the M2 phenotype exhibit increased expression of insulin-like growth factor (IGF)-1, arginase (Arg)-1, triggering receptor expressed on myeloid cells 2 (TREM2), mannose receptor (CD206), chitinase 3-like 3 (Ym-1) and FIZZ1 (Figure 1). CD86 has been mentioned to initiate the pro-inflammatory response in microglia (David & Kroner, 2011). In contrast, neuroglia-derived macrophage chemoattractant protein (MCP)–1 and tumour growth factor (TGF)– β1 were the most abundant chemokine and multifunctional cytokine in brain tissues, as determined by immunocytochemical analysis (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). MCP-1 affects neuronal loss, which is essential to the development of neurological disorders such as Parkinson's disease, and Alzheimer's disease, via BBB permeability and macrophage polarization (Sawyer et al., 2014). In the brains of AD-afflicted animals and humans, activated microglia and pro-inflammatory cytokines have been identified. Leucine-rich repeat-containing protein 3 (NLRP3) inflammasome, a key regulator of the innate immune system, is significantly upregulated in Alzheimer's disease brains with the emergence of amyloid and tau pathology. Furthermore, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a transcription factor that regulates numerous signals, is the most perturbed pathway in AD due to its essential role in neuroinflammation, which is closely associated with neuron growth and synaptic plasticity (C. Wang et al., 2022). The ability of nuclear translocation of cytoplasmic complexes to activate the NF-κB/Rel transcription family to induce transcription of proinflammatory genes plays a central role in inflammation. This pathway is triggered by pathogen- or stress-related signals (Paul P Tak & Gary S Firestein, 2001). Cyclooxygenases are enzymes (specifically Cox-1 and Cox-2) that catalyse the process of

converting arachidonic acid to bioactive prostaglandins (PGs). Cox-1 is the universal housekeeping enzyme, whereas Cox-2 is activated by mitogens to contribute to inflammation. Cox-2 is mainly expressed at the postsynaptic dendrites and excitatory terminals of the cortical and spinal cord neurons in the brain. In response to synaptic excitation, neuronal Cox-2 is activated to produce PGE2, the predominant Cox-2 metabolite in the brain, which stimulates glutamate release and neuronal firing in a retrograde manner. Cox-2 is also involved in the metabolism of new endocannabinoids derived from 2-arachidonoyl-glycerol, allowing them to be modulated on presynaptic terminals. Since neuroinflammation is initially triggered by the induction of glial Cox-2 expression, the homeostatic function of neuronal Cox-2 in synaptic transmission and plasticity is crucial for memory and anxiety (Rawat, Kukal, Dahiya, & Kukreti, 2019). In order to develop more effective therapies, current research focuses on identifying substances that prevent mitochondrial dysfunction, oxidative stress, and inflammation. The current neuroprotective agents have been evaluated for the clinical outcome, but the result is disappointing. The alternative or new therapeutics agent has been considered to be the new insight of scientific study.

Nutraceuticals are non-pharmaceutical substances with beneficial effects. They include such as herbal extracts, prebiotics, probiotics, and symbiotics. The field of nutraceuticals is currently selecting treatments for various diseases (Nasri, Baradaran, Shirzad, & Rafieian-Kopaei, 2014). Many compounds have been identified as constituting nutraceuticals, including vitamins, amino acids, minerals, trace elements, carbohydrates, herbs or herbal extracts, or other necessary nutrients (Kapoor, Jamwal, Shukla, & Gandhi, 2020). There are over 1000 compounds within the nutraceutical category (Williams, Mohanakumar, & Beart, 2015). Nutraceuticals have been investigated for their potential efficacy, especially in disease prevention. Some neurological disorders, such as Alzheimer's, lack precise pharmacological treatment. Neuro-nutraceuticals refer to a broad range of compounds derived from natural sources found in food, herbal products, and dietary supplements that include both nutritional and protective properties to enhance brain health and disease prevention. Overall, nutritional supplements are commercial products that are consumed in addition to food for the purpose of obtaining excess health-promoting nutrients. Brain supplements are used mainly for cognitive and memory improvement (Kumar, Konar, Garg, Kaul, & Wadhwa, 2021).

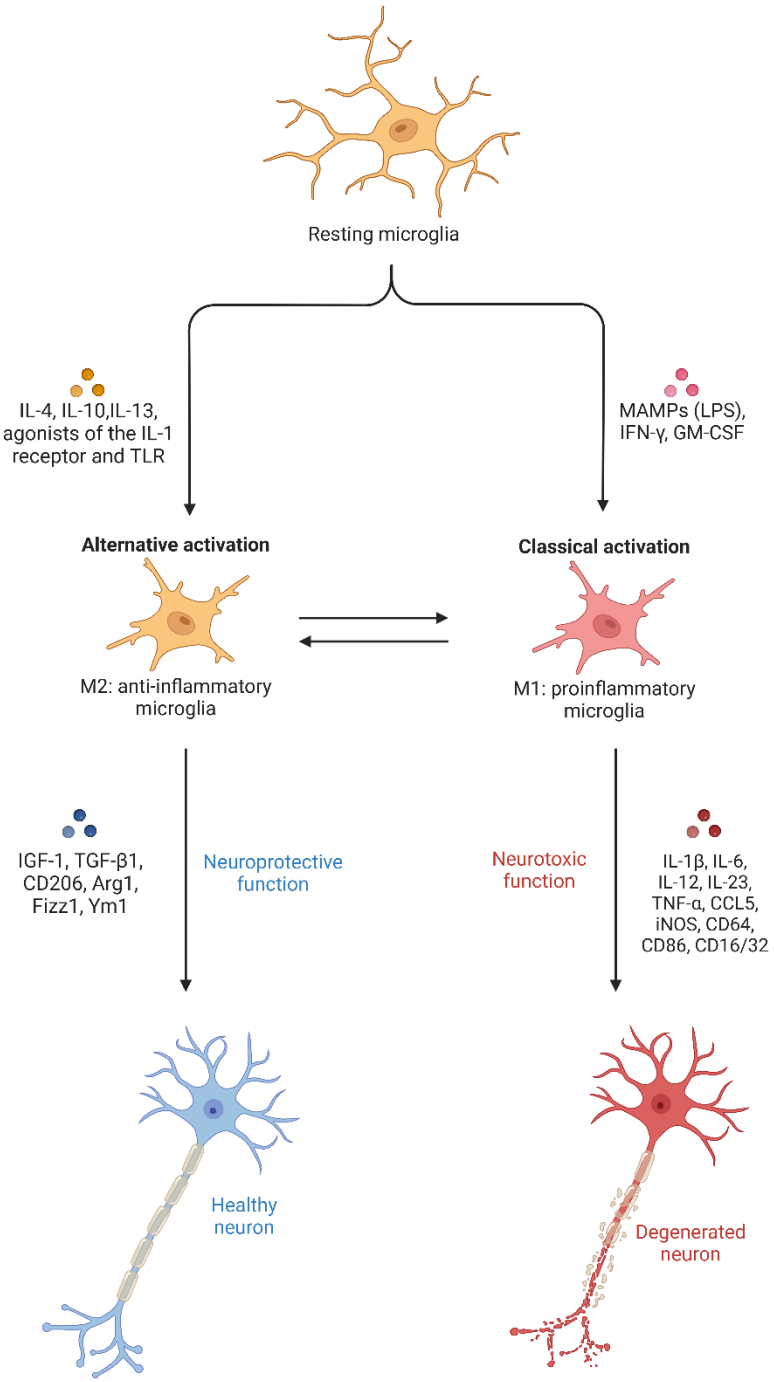


Figure1. The microglia activation pathway. Adapted from "Microglia Activation" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

Nutraceuticals are also considered an alternative as a protective agent. In Asia, traditional medicine has been used singly and in combination as the therapy of choice for traditional remedies. In regulating the central nervous system, herbal crude extracts and their bioactive phytochemicals, such as flavonoids, alkaloids, and saponins, play an important role. These phytoconstituents have anti-inflammatory, antioxidant properties, neuroprotective, and immunomodulatory, and they promote the growth of beneficial microorganisms that influence the gut-brain axis. Regulation of neurogenesis, neuritogenesis, redox signalling pathways, synthesis and degradation of neurotransmitters, and synaptic transmission is attributed to a direct effect on gene expression profile, epigenetic change, and metabolism (Howes, Perry, Vásquez-Londoño, & Perry, 2020). The intriguing herbal medicines that have shown neuroprotective effects are as follows: *Haematococcus pluvialis*, *Undaria pinnatifida*, *Helicteres angustifolia*, *Vitis vinifera*, *Curcuma longa*, *Withania somnifera*, *Bacopa monnieri*, and *Camellia sinensis* (Akbarialiabad, Dahroud, Khazaei, Razmeh, & Zarshenas, 2021) (Kumar et al., 2021) (Satyam & Bairy, 2022).

Bacopa monnieri (L.) Wettst. (BM) has been used to treat neurological disorders, specifically as a memory booster (Dubey & Chinnathambi, 2019). Asians believe BM is a miracle herbal medicine used as a brain tonic. BM has a report on antioxidant activity in the brain (Bhattacharya, Bhattacharya, Kumar, & Ghosal, 2000), preventing neurotoxicity (Jyoti, Sethi, & Sharma, 2007), and anti-inflammatory in microglia cells (R. Jeyasri, Muthuramalingam, Adarshan, Shin, & Ramesh, 2022). It is also known as a nootropic herb and improves brain function by aiding in the repair of injured neurons, neuronal synthesis, and the restoration of synaptic activity. BM contains the bacosides A and B, saponins A, B, and C, alkaloids brahmine, nicotinine, herpestine, triterpenoid saponins, stigmastanol, β-sitosterol, betulinic acid, D-mannitol, stigmasterol, α-alanine, aspartic acid, glutamic acid, serine, and pseudojujubogen (Rajendran Jeyasri, Muthuramalingam, Suba, Ramesh, & Chen, 2020). Bacopaside I, the chemical constituent of BM, has a neuroprotective effect against cerebral ischemia-induced injury. The protective mechanism may involve increasing antioxidant levels and enhancing cerebral energy metabolism (X. Liu et al., 2013). In subjects with memory loss, BM was able to produce modest improvements, whereas only a few neuropsychological tests demonstrated statistical significance. The ability of BM, in combination with other plant extracts, significantly reduces the effects of Alzheimer's disease and depression (Brimson et al., 2021).

Camellia sinensis (L.) Kuntze (CS), or green tea, is a herbal medicine containing high levels of catechin, an antioxidant-rich compound that benefits neurodegenerative and cognitive diseases. (Afzal et al., 2022). CS is famous as a drink in various areas in Asia, especially Japan, a country with a high rate of green tea consumption. Epigallocatechin gallate (EGCG) is the most important bioactive compound of catechins. Additionally, Japanese and Chinese green tea contains catechin, epigallocatechin (EGC), epicatechin gallate (ECG), and epicatechin (EC). These compounds can be found in abundance in unfermented teas such as green tea (Ide, Matsuoka, Yamada, Furushima, & Kawakami, 2018). EGCG interacts with misfolded proteins such as amyloid beta-peptide (Aβ), which is associated with Alzheimer's disease, and α-synuclein, which is associated with Parkinson's disease. EGCG may provide significant potential benefits for neurodegenerative disease drug discovery (Gonçalves, Sodero, & Cordeiro, 2021). The Ohsaki Cohort 2006 Study reported that the consumption of green tea significantly reduces the incidence of dementia. (Tomata et al., 2016).

According to a recent study, both BM and CS contain a chemical constituent that has the potential to be studied as a novel therapeutic for neurological disease as a single treatment but not in combination. The goal of this study was to assess the neurological potential of BM and CS alone and in combination against inflammation in an LPS-induced microglia activation model by measuring the presence of cytokines (IL-6, TNF-α), chemokines (MCP-1), Nitric Oxide content by Griess assay, and the expression of iNOS, COX-2, p38, p-JNK, and p-NF-κB p65 in western blots (Figure 1). Furthermore, investigate the neuroprotection in the hydrogen peroxide (H_2O_2)-induced neuronal injury model by measuring the cell viability on CCK8 assay and lactate dehydrogenase (LDH) activity to determine if the combination has a synergistic effect and can serve as potential neuroprotective agents.

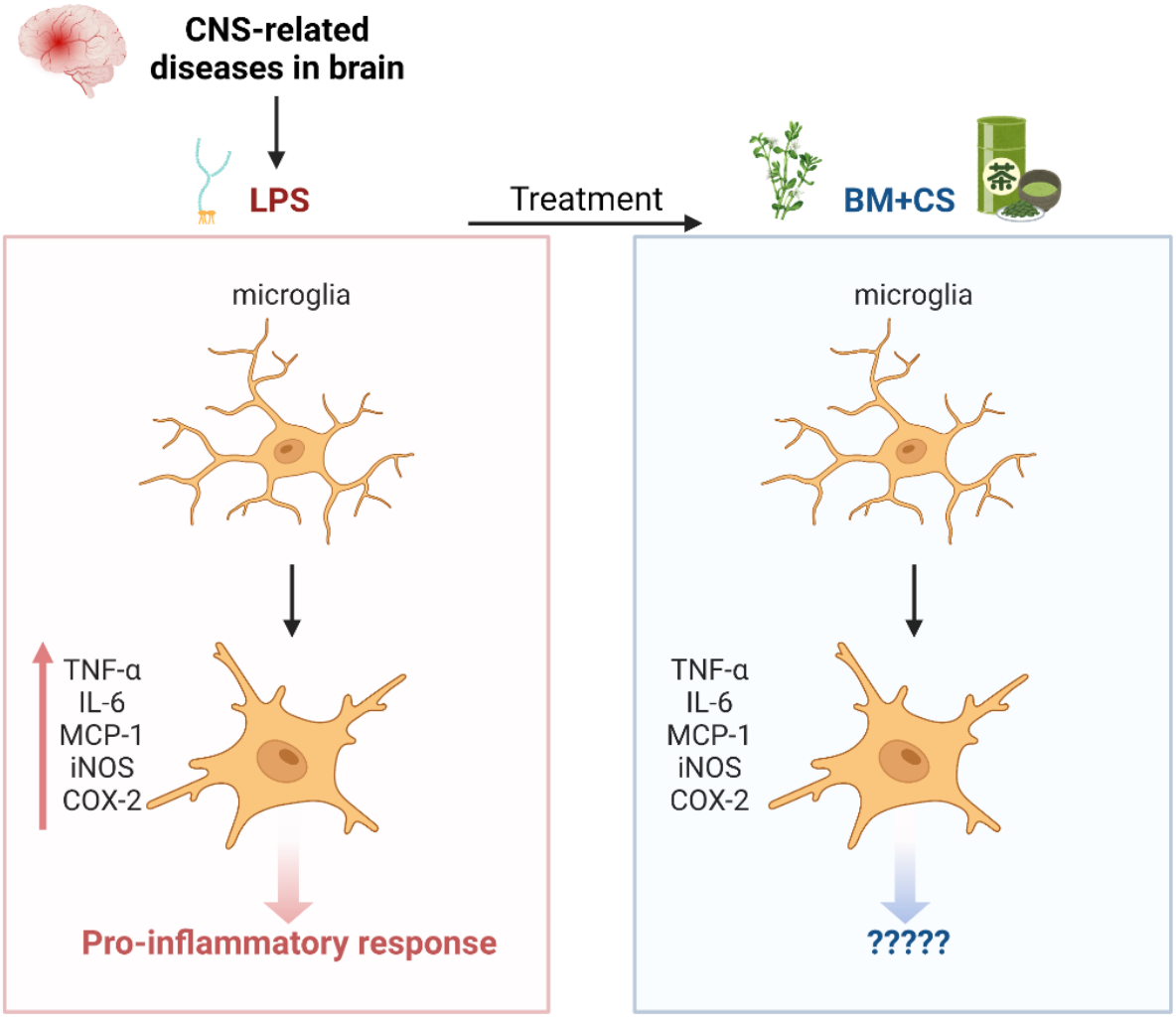


Figure 2. A conceptual framework of the neurological potential of BM and CS alone and in combination against the inflammation in an LPS-induced microglia activation model. Adapted from "Anti-inflammatory Drug Treatment" by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

Chapter 2

Literature Reviews

Nutraceuticals

Nutraceuticals are products that are used for medical purposes in addition to their nutritional value. A nutraceutical product is a substance that possesses physiological benefits or provides protection against chronic disease. Nutraceuticals may be used to enhance health, delay the ageing process, prevent chronic diseases, increase life expectancy, or support the body's structure or function. (Nasri et al., 2014) Nutraceuticals may include isolated nutrients, nutritional supplements, and diets in addition to genetically altered "designer" foods, herbal items, and processed foods, including cereals, soups, and beverages (Andlauer & Fürst, 2002). Sometimes, the line between nutraceuticals and functional foods is murky as an illustration when a phytochemical or phytochemical extract is added to a food formulation. The phytochemical or phytochemical extract in a capsule will constitute a new nutraceutical (Espín, García-Conesa, & Tomás-Barberán, 2007). Common methods of classifying nutraceuticals include food sources, mechanism of action, chemical composition, etc. Nutraceutical food sources are entirely natural and can be classified as (L. Das, Bhaumik, Raychaudhuri, & Chakraborty, 2012) natural or traditional; based on the natural source, it can be categorised as products derived from plants, animals, minerals, or microorganisms and unnatural or non-traditional refers to nutraceuticals that have been manufactured using biotechnology (Nwosu & Ubaoji, 2020).

Traditional nutraceuticals include a variety of naturally occurring substances that provide additional health benefits and can be classified according to the:

1. Chemical Constituents

    1.1. Nutrients: Amino acids, fatty acids, minerals, and vitamins identified as nutritional functions are the nutrients. The majority of foods contain vitamins that aid in the treatment of illnesses. Minerals are found in plants, animals, and dairy products. Foods containing fatty acids such as omega-3 PUFAs are potent inflammation regulators, maintain brain function, and reduce lipid deposition.

1.2. Herbals: The majority of these contain analgesic, anti-inflammatory, astringent, antipyretic, and antiarthritic properties. Several of the herbs contain flavonoids with a diuretic, carminative, and antipyretic properties, such as apiol and psoralen. Peppermint contains the active ingredient menthol, which helps treat colds and influenza. A number of plants contain tannin, which is believed to aid in the management of depression, common cold, anxiety, coughs, hypertension, and asthma, whereas proanthocyanadin (condensed tannin), which is present in some herbal remedies, is useful in the treatment or prevention of cancer, abscesses, and urinary tract infections (B. Chauhan, Kumar, Kalam, & Ansari, 2013).

1.3. Phytochemicals: Phytochemicals are naturally occurring bioactive compounds found in plants, and their presence and secretions vary from plant to plant. Terpenoids, polyphenols, phenolic constituents, alkaloids, carotenoids, phytosterols, saponins, and fibres are the most prevalent classes. The antioxidant activities, effect of cell differentiation, increased activity of detoxifying enzymes, effect of DNA metabolism, maintenance of DNA repair, increase in apoptotic cell death of cancer cells, decrease in cell proliferation, etc., play a crucial role in human health (Thakur, Singh, & Khedkar, 2020).

2. Nutraceutical Enzymes

These enzymes originate from the plant, animal, and microorganism sources. Without enzymes, our bodies would be unable to function at their optimum level. Enzyme supplements in the diet eliminate the symptoms of medical problems such as blood glucose disorders, gastrointestinal problems, and obesity.

2.1 Chemical Constituents

3. Probiotic Microorganisms.

Probiotics are "live microorganisms that confer a health benefit on the host when administered in adequate amounts." Therefore, probiotic cultures should be formulated so that they can reach the target site in the host after surviving processing, storage, and gastrointestinal transit while retaining high viability and adequate numbers. However, there

is no consensus on what an adequate intake of live microorganisms should be. Concerns have also been raised regarding probiotic side effects, particularly in immunocompromised individuals, those with an abnormal gastrointestinal mucosal barrier, patients with post-surgical procedures, and premature infants. If present in high concentration, probiotics can negatively affect the balance between anti- and pro-inflammatory cytokines and other cellular functions, resulting in altered long-term immune responses in individuals with immune disorders (Siciliano et al., 2021).

Non-Traditional Nutraceuticals, the bioactive molecules in food products are engineered to produce health-promoting products. They can be categorised as either fortified or recombinant nutraceuticals.

1. Fortified Nutraceuticals

   These are nutraceuticals derived from agricultural breeding or with additional nutrients and/or ingredients. Cereals with added vitamins or minerals, milk fortified with cholecalciferol for vitamin D deficiency, flour fortified with folic acid, prebiotic and probiotic fortified milk with *Bifidobacteriumlactis* HN019 for diarrhoea, respiratory infections, and severe illnesses in children, and orange juice fortified with calcium are examples (Sazawal et al., 2010).

2. Recombinant Nutraceuticals

   Recombinant nutraceuticals include the production of probiotics and the extraction of bioactive components using enzyme/fermentation technologies in addition to genetic engineering. Biotechnology is also used to produce energy-rich foods such as bread, alcohol, fermented starch, yoghurt, cheese, and vinegar. Examples include cows engineered with recombinant human lactoferrin (rhLf) to combat lactoferrin deficiency (Hyvönen et al., 2006).

Nutraceuticals have a critical role in neurological symptoms. Multivitamins, zinc, polyphenols, omega fatty acids, and probiotics have all been linked to improvements in cognitive function, spatial memory, and learning. It is important to determine the optimal combination of antioxidants and/or probiotics because it may not be practical to consume all nutraceuticals on a regular basis.

It was determined that the combination of multivitamins, zinc, and omega-3 fatty acids is the most effective method for enhancing memory and cognitive performance (Madireddy & Madireddy, 2021). Misfolded proteins are the primary cause of neurodegenerative diseases. Abnormal misfolding of the proteins τau and amyloid-β (Aβ) promotes the progression of Alzheimer's disease; traumatic brain injury can be induced by modifying the τau, transactive response DNA-binding protein-43 (TDP-43), and A proteins; and τau and TDP-43 dysfunction can subsequently induce epilepsy and other tauopathies. The cytotoxic cascade of molecular and cellular events is primarily triggered by protein Aβ in Down syndrome and α-synuclein in Parkinson's disease, resulting in detrimental outcomes and further degeneration (Colín-González, Ali, Túnez, & Santamaría, 2015) (Saldanha & Tollefsbol, 2012). These misfolded proteins further stimulate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation, leading to the production of inflammatory cytokines such as tumour necrosis factor-α (TNF-α), interleukins-6 (IL-6), and the activation of destructive molecules, cyclooxygenase (COX-2), and inducible nitric oxide synthase (iNOS) (Kelsey, Wilkins, & Linseman, 2010). In addition, misfolded proteins dysregulate multiple signalling pathways, including extracellular signal-regulated kinase (ERK), cyclic adenosine monophosphate (cAMP) response-element binding signalling (CREB), and protein kinase A/protein kinase B (PKB/PKA), and cholinergic functions, leading to defects in cognitive functions and degradation of the synaptic process (Gonsette, 2008; Lin & Beal, 2006). As a supplementation therapy, nutraceuticals can prevent neurodegeneration by targeting proteins that are misfolded practically at all levels and allowing them to act as a target for the prevention of neurodegeneration.

Bacopa Monnieri (L.) WETTST. (BM)

The medicinal plant known as *Bacopa monnieri* (L.) WETTST. is commonly used in preparing traditional medicines across Asia for treating neurological conditions, particularly as a memory enhancer (Dubey & Chinnathambi, 2019). *Bacopa monnieri* is a perennial weed commonly found in rice fields with small oblong leaves and purple flowers and belongs to the family Scrophulariaceae, known by various local names across Asia (Aguiar & Borowski, 2013). In India, it is known as Brahmi (Mathur, Goyal, Koul, & Anand, 2016); in Thailand, it is known as Prommi. BM has a report on antioxidant activity in the brain (Bhattacharya et al., 2000), preventing neurotoxicity (Jyoti et al., 2007), and anti-inflammatory in microglia cells (R. Jeyasri et al., 2022). Numerous phytochemical analyses have revealed that its chemical constituents are the alkaloids brahmine, herpestine, nicotine, saponin, monierin, hersaponin, Bacosides A1, A2, A3, and B, bacogenins A1 to A4, steroids triterpene, and bacosine. In addition to Bacopasaponins A–F, HPLC analysis has identified three new triterpenoid glycosides, Bacopasides III–V (figure 3) (Sharma, Yelne, Dennis, Joshi, & Billore, 2000) (Subha Rastogi, Pal, & KULSHREsHTHA, 1994) (S. Rastogi, Pal, & KulshreshthaDk, 1994) (Basu, 1967) (Chandel, Kulshreshtha, & Rastogi, 1977) (Garai, Mahato, Ohtani, & Yamasaki, 1996) (Mahato, Garai, & Chakravarty, 2000) (Chakravarty, Garai, Masuda, Nakane, & Kawahara, 2003) (Sivaramakrishna, Rao, Trimurtulu, Vanisree, & Subbaraju, 2005).

The plant's beneficial effects can be attributed to its vast array of structurally similar, closely related saponins. Some of the earliest research on this topic reported the cognitive effects of BM. Consequently, its effects on cognitive performance hold great promise for the amelioration of age-related cognitive decline and cognitive improvement in the young (Dhawan & Singh, 1996) (Vohora, Pal, & Pillai, 2000) (Scholey & Stough, 2011).

Both bacoside A and bacoside B enhance memory and decision-making in addition to promoting calmness. It has been demonstrated that *Bacopa monnieri* leaf extract improves memory functions in hypobaric hypoxia-induced brains. The effects of bacoside A on cytochrome C oxidase activity, ATP levels, apoptosis, plasma corticosterone levels, and oxidative stress markers were studied in male Sprague Dawley rats. Incorporation of bacoside increased cytochrome C activity and ATP levels, and along with memory retrieval, bacoside was observed to improve learning ability in rats

and prevent dendritic atrophy, as revealed by studying its molecular mechanisms: cAMP response element-binding protein (CREB) phosphorylation, neuronal cell adhesion, and expression of NR1 subunit of NMDA receptors (Hota et al., 2009). Neonatal hypoglycemia disrupts the dopamine D1 and D2 receptors, decreasing the amount of cAMP ($p < .001$) and the number of D1 receptors ($p < .001$), thereby impacting memory and cognition. SOD gene expression decreased in the cortex. Bacopa extract effectively improved the dopaminergic and cAMP imbalance(Thomas, Joy, Ajayan, & Paulose, 2013). Additionally, it improves total memory, logical learning, and phenytoin-induced memory impairment (Chaudhari, Tiwari, Tiwari, & Sharma, 2017). On a mouse model of the water maze, experiments were conducted to determine if BM improved scopolamine-induced spatial memory impairment. The Morris water maze (MWM) scale was utilised to evaluate the amnesic effect, while the rotarod test was utilised to evaluate the muscle coordination of mice. The BM extract reversed the scopolamine-induced anterograde and retrograde amnesia by increasing calmodulin and decreasing phosphorylated CREB and kinase C. (Anand, Saraf, & Prabhakar, 2010). Comparing the neuroprotective effects of a bacoside-rich fraction of BM (bacoside) and bacoside encapsulated in lactoferrin conjugated PEG–PLA-PCL-OH-based polymersomes (BAN) against scopolamine-induced amnesia, another study found that BAN was more effective due to the specific delivery of bacoside to overcome the blood-brain barrier (BBB) via polymersomes, via the receptor. BM also altered the amnesia induced by diazepam (DP) by decreasing the escape latency time (Saraf et al., 2008). Bacopa monnieri can reverse scopolamine and DP-induced amnesia in mice. BM inhibited N_w-Nitro-l-arginine (l-NNA)-induced anterograde amnesia but did not reverse dizocilpine (MK-801)-induced anterograde amnesia. The antiamnesic effect of BM against l-NNA-induced amnesia occurred via the NO pathway and CaM (calmodulin) (Anand et al., 2010). On the other hand, Brahmi Vati, an Ayurvedic polyherbal formulation, was superior to a bacoside A rich fraction (prepared by extraction, fractionation, and HPLC standardisation) for enhancing memory and treating seizures associated with memory loss because it results in significantly higher glutathione levels in the brain(Mishra, Mishra, & Jha, 2018). CDRI-08, a fraction of BM extract, plays a role in memory enhancement and as an antidiabetic in streptozotocin (STZ)-induced diabetes mellitus type 2 (DM2) mice, accompanied by a decrease in oxidative stress and an increase in glutamate ionotropic receptor AMPA type subunit 2 (GLUR2) subunit gene expression in the hippocampal region (Pandey, Singh, & Prasad, 2015). An experiment was conducted in which subjects without signs

of psychiatric disorder or dementia were treated with a placebo or standardised BM extract (SBME) twice a day for 12 weeks, followed by an additional placebo period of 4 weeks. SBME was found to enhance logical memory, associated learning, and mental control (Raghav, Singh, Dalal, Srivastava, & Asthana, 2006). *In vitro* studies with a standardised extract of BM screened in a panel of cell-free and receptor-transfected cell assays suggested that BM has an antagonistic effect on serotonin 2A (5-HT2A) and serotonin 6 (5-HT6) receptors and that it inhibits prolyl endopeptidase (PEP), catechol-O-methyl transferase (COMT), and poly (Dethe, Deepak, & Agarwal, 2016).

Neurodegenerative disorders, which are characterised by the progressive loss of neurons for various reasons, are caused by a decrease in acetylcholine (ACh) levels and choline acetyltransferase activity. Inhibition of acetylcholinesterase (AChE) has led to the replacement of acetylcholine (ACh). However, the use of synthetic drugs such as galantamine, rivastigmine, tacrine, and donepezil resulted in severe side effects such as gastrointestinal disorders and hepatotoxicity, so bacosides, a natural product, are favoured (Yadav, Parle, Sharma, Ghimire, & Khare, 2016). According to (von Bernhardi, Tichauer, & Eugenín, 2010), ageing, a major risk factor for neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD), is caused by cytotoxicity in microglial cells. NO, produced by glial cells in response to stimulation by superoxide radicals, is believed to be involved in neurodegenerative disorders such as Alzheimer's disease, ischemia, and epilepsy. *In vitro* (model of ischemia) experiments where organotypic hippocampal slice cultures (OHSCs) were incubated with triterpenoid saponins from bacosides and *in vivo* (transient two vessels occlusion-induced mice) experiments where the mice were treated daily with Bacopa extract revealed that a 25 M concentration of bacopaside I, exhibited an enhanced neuroprotective effect against oxygen-and glucose-deprivation (OG Bacopaside I was blocked by PKC and phosphoinositide 3-kinase (PI3K) inhibitors, but not by extracellular signal-regulated kinases (ERK) inhibitor, which helped reverse the decrease of phosphorylated protein kinase B (PKB), also known as Akt (caused by OGD), an antiapoptotic factor, thus suggesting the use of bacosides against apoptosis (Le et al., 2015). Experiments were conducted in a culture of purified rat astrocytes to determine the effect of a methanolic Bacopa extract on the toxicity induced by S-nitroso-N-acetyl-penicillamine (SNAP). The results indicated that SNAP induced an increase in the production of reactive species but did not destroy the cell

membrane, indicating that BM can be effective against neurodegenerative disorders (Russo, Borrelli, et al., 2003).

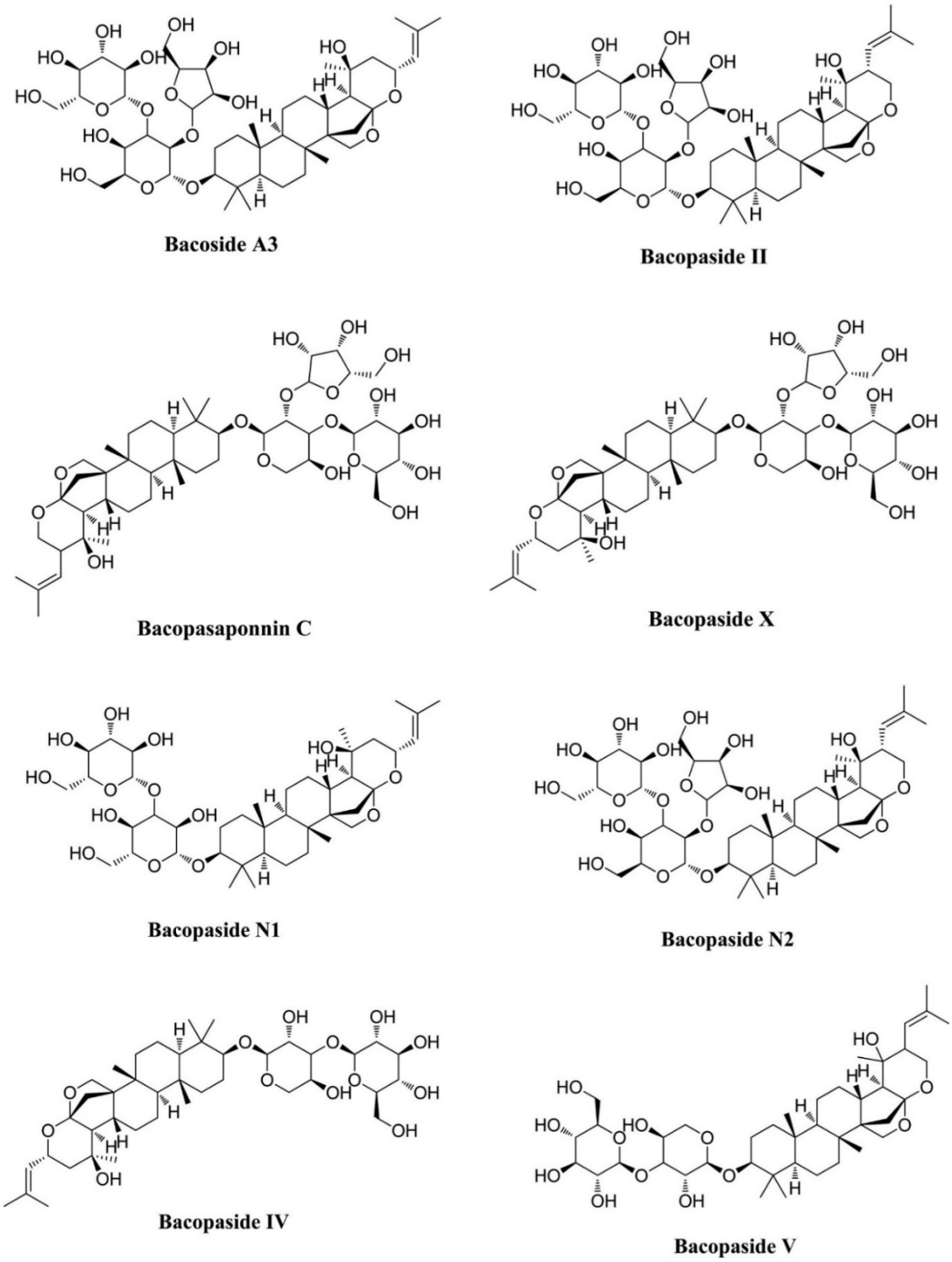


Figure 3. Chemical structure of *Bacopa Monnieri*. Retrieved from (Sukumaran, Amalraj, & Gopi, 2019)

Several studies have recommended the use of nonsteroidal anti-inflammatory drugs such as donepezil, tacrine, rivastigamine, and glantamine to treat the progressive neurodegenerative disorder Alzheimer's disease (AD) (Apetz, Munch, Govindaraghavan, & Gyengesi, 2014). However, bacosides have been hypothesised to protect the cholinergic neurons, thereby slowing the progression of the disease in a patient who has already been diagnosed (N. B. Chauhan & Mehla, 2015). Current monotherapeutic drugs are unable to treat the heterogeneity of the ageing brain, adding to the list of synthetic drugs' disadvantages (Sivaraman et al., 2019). Due to the irreversibility of already degenerated neurons, there is no permanent cure for Alzheimer's, but herbal supplements can delay and reduce the progression of AD. Several in-vitro and in-vivo studies indicate that bacosides not only delay the onset of Alzheimer's disease but also regulate other pathophysiological activities of the disease. AD, associated with memory loss and mental decline, is the specific cause of dementia after age 70. Male Wistar rats were administered ethylcholine aziridinium ion (AF64A) to induce Alzheimer's disease at doses of 20, 40, and 80 mg/kg body weight, and their spatial memory, the density of neurons, and cholinergic neurons were evaluated using the MWM test. BM extract improved escape latency ($p < 0.01$) and reversed the reduction of cholinergic neuron densities and neurons, suggesting that BM is a neuroprotectant against Alzheimer's disease (Uabundit, Wattanathorn, Mucimapura, & Ingkaninan, 2010). It is possible to treat Alzheimer's disease by inhibiting AChE and monoacylglycerol lipase (MAGL). Molecular docking studies suggest that the low binding energy of bacoside A on the targets of AChE and MAGL was 20.1 and 12.7 kcal/mol, respectively(Leonard, 2016). Using the MGL tools Autodock 4.2 module, another docking analysis was conducted with BuChE and AChE as receptors and bacosides as ligands. After an initial analysis of their hydrophobic interaction, inhibition constants, and hydrogen bonds, it was determined that bacosides find the interacting position at the active site. However, binding energy studies revealed that bacoside X, bacoside A, daucosterol, and 3-beta-D-glucosylstigmasterol inhibit the BuChE and AChE activities (Jamal et al., 2019). In another study, it was found that *B. monnieri* extracts reduced the neurotoxicity induced by low-density lipoprotein (LDL), with Lut (luteolin or 3′,4′,5,7-tetrahydroxy flavone) playing the most important role among the four compounds (bacoside A3, bacopaside I, bacopaside II, and luteolin). The compounds successfully increased cellular AChE activity, which is mediated by LDL oxidation (Yamchuen, Chaiwiang, Lapphanichayakool, Ingkaninan, & Limpeanchob, 2017). Bacoside A has been suggested to inhibit the formation of mature fibrils and

prevent cytotoxicity caused by amyloid-beta (1–42), the peptide accountable for the toxicity and progression of Alzheimer's disease. Nevertheless, microscopic and spectroscopic analyses revealed that although bacoside A blocked membrane interactions of A-beta42, it did not disrupt the formation of A-beta42 oligomers, indicating that blocking membrane interactions of A-beta42 is the underlying molecular mechanism for reducing amyloid toxicity (Malishev et al., 2017). In addition, it inhibits lipid peroxidation, and amyloid-beta deposition in the hippocampus, increases glutathione peroxidase, and chelates iron (Chaudhari et al., 2017). Crude Brahmi extract containing bacopasaponins, betulinic acid, bacoside A, bacoside B, etc., also inhibited tau-mediated toxicity (Tau is a microtubule-associated protein responsible for Alzheimer's) by inhibiting caspase-1, matrix metalloproteinase-3 and caspase-3 and reducing reactive oxygen species (ROS) and neuroinflammation (Dubey & Chinnathambi, 2019). One-month-old Mus musculus mice were divided into four groups: group 1: the control mice, group 2: mice treated with BME (Bacopa monnieri extract), group 3 (AD induced): mice treated with D-GAL and sodium nitrite ($NaNO_2$); and group 4 (AD induced): treated with BM. The results were analysed using the MWM technique, and biochemical assays were used to determine the ATPase system constituents. The results demonstrated that D-GAL and $NaNO_2$ caused learning and memory deficits, whereas BME had positive effects on body weight, learning, memory, and concentration and reversed all the constituents of ATPase to normal levels in AD-induced mice, suggesting that it can be used as a neuroprotective agent against AD (Kunte & Kuna, 2013). Examining the dosage of bacosides (200 mg/kg), determined by ageing biomarker senile dementia of Alzheimer's type (SDAT) and lipofuscin biomarker neurotransmitter acetylcholine in aged female Wistar rat brain, it was determined that bacosides prevented the degenerative effects of ageing and the age-related pathologies such as SDAT (M. Rastogi et al., 2012).

Parkinson's disease (PD) is a neurodegenerative disorder characterised by the death of selective dopaminergic neurons and the accumulation of protein α-synuclein (a presynaptic protein that regulates release). Protein α-synuclein interacts with the active site of delicate protein DJ-1, also known as Parkinson's disease protein 7 (coded by the PARK7 gene), which is responsible for the formation of Lewy bodies and oxidative stress (Javed et al., 2018). Molecular docking studies have also revealed interactions of 10 H-bonds between LRRK2 (a significant Parkinson's disease marker) and Bacopa saponin (an important constituent of bacoside A) with a binding affinity of 7.5 kcal at the ligand-receptor site, proving Bacopa saponin to be a major inhibitory component

for Parkinson's disease (Jain et al., 2013). Two *Caenorhabditis elegans* models: a pharmacological model expressing green fluorescent protein (GFP) specifically in dopaminergic neurons [BZ555(Pdat-1:GFP)] and a model expressing GFP in all neurons. A transgenic model expressing "human" α-synuclein [NL5901(Punc-54: alpha synuclein:YFP + unc-119)] and the neurotoxin 6-hydroxy dopamine (6-OHDA) were administered to mice expressing α-synuclein. *Bacopa monnieri* was effective at preventing dopaminergic neurons by boosting the antioxidant enzymes SOD and catalase, restoring lipid levels, and decreasing α-synuclein accumulation (BABITA Singh, Mahdi, & Pandey, 2014). The modulation of gene expression of GluR1, an alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptor, and NR1, an N-methyl-D-aspartate (NMDA) receptor in the striatum of rats with 6-hydroxydopamine-induced Parkinson's disease (PD) was investigated. GluR1 mRNA expression was found to decrease, while NR1 mRNA expression increased. Bacoside A administration reversed alterations, with elevated levels of TNF-α and IL-6 (Chandrasekar, 2020). In mice with Parkinson's disease, *B. monnieri* plant extract significantly improved caspase-3, hydroxylase activity, expression of a neurogenic gene in the substantia nigra region of the brain, and decreased oxidative stress (B. Singh, Pandey, Verma, Ansari, & Mahdi, 2016). In a Drosophila model of PD, loss of dopaminergic neurons and aggregation of Lewy bodies were observed. The flies were fed leaf extract of *B. monnieri* prepared in acetone at concentrations of 0.25, 0.50, and 1.0 l/ml for 24 days. Parkinsonian flies exhibited improved behavioural abnormalities and decreased apoptosis and oxidative stress (Siddique, Mujtaba, Faisal, Jyoti, & Naz, 2014). To examine the effect of Parkinson's disease on Na+/K+, Ca2+, and Mg2+ ATPase activities, rats were separated into four groups: Group A: Rats administered Rotenone (RT) intraperitoneally to induce Parkinson's disease; Group B: Rats administered BM extract (180 mg/kg/day) orally for 20 days; Group C: Rats administered saline water; and Group D: Rats administered levodopa (LD) orally as a drug control. RT-induced PD rats exhibited significantly lower levels of Na^{+}/K^{+}, Ca^{2+}, and Mg^{2+} ATPase activities in various brain regions. BM and LD, on the other hand, altered the changes, leading to the conclusion that BM is an effective treatment for PD (Swathi, Bhuvaneswar, & Rajendra, 2013). Mild cognitive impairments were observed in PD-induced animals, which were ameliorated by BM treatment in the T-maze test while working, and reference memories were significantly enhanced in the eight arm-radial mazes. In conclusion, it was hypothesised that BM ameliorates both nonmotor and motor symptoms of PD in rat models.

Epilepsy, a chronic disorder of the central nervous system, is characterised by cognitive, memory, and learning deficits. It is caused by abnormal central cholinergic neuronal regulation, glutamatergic neuronal imbalance, and gamma-aminobutyric acid (GABA) neuronal imbalance (E. Komali, Venkataramaiah, & Rajendra, 2021). GABA, the principal inhibitory neurotransmitter, counteracts neuron excitation, and its general receptors, including GABA, GABAA, and GABAB, decrease with the onset of epilepsy. Scatchard analysis of [3H] GABA, [3H] bicuculline, and [3H] baclofen revealed a decrease in B_{max} ($p < 0.001$) in the cerebral cortex of an epileptic rat. Furthermore, GABA receptor subunits such as GABAAa′1, GABAAy, GABAAd, GABAB, and GAD were downregulated ($p < 0.001$), whereas GABAAa′5 subunit and cycle (Hsieh et al., 2012). Similar alterations in GABA receptors were also observed in the hippocampus of rats with an epileptic temporal lobe induced by pilocarpine. However, the administration of bacoside A modifies these changes, demonstrating its therapeutic value against epilepsy (Mathew, Gangadharan, Kuruvilla, & Paulose, 2011). During epilepsy, metabotropic glutamate-8 receptor (mGluR8 gene expression) decreased ($p < 0.01$), and *B. monnieri* administration increased ($p < 0.05$) mGluR8 gene expression (Paulose, Chathu, Khan, & Krishnakumar, 2008). It has been suggested that nerve tonic derived from B. monnieri can cure epilepsy, a neuronal disorder characterised by memory loss and learning disabilities (Mathew, Paul, Nandhu, & Paulose, 2010). Upon evaluation, elevated levels of acetylcholine esterase, malate dehydrogenase, insulin, and T3 were discovered in the serum. Epilepsy, which causes convulsions, is prevented by bacoside A by reducing peripheral nervous system (PNS) impairment (Mathew et al., 2010). BM extract was given to eight groups of rats (a: epileptic group treated with n-hexane extract (nHE), b: epileptic group pretreated with ethyl acetate extract, c: epileptic group pretreated with chloroform extract (CE), d: epileptic group pretreated with DP, e: epileptic group pretreated with n-butanol extract (n-BE), f: PTZ-induced epilepsy BM administration altered the increased acetylcholine content and low acetylcholinesterase activities during PTZ-induced seizures induced by the drug DP. In PTZ-induced epilepsy, glutaminase, alanine aminotransferase, and aspartate activities were elevated and reversed by pretreatment with BM (E Komali, Venkataramaiah, & Rajendra, 2018; Suseela, Swathi, & Rajendra, 2013).

To determine the effect of bacoside on early prenatal or postnatal exposure to valproic acid (VPA), thalidomide, and ethanol-induced autism-like behavioural changes, a group of 12.5-day pregnant

female rats, male pups of saline-treated mothers, and VPA-induced autistic male pups were utilised. The group of pregnant female rats was treated with saline/VPA (600 mg/kg, I.P.), and the male autistic pups were divided into two groups, one receiving saline and the other receiving B. monnieri extract. In addition to behavioural tests at the adolescent and adult stages, animals were sacrificed, and their brains were isolated so that researchers could observe increased oxidative stress, serotonin level, decreased number of Purkinje cells, and neurodegeneration. Incorporating B. monnieri extract, however, reversed the alterations and improved autistic symptoms, demonstrating its antioxidant, antianxiety, and neuroprotective properties. In addition to behavioural abnormalities, downregulation of neurotransmitters was observed in children with an autism spectrum disorder. IMR32 cells were supplied with 50 M solutions of Brahmi, Brahmi ghrita, Brahmi vati, and Saraswati ghrita and allowed to grow for 24 hours. The mobile phase consisted of 0.1% formic acid with 15 g/ml Na_2-EDTA and 0.1% formic acid in acetonitrile in a ratio of 92:8, while the cell secretion was observed using ultra-fast liquid chromatography/mass spectrometry with electrospray ionisation. The herbal remedies increased the levels of neurotransmitters, but Brahmi vati had an even greater effect. By regulating neurotransmitter homeostasis, these medications can therefore be used to alleviate abnormal symptoms (Mamidala, Rajesh, & Rajesh, 2016). Affecting dystrophin and a few other proteins can result in autism by inhibiting signalling molecules such as TGF-β, RhoGAP, and CAM-mediated signalling molecules. This issue can be resolved, as well as cognitive repair, by BM (Anand et al., 2015).

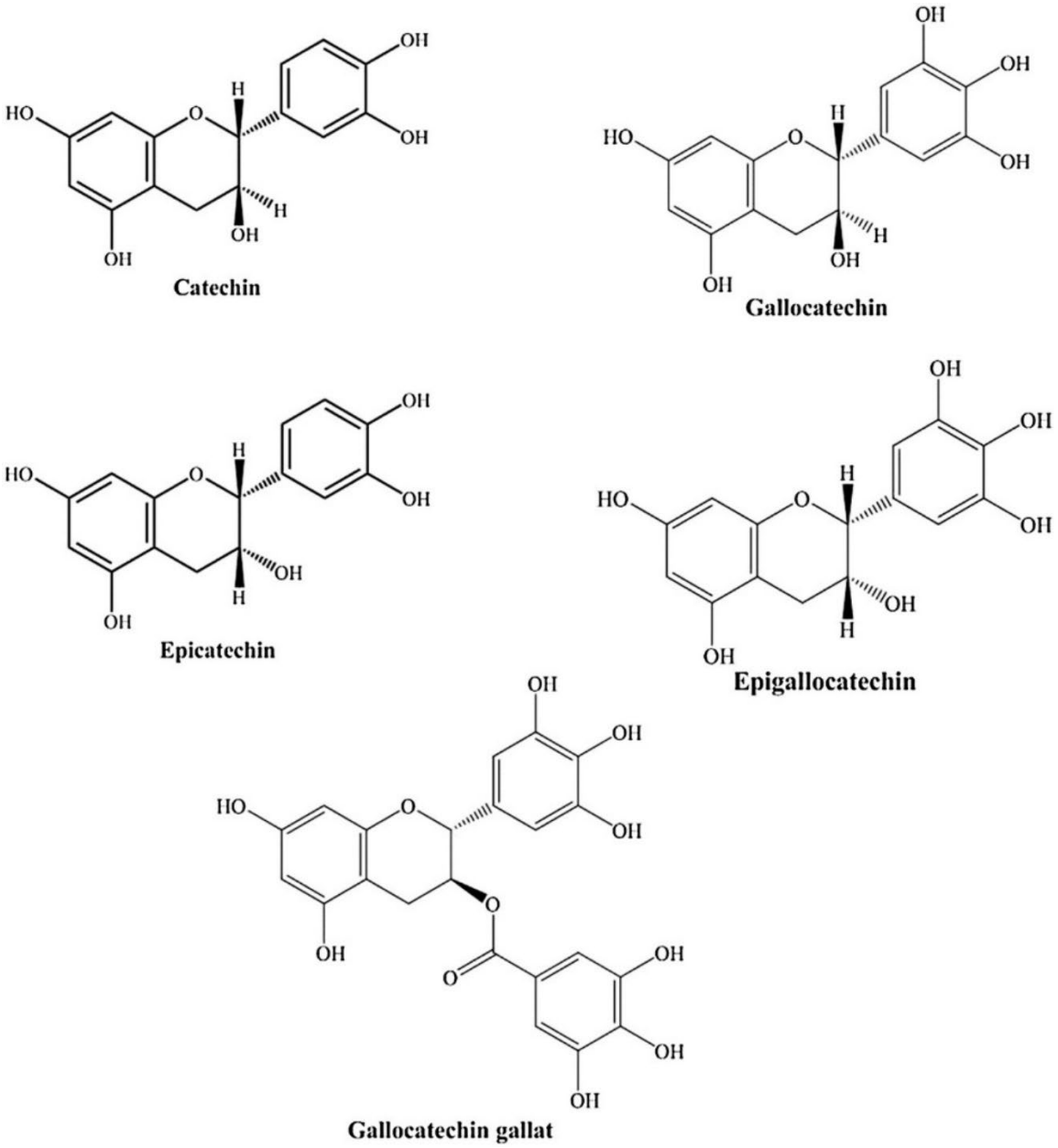
A study was conducted to determine the impact of bacoside A on acute and chronic models of experimental autoimmune encephalomyelitis, a model that aids in the identification of disease targets in animal models of multiple sclerosis. By inhibiting chemokine and inflammatory cytokines in both the acute and chronic models, the results suggested a decrease in disease progression and severity. In addition, bacoside A helped reduce neutrophil infiltration, cellular changes, and demyelination in the brain by inhibiting inflammatory cytokines (IL-6, IL-17a, CCL5, and TNF-α) and promoting expressions of brain-derived neurotrophic factor, neural cell adhesion molecule, and forkhead box P3. Similarly, in another experiment, the effects of bacoside-A on lipopolysaccharide (LPS)-activated microglia and astrocyte cultures were investigated. Observations revealed that bacoside-A promoted cell proliferation and decreased NO and TNF-alpha production in cultures, thereby preventing the LPS-induced activation of microglia and astrocytes (K. Das, 2019).

Camellia sinensis (CS)

Camellia sinensis (CS), or green tea, is a herbal medicine containing high levels of catechin, an antioxidant-rich compound that benefits neurodegenerative and cognitive diseases. (Afzal et al., 2022). CS is famous as a drink in various areas in Asia, especially Japan, a country with a high rate of green tea consumption. The Ohsaki Cohort 2006 Study reported that the consumption of green tea significantly reduces the incidence of dementia. (Tomata et al., 2016). Green tea leaf analysis reveals that it contains polyphenols (36% of dry weight), methylxanthines (3.5%), amino acids (4%), organic acids (1.5%), carotenoids (0.1%), volatiles (0.1%), carbohydrates (25%), protein (15%), lignin (6.5%), lipids (2%), ash (5%), chlorophyll, and a small amount of other substances (0.5%), the proportions of which vary depending on the season, climate, horticultural practices, and age of the leaf (Graham, 1992). The most potential biological phytochemicals of the CS are catechins, which include epigallocatechin gallate (EGCG), catechin (C), epicatechin (EC), gallocatechin (GC), gallocatechin gallate (GCG), epigallocatechin (EGC), and epicatechin gallate (ECG) (Figure 4) (Cooper, Morré, & Morré, 2005). They consist of a 2-phenylchromane skeleton with hydroxyl groups at the 3, 5, 7, 3′, and 4′ positions. During biosynthesis, if the B-ring is derived from the gallic acid synthon, the 5′ position of catechin is also substituted with a hydroxyl group, resulting in the name "gallo" catechin. Additionally, the hydroxyl group in the 3′ position can be esterified with gallic acid, forming catechin "gallate." Lastly, levorotatory (2r, 3r) compounds are referred to as "epi" catechins, whereas dextrorotatory (2s, 3r) compounds are referred to as "catechins." Thus, these combinations distinguish eight distinct molecular structures. Only EGCG is of interest in the field of medicinal chemistry among catechins (Legeay, Rodier, Fillon, Faure, & Clere, 2015).

Epigallocatechin gallate (EGCG), the principal catechin found in green tea, has the potential to impact various human diseases, according to accumulating preclinical evidence. EGCG appears to function as a potent antioxidant, preventing oxidative damage in healthy cells, but other mechanisms have been proposed to explain the health benefits of EGCG and tea, mainly green tea. Specific mechanisms and final effects are not unique to catechins, and numerous in vitro and in vivo studies demonstrate the health benefits of polyphenols. With over 8000 polyphenols found in foods and numerous epidemiological studies suggesting that the consumption of polyphenol-rich foods positively affects many disease risk factors, polyphenol-rich foods are recommended

(Danesi et al., 2013). EGCG inhibited the neuroinflammatory response of microglia induced by Aβ, protected against indirect neurotoxicity (Cheng-Chung Wei et al., 2016), and prevented neuronal cell death caused by Aβ in Tg2576 mice (Rezai-Zadeh et al., 2005).



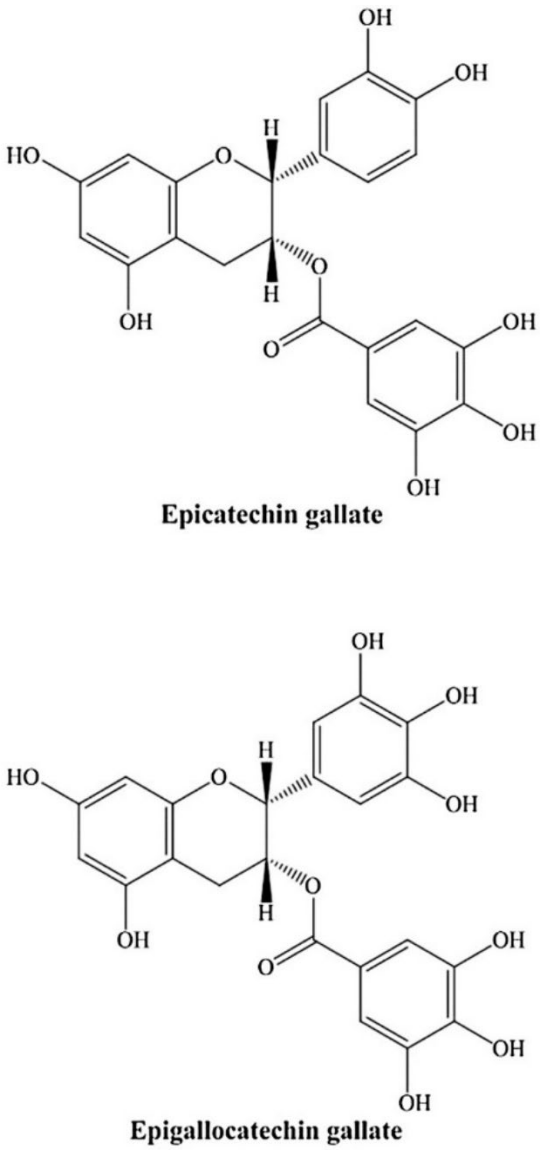


Figure 4. Chemical structure of green tea (*Camellia sinensis*). Retrieved from (Bedrood, Rameshrad, & Hosseinzadeh, 2018)

α-synuclein is a protein composed of 140 amino acids that are predominantly expressed in the presynaptic cleft of nerve cells. The alpha-helical structure of α-synuclein is typically involved in neuronal differentiation, regulation of dopamine synthesis, and inhibition of apoptosis in neurons. Under physiological conditions, α-synuclein cannot form a fibrillar structure because its monomeric and oligomeric forms are in equilibrium. Additionally, ubiquitin-proteasome machinery and lysosomal autophagic pathways remove excess and oligomeric α-synuclein from a healthy brain. Nevertheless, an increase in α-synuclein level, impaired mitochondrial function, and disruption in the association of α-synuclein with membrane increase the propensity of this non-toxic structure to aggregate and disrupt the normal mechanism in neurons, resulting in neuronal death. Consequently, preventing the formation of aggregates is a crucial measure in preventing PD pathology (Ghiglieri, Calabrese, & Calabresi, 2018) (Burré, Sharma, & Südhof, 2015) (Conway, Harper, & Lansbury, 1998).

In a cell-free *in vitro* system, the green tea polyphenol epigallocatechin-3-gallate (EGCG) inhibited α-synuclein aggregation and PC12 cell toxicity mediated by α-synuclein. EGCG could bind to native unfolded -synuclein polypeptide chains favourably and prevent the formation of toxic structures by mediating the formation of an unstructured oligomer. Additionally, it can prevent the addition of monomeric α-synuclein to fibrillar intermediates. Epitope mapping on peptide membrane revealed that EGCG could bind with aggregation-prone sites via intermolecular hydrophobic interaction to prevent -synuclein fibrillization. In addition, NMR studies indicate that EGCG may inhibit the transformation of α-synuclein to the β-sheet structure by binding with Ile, Phe, and Tyr residues. Furthermore, EGCG can disaggregate preformed fibrillar structures into non-toxic amorphous protein structures, which may be due to its interaction with Leu, His, Phe, and Tyr residues (Bieschke et al., 2010). A study conducted by Lorenzen and his colleagues (Lorenzen et al., 2014) demonstrates that EGCG inhibits the interaction between -synuclein oligomers and cell membranes, thereby protecting rat neuronal cells from toxicity.

Parkinson's disease primarily affects the region of the brain containing dopaminergic neurons, the Substantia Nigra pars compacta (SNpc). <80% of dopaminergic neurons are predicted to be lost during the progression of PD, resulting in a decrease in dopamine levels and availability in the brain. Dopamine is a neurotransmitter that, under normal physiological conditions, regulates the excitability of striatal neurons to transmit the signals required for the proper coordination of muscle

movement. However, the absence of dopamine results in an irregular pattern of nerve firing and a loss of motor control. Neurotoxins 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 6-hydroxydopamine (6-OHDA) selectively damage the substantia nigra region of the brain, resulting in the loss of dopaminergic neurons, and are widely used to identify the underlying molecular mechanism in the progression of Parkinson's disease (PD) (Barber, Klein, Mackay, & Hu, 2017) (Magrinelli et al., 2016) (Salari & Bagheri, 2019).

In cynomolgus monkeys administered MPTP, catechin-rich tea polyphenol extracts improved motor impairments, restored tyrosine hydroxylase (Th) and dopamine levels, and decreased the level of -synuclein oligomers and their aggregation (M. Chen et al., 2015). Significant levels of striatal antioxidants, superoxide dismutase (SOD), and catalase were significantly modulated by pretreatment with green tea extract and EGCG, protecting male C57/BL mice from dopamine loss. In addition, they prevented the reduction of TH, the enzyme that catalyses the formation of L-dihydroxyphenylalanine (L-DOPA) from tyrosine in the biosynthetic pathway of dopamine, thereby preventing the death of dopaminergic neurons (Levites, Weinreb, Maor, Youdim, & Mandel, 2001). EGCG pretreatment of C57/BL mice administered MPTP decreased the expression of α-synuclein and prevented neuronal apoptosis by increasing the expression of Bcl-2 and decreasing the expression of Bax. In addition, it provided neuroprotection by elevating protein kinase C-α (PKC-α) in the striatum (Mandel, Maor, & Youdim, 2004). In human NB SH-SY5Y cells, EGCG also induced PKC expression, which mediated the degradation of the pro-apoptotic protein Bad and offered neuroprotection. PKC activity promotes neuronal survival by activating multiple protein phosphorylation pathways, including ERK and JNK (Maher, 2001) (Kalfon, Youdim, & Mandel, 2007). Additionally, EGCG prevented neurodegeneration in CHO cells expressing DAT by inhibiting the ability of dopamine transporters (DAT) to actively uptake MPP+ and transport it to presynaptic dopaminergic neurons (Pan, Fei, Zhou, Jankovic, & Le, 2003). DAT is involved in the reuptake of dopamine from the synaptic cleft and the termination of signalling under physiological conditions. However, in PD, where dopamine availability in nerve terminals is diminished, inhibiting DAT is one strategy for preventing disease progression (Huot, Fox, & Brotchie, 2016). Li et al. (Li, Peng, Li, & Le, 2006) found that EGCG prevents the reuptake of dopamine from the synaptic cleft by inducing its internalisation through the activation of PKC in DAT-PC12 cells without affecting DAT expression. Long-term use of L-DOPA for the treatment of PD is associated with adverse effects that may be due to the formation of 3-O-methyl dopa,

which inhibits dopamine transporter and uptake and further reduces dopamine turnover. Catecholamine-O-methyltransferase (COMT) inhibitors are used in combination for the treatment of PD to prevent the conversion of L-DOPA to 3-O-methyl dopa. EGCG has been shown to inhibit COMT both in vitro and in vivo, which could effectively prevent further methylation of L-DOPA. This suggests that EGCG can also be given in combination with existing drugs to increase their availability and effectiveness in the brain (Kang et al., 2010).

Dopaminergic neurons are killed by chronic inflammatory processes in the SNpc of PD brains, according to a number of studies. Even though the central nervous system (CNS) has been considered an immune-privileged site, the breakdown of the blood-brain barrier (BBB) during PD activates the innate immune system, which then secretes and triggers a series of inflammatory events, including cytokines, ROS, and RNS, that contribute to the progression of PD. In response to the degeneration of dopaminergic neurons, immune cells from the periphery enter the brain parenchyma, where they interact with microglial cells to exacerbate the inflammatory condition. During this process, it has been reported that the lymphocyte populations of PD patients and the PD rat model change (Bas et al., 2001). EGCG was found to modulate peripheral immunity by decreasing the CD4+/CD8+ ratio and downregulating the expression of TNF-α, and IL-6, thereby protecting C57BL/6 J mice from MPTP-induced neurotoxicity (Zhou, Zhu, & Liang, 2018). Neuroinflammation associated with Parkinson's disease induces the expression of inflammatory mediators such as TNF-α and interleukins and plays a significant role in the initiation of apoptosis. In the rotenone-induced Parkinson's disease (PD) model in rats, EGCG treatment significantly reduced the expression of TNF-α, IL-1, and IL-6 and had a neuroprotective effect. (Tseng et al., 2020)

TNF-related apoptosis-inducing ligand (TRAIL) is a member of the tumour necrosis factor (TNF) superfamily that induces apoptosis through death receptors. Even though the intrinsic apoptotic pathway mediates cell death, there is also evidence of death receptor-mediated apoptosis in Parkinson's disease. In SH-SY5Y neuroblastoma cells, EGCG inhibits the expression of TRAIL ligand and TRAIL receptor DR5 (Weinreb, Mandel, & Youdim, 2003). Even though TRAIL expression in the CNS is relatively low, the pro-inflammatory cytokine interferon- can stimulate its production. Individuals with Parkinson's disease have elevated levels of IFN-ɣ in their blood plasma, highlighting the relationship between TRAIL and IFN-ɣ (Mount et al., 2007). Liu et al.

have revealed that EGCG has neuroprotective properties by inhibiting IFN- in human macrophages exposed to LPS-induced toxicity. EGCG may inhibit death receptor-mediated apoptosis due to its inhibitory effect on IFN-, which in turn inhibits TRAIL activity (J. B. Liu et al., 2016).

The treatment of male Wistar rats with a standardised extract of green tea and its active constituents epicatechin and EGCG decreased the oxidation capacity and reversed a dramatic increase in the level of inflammatory mediators COX-2 and iNOS induced by 6-OHDA neurotoxicity (Bitu Pinto et al., 2015). Though iNOS is expressed less in the brain than nNOS, during pathological conditions of Parkinson's disease, there is a sharp increase in both of their levels in the SNpc region, which contributes to the production of NO that causes dopaminergic neuron degeneration. EGCG has also been observed to inhibit iNOS and nNOS in the MPTP mouse model of PD. This may be due to EGCG's direct inhibition of MPP^+ uptake or its ability to inhibit iNOS transcription by preventing NF-κB from binding to the iNOS promoter (Choi et al., 2002) (S. Singh et al., 2005).

Furthermore, there is compelling evidence that iNOS can induce the expression of COX-2 and vice versa, indicating that inflammatory processes are linked to PD. The increased expression of COX-2 and iNOS in the brains of PD postmortem specimens lends credence to this theory. Selective inhibition of COX-2 inhibits the formation of oxidation species, dopamine-quinone, and protects against dopaminergic neurodegeneration as a result (Teismann et al., 2003) (Sánchez-Pernaute et al., 2004) (Mollace, Muscoli, Masini, Cuzzocrea, & Salvemini, 2005) (Bortolanza et al., 2015).

The expression factor as the master switch required for the expression of numerous inflammatory genes, NF-κB regulates the expression of numerous inflammatory genes. Targeting and inhibiting NF-κB activity has therefore emerged as a therapeutic option for the treatment of PD. It has been discovered that green tea extract protects neuronal cultures from 6-OHDA-induced toxicity by preventing nuclear translocation and NF-κB activation (Levites, Youdim, Maor, & Mandel, 2002) (Tiwari & Pal, 2017).

Microglia, neuroinflammation and related responses

The central nervous system (CNS) is comprised of neurons and neuroglial cells, two major cell types. In addition to astrocytes, oligodendrocytes, microglia, and ependymal cells, neuroglial cells include astrocytes. Specifically, 10-15% of the glial cell population in the adult brain are microglia (Nayak, Roth, & McGavern, 2014). Neuroinflammation is the third pathological characteristic of Alzheimer's disease. Several factors, including traumatic brain injury, exposure to toxins, and protein accumulation, trigger the CNS's inflammatory responses, which are mediated by microglia, the CNS's resident macrophage. The primary modulators of neuroinflammation in the CNS are microglia. Microglia, which are effector cells of the innate immune system that reside in the CNS, play crucial roles in brain homeostasis. In a healthy brain, microglia exert neuroprotective functions by providing innate immunity, eliminating apoptotic cells, and modifying synaptic connectivity (Leng & Edison, 2021). They reside in the brain parenchyma and are identified as the brain's resident immune cells. Microglia, unlike neurons and other neuroglial cells, which originate from the neuroectoderm, originate as primitive macrophages from the embryonic yolk sac and migrate to the CNS via the circulatory system (Ginhoux et al., 2010). During embryonic development, microglia initially surround the neuroepithelium before entering it to occupy the brain parenchyma and assume an amoeboid shape with short processes. In the adult brain, unlike macrophages and dendritic cells, resting microglia have a ramified morphology (Kaur & Ling, 1991). They help maintain tissue homeostasis by supporting neuronal survival, cell death, and synaptogenesis, thereby contributing to CNS development. Intriguingly, various pathological stimuli in the CNS, such as infection, brain trauma, stroke, and neurodegeneration, can activate these resting cells (Ling, Ng, Wu, & Kaur, 2001). The ramified cells transform into amoeboid reactive cells during activation. In response to the insult, they undergo rapid proliferation, production, and secretion of a broad spectrum of cytokines, chemokines, and other immune mediators. On the other hand, cellular experiments, preclinical models, and clinical studies suggest that chronic activation of microglia has deleterious effects on the survival and normal function of neurons, as well as on neuroregeneration via stem cells (Banati, Gehrmann, Schubert, & Kreutzberg, 1993). Under a stable microenvironment, microglia maintain their resting state and normal CNS function. Microglia can transform from a quiescent to an activated state in response to stimuli that alter the microenvironment (Saitgareeva, Bulygin, Gareev, Beylerli, &

Akhmadeeva, 2020). A new schema was required to describe the phenotype of microglia. The M1 phase, which can be induced *in vivo* and *in vitro* by microbe-associated molecular patterns (MAMPs) such as LPS, granulocyte-macrophage colony-stimulating factor (GM-CSF) and inflammatory cytokine such as IFN-ɣ, has been established as a descriptor for classically activated microglial cells. Microglia cannot return to a resting state unless anti-inflammatory triggers released by other microglia are first received. These additional stages have been categorised as alternative activation and have a number of healing responses (Xu et al., 2003). The M2a phenotype is stimulated by IL-4 or IL-13 and participates in Type 2 T helper cell (Th2) responses to allergens or parasite killing and encapsulation. Agonists of the IL-1 receptor and Toll-like receptor (TLR) induce the M2b phenotype, which promotes Th2 differentiation and antibody production. IL-10 and glucocorticoid hormones induce the M2c phenotype, which plays a role in immunoregulation, matrix deposition, and tissue remodelling (Franco & Fernández-Suárez, 2015). Microglia with the M1 phenotype exhibits upregulation of CD16 Fc receptors, CD32, CD64, CD86, interleukin (IL)-1β, IL-6, IL-12, IL-23, tumour necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS), and chemokine (C-C motif) ligand 5 (CCL5). Microglia with the M2 phenotype exhibit increased expression of insulin-like growth factor (IGF)-1, arginase (Arg)-1, triggering receptor expressed on myeloid cells 2 (TREM2), mannose receptor (CD206), chitinase 3-like 3 (Ym-1) and FIZZ1. This activated microglia produces increased proinflammatory proteins such as IL-6 and TNF-α, leading to prolonged neuroinflammation. The activation causes critical processes associated with neurodegenerative diseases that directly or indirectly induce clustering of the proteins τau and amyloid-β (Aβ), loss of synaptic function, declines in cognition and memory, neuronal cell death, and mitochondrial damage (Smith, Das, Ray, & Banik, 2012).

The study investigated the function of monocyte chemoattractant protein 1 (MCP-1, also known as CCL-2). MCP-1 is a chemokine whose secretion is stimulated by inflammatory signals, thereby producing a gradient that attracts monocytes to sites of inflammation. Although MCP-1 is expressed by numerous cell types, the primary sources in the CNS are microglia and macrophages. Importantly, the expression of its receptor, CCR2, regulates the MCP-1 response. The primary functions of MCP-1 in the CNS are the induction of cellular migration, the modification of the BBB, and the propagation of inflammation. When MCP-1 is inhibited or deleted, compromised monocyte migration, recruitment, and cytokine expression are diminished. MCP-1 has been shown

to alter BBB permeability by altering tight junction and adherens junction proteins-claudins, cadherins, and zona occludens—in accordance with its role in monocyte transmigration. MCP-1 is also a crucial mediator in the inflammatory process, where MCP-1 signalling induces the production of a variety of pro-inflammatory cytokines (Sawyer et al., 2014).

The role of neuroinflammation in the pathogenesis of Alzheimer's disease may provide a rationale for the search for novel therapeutic approaches. In addition, studies have demonstrated that A inhibits the growth of bacteria and fungi, possesses antiviral properties, and modulates viral interactions with phagocytes (Morales, Guzmán-Martínez, Cerda-Troncoso, Farías, & Maccioni, 2014). The possible scenario for the involvement of microorganisms in AD is already acknowledged as the "infectious aetiology" of AD and has been supported by a multitude of reports and proposed mechanisms of implication. Numerous bacteria have been linked to Alzheimer's disease, including spirochetes and other pathogens such as *Chlamydia pneumoniae* and *Helicobacter pylori*. In numerous ways, microbial virulence factors are associated with AD and neuroinflammation. Such virulence factors include rhamnolipids, which were detected in the CSF and serum of Alzheimer's disease (AD) patients, and lipopolysaccharides (LPS), molecules of the outer membrane of Gram-negative bacteria that activate the LPSs/Toll-Like Receptor 4 (TLR4) signal transduction pathway and induce the release of essential pro-inflammatory cytokines required to activate potent immune responses.

LPS are made up of a core oligosaccharide, a hydrophilic O-antigen, and their lipid component, Lipid A. Lipid A is composed of a disaccharide bound to multiple fatty acid chains and is highly conserved. Lipid A is likely the primary inflammatory component of LPS, and its structure is crucial for inducing the TLR4 – Nuclear Factor kappa beta (NF-κB) signalling pathway and, consequently, the expression of cytokines (interleukins – IL, tumour necrosis factor – TNF, etc.), inducible nitric oxide synthases (iNOS), and cyclooxygenase-2 (COX-2). Following shedding, multiplication, and lysis, LPS appear to exert their toxic effects after being released by bacteria. LPSs have been identified as a factor that increases Aβ production and accumulation, as well as the hyperphosphorylation of tau protein. LPS levels were previously found to be elevated in the brain and blood plasma of AD patients, as well as in the vicinity of AD amyloid plaques. Chronic activation of TLR4 by LPSs can increase cytosolic Ca2+ levels, resulting in apoptosis, and inhibiting A42 recognition and clearance. LPSs can also promote in vitro blood clotting and

amyloidosis, but this effect is reversed by LPSs Binding Protein, an acute phase inflammatory component.

COX, also known as prostaglandin H synthases (PGHS), are rate-limiting enzymes in the production of prostaglandins by converting arachidonic acid into bioactive prostanoids. COX-1 and COX-2 are strongly implicated in neuroinflammation and Alzheimer's disease (Ad and Bosetti, 2011). COX-1 is expressed constitutively in many tissues to maintain homeostasis, whereas COX-2 is an inducible isozyme that is upregulated by pro-inflammatory cytokines, mitogens, and endotoxins, among others. However, both COX-1 and COX-2 are continuously expressed in the brain. Also found to be overexpressed in the AD brain was COX-1. In numerous studies, COX-2 levels appear to be elevated in neurons during the early stages of Alzheimer's disease but not in later stages. In the context of identifying specific preventive targets for Alzheimer's disease, which is a central challenge in contemporary medicine, the Achilles' heel is COX inhibition, which has been reported to target neurons to halt an early behavioural decline in AD model mice. Data indicate that COX inhibition has extensive effects on multiple neuronal pathways that counteract the neurotoxic effects of early A oligomer accumulation. Describes the role of COX-2 in inflammatory and degenerative brain disorders ed pro-resolution mediators oversee the resolution process (Andreadou, Katsipis, Tsolaki, & Pantazaki, 2021).

In Parkinson's disease (PD), one of these factors, resolving D1, has been observed to decrease. It has been demonstrated that activating this mechanism improves an experimental rat model of PD. As with other neurodegenerative disorders, awareness of the role of inflammation and the immune system in the pathogenesis of Parkinson's disease (PD) has increased dramatically over the past decade, from a secondary effector of the neurodegenerative process to a primary, essential factor in the initial pathogenesis. The capacity of immune-competent cells resident in the CNS to influence the fine-tuning of synaptic function and neuronal survival has also received increased attention (C. Wang et al., 2020). As anticipated, postmortem examinations have revealed gliosis in α-synucleinopathies (Fellner, Jellinger, Wenning, & Stefanova, 2011) and, more specifically, in the dopaminergic degeneration of the α-synuclein (Chao, Wong, & Tan, 2014). PET analysis using specific tracers has revealed the early activation of microglial cells in PD patients (Nicastro, Surendranathan, Mak, Rowe, & O’Brien, 2019). Genetic Parkinson's disease cases are caused by dominant (LRRK2, SNCA, VPS35) or recessive (PRKN, PINK1, DJ1) mutations (Zhao et al.,

2013). Several of these genes also play essential roles in the immune system. The majority of autosomal dominant PD is caused by LRRK2 mutations, which are also involved in autoimmune diseases. 15 Genetic studies have found an association between the human leukocyte antigen and increased susceptibility to PD, indicating that immune mediators may play a role in initiating the onset of the disease (Blauwendraat et al., 2019). Numerous immune-related loci have been identified as being associated with sporadic Parkinson's disease through genome-wide association studies. Immune factors detected in the plasma or cerebrospinal fluid (CSF) of PD patients are indicative of an inflammatory profile, possibly associated with disease pathogenesis. In various PD populations, the levels of pro-inflammatory cytokines IL-6, IL-1β, and TNF-α are altered (Usenko et al., 2020).

SARS-CoV-2 was discovered to infect and traverse brain microvascular endothelial cells (BMECs) via a transcellular pathway accompanied by basement membrane disruption without apparent modification of tight junction ultrastructure (TJs) (Figure 5) (L. Zhang et al., 2021), that is extremely susceptible to peripheral immune changes, and obviously under the cytokine storm typical of COVID-19 (Schwartz, Kipnis, Rivest, & Prat, 2013). Especially, IL-1β, TNF-α, and IL-6 play a role in synapse formation, neuroplasticity, and cognition at the morphological, functional, and cognitive levels. A further pathway of serious concern is that COVID-19-induced proinflammatory cytokines upregulate indoleamine 2,3-dioxygenase (IDO), the preliminary and rate-limiting enzyme of the tryptophan degradation pathway. The stimulation of indoleamine 2,3-dioxygenase reduces the amount of tryptophan and increases the amount of tyrosine. Following this, tyrosine is converted into many metabolites that, include tyrosine acid and quinoline acid, which respectively suppress and stimulate NMDA neurotransmission (Hori & Kim, 2019).

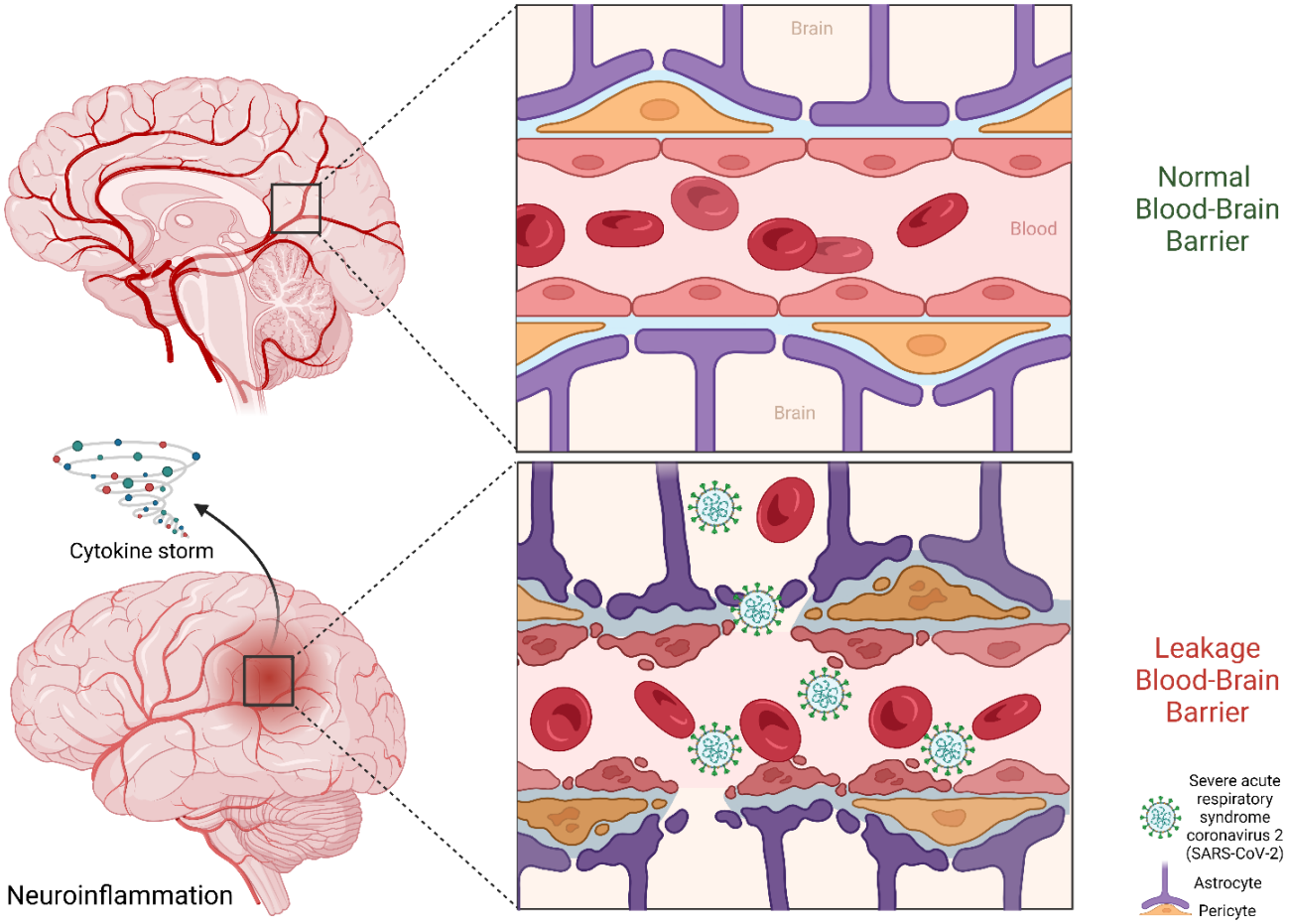


Figure 5. SARS-CoV-2 infects brain vascular endothelial cells and causes cytokine storms lead to brain damage. Created with BioRender.com (2023).

NF-κB signalling in inflammation

Nuclear factor- κB (NF-κB) is a family of inducible transcription factors that regulates a vast array of genes involved in various immune and inflammatory response processes. This family consists of five structurally related members, NF- κB 1 (also known as p50), NF- κB 2 (also known as p52), RelA (also known as p65), RelB, and c-Rel, which mediates transcription of target genes by binding to a specific DNA element, B enhancer, as hetero- or homo-dimers. The NF- κB proteins are sequentially sequestered in the cytoplasm by a family of inhibitory proteins, including members of the IκBα family and proteins with ankyrin repeats. IκB is the most studied and significant member of the IκB family. Moreover, the precursor proteins of NF-κB1 and NF-κB2, p105 and p100, function as IκB-like proteins because their C-terminal portion resembles the structure of IκB and possesses NF-κB inhibitory properties(Beinke & Ley, 2004; Oeckinghaus & Ghosh, 2009; Sun, Chang, & Jin, 2013). Inflammation is a host's protective response to infections and tissue damage, characterised by a series of reactions, including vasodilation and the recruitment of immune cells and plasma proteins to the site of infection or tissue injury. Deregulated inflammatory responses can contribute to the development of acute or chronic inflammatory diseases by causing excessive or long-lasting tissue damage. NF-κB is a key mediator of the induction of pro-inflammatory genes in both innate and adaptive immune cells (H. Zhang & Sun, 2015). Despite their distinct signalling mechanisms, the activation of NF- κB is essential for regulating immune and inflammatory responses. Regulation of inflammatory responses is a well-established function of NF-κB. NF-κB regulates inflammatory T cells' activation, differentiation, and effector function, mediating the induction of numerous pro-inflammatory genes in innate immune cells. Important players in innate immunity and inflammation are innate immune cells, such as macrophages, dendritic cells, and neutrophils. These cells express pattern recognition receptors (PRRs), also known as pathogen-associated molecular patterns (PAMPs), that detect various microbial components. PRRs also recognise damage-associated molecular patterns (DAMPs), which are molecules that necrotic cells and damaged tissues release. Five families of PRRs are expressed by mammalian cells, including toll-like receptors (TLRs), RIG-I-like receptors, NOD-like receptors (NLRs), C-type lectin-like receptors, and cytosolic DNA sensors. Different families of PRRs have distinct structural properties and respond to different PAMPs and DAMPs, but their downstream signal transduction pathways are remarkably similar. Activation of the canonical NF-κB pathway, which is

responsible for transcriptional induction of pro-inflammatory cytokines, chemokines, and other inflammatory mediators in diverse types of innate immune cells, is a common PRR signalling event. These inflammatory mediators can act both directly and indirectly by promoting the differentiation of inflammatory T cells to induce inflammation. Transforming growth factor-activated kinase 1 (TAIK1) is a signalling component that integrates the various PRR pathways for NF-κB activation. TAB2 can bind poly-ubiquitin chains, which is necessary for TAK1 activation. After being activated, TAK1 causes the downstream kinase IKK to become active. IKK then phosphorylates IκB, which in turn activates NF-κB. Recent research suggests that NF-κB might also be responsible for regulating the activation of inflammasomes (Sato et al., 2005). Chronic inflammatory diseases are characterised by their dysregulated NF-κB activation. Consequently, a better understanding of the mechanism underlying NF-κB activation and pro-inflammatory function is crucial for developing therapeutic strategies to treat inflammatory diseases (Lawrence, 2009) (P. P. Tak & G. S. Firestein, 2001) (Sutterwala, Haasken, & Cassel, 2014).

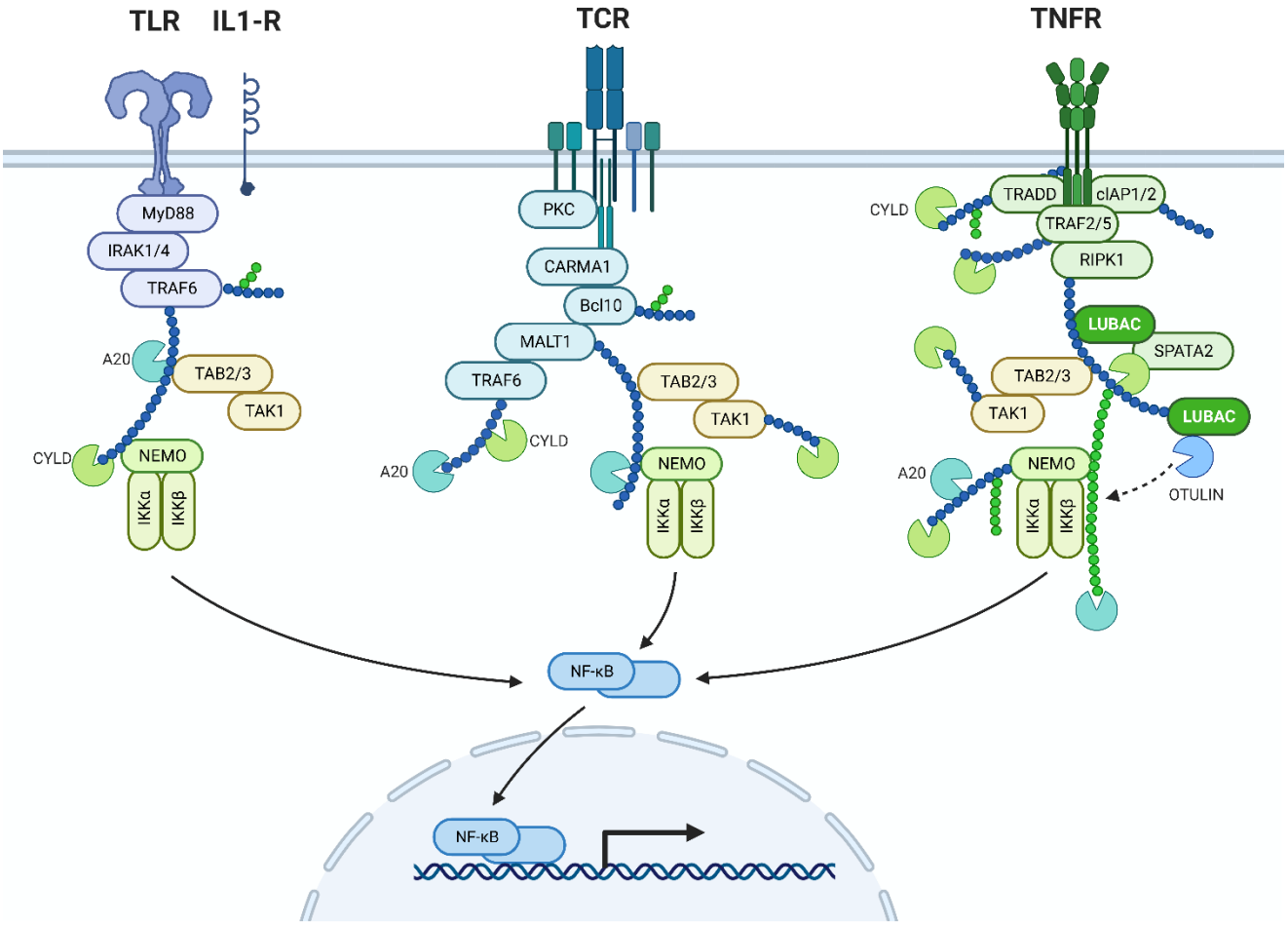


Figure 6. NF-kB Signaling Pathways - BioRender (2023). NF-kB Signaling Pathways. Retrieved from https://app.biorender.com/biorender-templates/figures/all/t-5e15f4b19f4e0c00852dfe19-nf-kb-signaling-pathways

MAP Kinase Pathways

In eukaryotic organisms, cell proliferation, cell differentiation, and cell death are regulated by a series of essential signal transduction pathways containing key components called mitogen-activated protein kinase (MAPK) modules. MAPKs are a family of serine/threonine protein kinases that regulate the production of inflammation mediators and are responsible for most cellular responses to cytokines and external stress signals (Kaminska, Gozdz, Zawadzka, Ellert-Miklaszewska, & Lipko, 2009). These MAPK modules each contain three sequentially activated protein kinases. Each cascade is triggered by distinct extracellular cues and culminates in the activation of a specific MAPK through the sequential activation of a MAPK kinase kinase (MAPKKK) and a MAPK kinase (MAPKK) (Keshet & Seger, 2010). Typically, interactions with a small GTPase and/or phosphorylation by protein kinases downstream of cell surface receptors activate the MAPKKK. The MAPKKK phosphorylates and activates the MAPKK directly, activating the MAPK by dual phosphorylation of a conserved TxY motif in the activation segment. Once activated, the MAPK phosphorylates various substrates in the cytosol and nucleus to induce changes in protein function and gene expression that are necessary for the proper biological response. MAPKs typically contain docking sites for MAPKKs and substrates, which permit high-affinity protein–protein interactions to ensure both that a specific upstream MAPKK activates them and that they are activated by a specific downstream substrate (Cuevas, Abell, & Johnson, 2007). There are three distinct families of MAP kinases. ERKs (extracellular-signal-regulated kinases), JNKs (Jun amino-terminal kinases), and p38/SAPKs are examples of these enzymes in mammals (stress-activated protein kinases) (Y. Zhang & Dong, 2007).

MEK1 and MEK2 are the MAPKKs for the classic ERK1/2 module, while the MAPKKKs include members of the Raf family, Mos, and Tpl2 (Shaul & Seger, 2007). JNK1, JNK2, and JNK3 are members of the JNK family that possess a TPY motif in the activation segment. Environmental stresses (ionising radiation, heat, oxidative stress, and DNA damage), inflammatory cytokines, and growth factors activate the JNK module, and the Rho family GTPases Cdc42 and Rac are frequently involved in signalling to the JNK module (Johnson & Nakamura, 2007). Apoptosis, inflammation, cytokine production, and metabolism are all significantly affected by the JNK module. MAPKKs for the JNK module are MKK4 and MKK7, and MAPKKKs consist of MEKK1 and MEKK4, MLK2 and MLK3, ASK1, TAK1, and Tpl2 (Huang, Shi, & Chi, 2009). Members

of the p38 family with a TGY motif in the activation segment include p38, p38, p38, and p38. Environmental stresses and inflammatory cytokines similarly activate p38 modules as JNK modules. Inflammation, apoptosis, cell differentiation, and cell cycle regulation are all influenced by p38 activation. MKK3 and MKK6 are the primary MAPKKs for p38 modules, while the MAPKKKs include MLK2 and MLK3, MEKKs, ASKs, TAK1, and TAO1 and TAO2. Important substrates in p38 signalling include the downstream kinases MK2/3, PRAK, MSK1 and MSK2, in addition to a number of transcription factors (Cuadrado & Nebreda, 2010).

Neuronal cell death

Neuronal cell death occurs during normal development, as a result of injury, and as neurodegenerative diseases such as Alzheimer's disease progress. Although developmental neuronal cell loss is necessary for the proper functioning of the nervous system, pathological forms of neuronal cell death can severely impair mental, cognitive, and motor abilities. Considerable attention has been devoted to the pathways that regulate cell death in the nervous system (Savitz & Rosenbaum, 1998). Traditionally, necrosis and apoptosis have been described as two distinct mechanisms of cell death; however, they may represent opposite ends of a continuum. Apoptotic cell death is a tightly regulated, energy-dependent process characterised by a series of morphological and biochemical alterations. Chromatin condensation, chromosomal and nuclear fragmentation, cellular shrinkage, and membrane blebbing characterise apoptosis through the formation of apoptotic bodies. Biochemically, the apoptotic cascade involves the activation of multiple enzymes and proteins, culminating in the activation of DNA-degrading caspase enzymes. Rapid loss of membrane and organelle integrity, resulting in cell swelling and lysis, defines necrotic cell death. In necrosis, the release of cellular contents triggers an inflammatory response, whereas, in apoptosis, this response is typically absent.

The regulation of neuronal cell death may be further complicated by the fact that the extracellular factors that promote survival and differentiation can also initiate apoptotic death cascades. In the presence of TrKA receptors and the low-affinity neurotrophin receptor p75, for instance, nerve growth factor (NGF) treatment of neuronal cultures increases neurotrophin responsiveness and promotes neuronal survival and differentiation. In the absence of TrkA receptors, the activation of p75 by NGF initiates a cascade of cell death (Yano & Chao, 2000). Thrombin, a multifunctional

serine protease, also appears to have a dual effect on neuronal survival, acting as both a neuroprotective agent and a mediator of neurotoxicity under certain conditions. In addition, the complexity and heterogeneity of the neuronal phenotype in terms of transmitter content, receptor expression, and morphology imply that there may be subtle differences in cell death pathways between neuron types.

As indicators of necrosis and apoptosis, respectively, the release of lactate dehydrogenase (LDH) and the accumulation of nucleosomes within cells was used. Both neuronal cell types released significant amounts of LDH; however, the pattern of LDH release was variable and agonist-dependent. In response to the nitric oxide generator sodium nitroprusside (SNP), cortical cells displayed pronounced LDH release and dramatic morphologic changes, whereas in differentiated PC12 cells, TNF-α induced LDH to release without any accompanying morphologic changes. TNF and thrombin-induced an increase in apoptosis in both types of neuronal cells but not in undifferentiated PC12 cells. Caspase inhibition, but not antioxidant treatment, decreased the accumulation of nucleosomes in primary cortical cells (Reimann-Philipp, Ovase, Weigel, & Grammas, 2001).

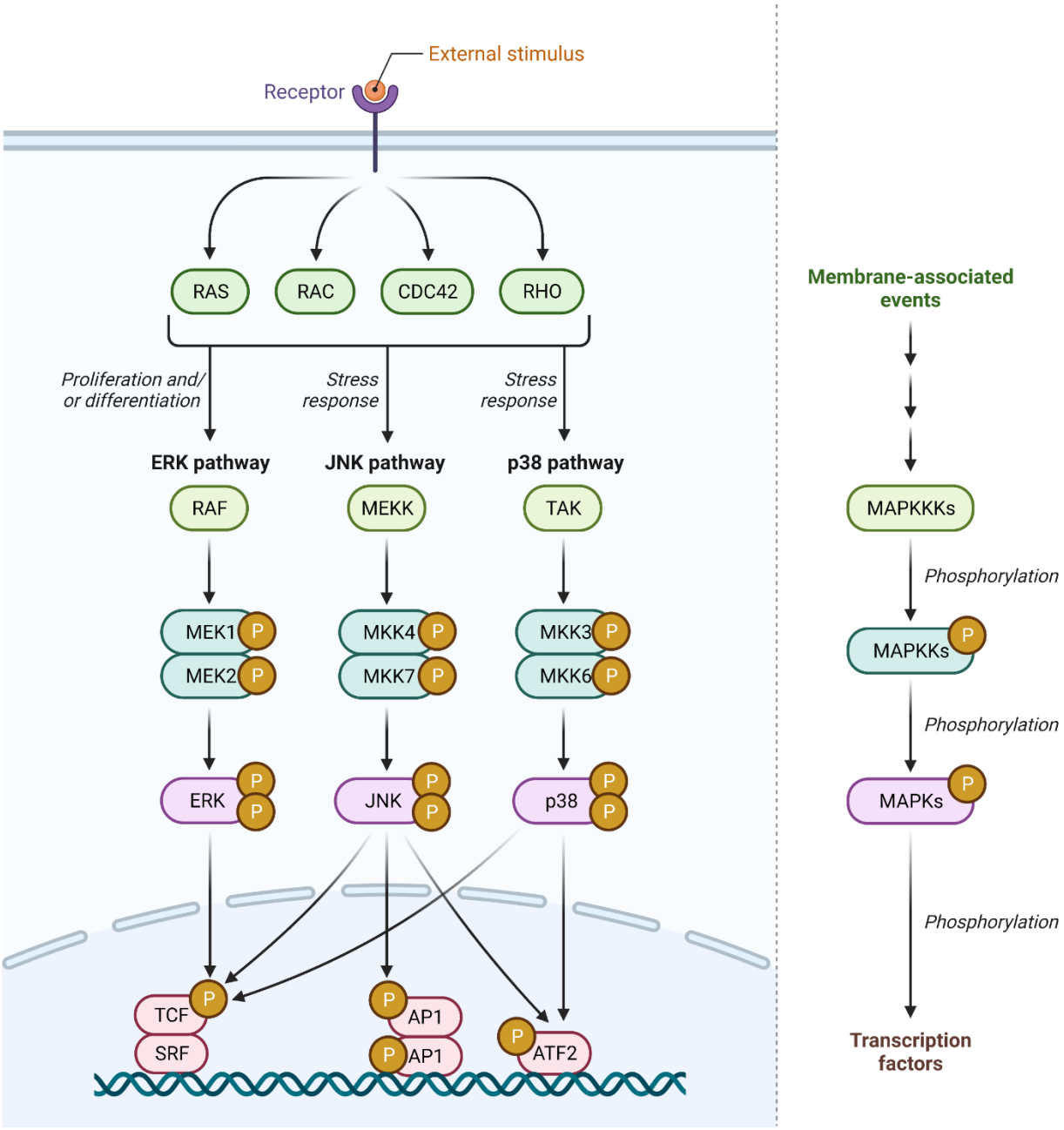


Figure 7. Three Major MAP Kinase Pathways: ERK, JNK, p38 - BioRender (2023). Three Major MAP Kinase Pathways: ERK, JNK, p38. Retrieved from https://app.biorender.com/biorender-templates/figures/all/t-5f6165506968d600b2d01325-three-major-map-kinase-pathways-erk-jnk-p38

Chapter 3

Materials and Methods

Materials and reagents

Ethanolic extracts of *Bacopa monnieri* herb (BM) and *Camellia sinensis* dry extract (CS) were obtained from ACEF Spa. (Piacenza, Italy) and dissolved in DMEM (Dulbecco's Modified Eagle's Medium)/Hams F-12 50/50 Mix with L-glutamine and 15 mM HEPES for all cell culture experiments. All the reagents used to measure antioxidant activity, including 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate ($K_2S_2O_8$), Trolox ($C_{14}H_{18}O_4$), gallic acid ($C_6H_2(OH)_3COOH$), the Folin–Ciocalteu reagent, and sodium carbonate (Na_2CO_3), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipopolysaccharide (LPS) from Escherichia coli, Serotype O55:B5 S-form, was obtained from Enzo Life Sciences (Exeter, UK). Hydrogen peroxide solution (H_2O_2) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The Cell Counting Kit (CCK) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Enzyme-linked immunosorbent assay (ELISA) MAX™ Standard Set Mouse MCP-1 and TNF-α were obtained from BioLegend (San Diego, CA, USA). The Mouse Interleukin-6 (IL-6) ELISA kit was obtained from Invitrogen (Waltham, MA, USA).

Chemical profile of BM and CS

The analyses were carried out on Thermo Ultimate RS 3000 coupled online to a Q-Exactive hybrid quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a heated electrospray ionisation probe (HESI II). For RP-UHPLC analysis, a Kinetex Biphenyl 100 mm × 2.1 mm, 2.6 µm (L × ID, particle size, Phenomenex®, Bologna, Italy) column was employed at a flow rate of 0.4 mL/min. The mobile phases consisted of A) 0.1% CH_3COOH in H_2O and B) ACN plus 0.1% CH_3COOH. Analysis was performed in gradient as follows: 0-30.0 min, 2-30% B; 30-38 min, 30-98% B; 99% B hold for 2 min; returning to initial conditions in 0.1 min. The column oven was set to 40 °C, 5 µL sample was injected. HRMS analysis was performed with Full MS (m/z 100-850) and data-dependent acquisition (dd-MS2 topN=5). A resolution of 35.000 and 15,000 FWHM at m/z 200 was selected, respectively. Stepped normalised collision energy (NCE) with values of 15, 25 and 30 were used. Negative ESI- was employed. Source

parameters were: sheath gas pressure, 50 arbitrary units; auxiliary gas flow, 13 arbitrary units; spray voltage, -2.50 kV; capillary temperature, 260 °C; auxiliary gas heater temperature, 300 °C, S-lens RF value: 30 arbitrary units. Metabolite annotation was performed by comparison with the *in silico* MS/MS Natural Product Library of MS DIAL v4.80, as previously reported (Sommella et al., 2018).

Determination of Total Phenolic Content (TPC) of the BM or CS

In separate 2 mL Eppendorf microtubes, 10 µl aliquots of each extract (BM or CS), gallic acid as a standard, and a blank were added. Each microtube received 50 µl of 10% Folin–Ciocalteu (F–C) reagent, which was vortexed thoroughly. Then, 250 µl of 15% (w/v) sodium carbonate (Na_2CO_3) was added to each test tube. The reactants were incubated in the dark at room temperature for 2 hours. The absorbance was measured at 750 nm after transferring 200 µl of the reaction mixture from the assay tube to a microplate with 96 clear wells. The gallic acid standard curve was utilised to determine the total phenolic content, which was expressed as milligrams of gallic acid equivalent per gram (mg GAE/g) of dry extract.

Determination of Radical Scavenging Activity ($ABTS^+$) of the BM or CS

The $ABTS^+$ radical scavenging capacity of each extract was determined by combining 2.5 mL of a 0.01 M $ABST^+$ stock solution with 44 µl of potassium persulfate ($K_2S_2O_8$) to produce a 0.01 M ($ABTS^+$) working solution and then incubating the solution in the dark for 12 to 16 hours. Before measuring the absorbance at 734 nm, the mixture of 100 µl of the extract or Trolox and 1mL of the working solution was left in the dark for an additional 30 minutes. The standard was the Trolox solution, and the results are reported as mmol TEAC per gram of dry biomass (mmol TEAC/g dw) and $ABTS^+$ inhibition (%).

Cell Culture

Murine BV2 and neuroblastoma 2a (N2a) cell lines were used as models of microglia and neurons, respectively, and were cultured in DMEM (Dulbecco's Modified Eagle's Medium)/Hams F-12 50/50 Mix with L-glutamine and 15 mM HEPES, supplemented with foetal bovine serum (FBS; 10%; Gibco, UK) and penicillin–streptomycin (100 U/ml; Gibco, UK). Cells were grown in a sterile, humidified environment (37 °C; 5% CO_2) until the cell reached 80–90% confluence in

flasks. Using cell scrapers, adherent cells were removed, centrifuged (2,000 rpm; 3 minutes), and reseeded ($1.5x10^5$ cells/well) in 24-well culture plates. The cells were incubated in DMEM with 5% CO_2 at 37 °C for an additional 24 hours.

Cell Viability

In a 96-well plate, murine BV2 and Neuroblastoma 2a (N2a) cells ($2 x10^4$ cells/well) were seeded and cultured for 24 hours. The cells were pre-incubated, and then BM or CS was added. The cells were then incubated at 37°C with 5% CO_2 for a further 24 hours. 10 **µl** of CCK solution was added to each well, which was then incubated for one to four hours at 37 °C with 5% CO_2. Using a microplate reader, the absorbance of each well at 450 nm was determined (SpectraMax i3, Molecular Devices, San Jose, CA, USA).

Measurement of Nitric Oxide (NO) production by Griess assay

Murine BV2 cell 15×10^4 cells were seeded in a 24-well plate for 24 h followed by pretreatment with BM (100, 50, 10 µg/mL) or CS (1, 0.5, 0.1 µg /mL) or combination (BM 100 µg/mL+ CS 1 µg/mL) for 3 hours prior to LPS exposure (10 ng/mL) for 24 h. After the incubation period was complete, 50 µl of culture supernatant was transferred to a 96-well plate, mixed with 50 µl of Griess reagent (Sigma-Aldrich, MO, USA), and incubated for 20 min at 25 °C. $NaNO_2$ (5-180 µM) was provided as the reference standard. SpectraMax i3, manufactured by Molecular Devices (San Jose, CA, USA), was used to measure and quantify absorbance at 540 nm. Based on the nitrate standard curve, the NO concentration was assessed.

Measurement of Cytokine and chemokine Release

BV2 microglial cells were seeded $15x10^4$ cells in a 24-well plate for 24 hours, and they were preincubated with BM (100, 50, 10 µg/mL) or CS (1, 0.5, 0.1 µg/mL) or combination (BM 100 µg/mL+ CS 1 µg/mL) for 3 hours prior to LPS (10 ng/mL) exposure for 24 h. Using a commercial enzyme-linked immunosorbent assay (ELISA) kit for MCP-1, TNF-α, and IL-6, the level of cytokine in cultured media was measured following treatment, per the manufacturer's instructions.

Western immunoblotting

Cell proteins were extracted using radioimmunoprecipitation (RIPA) assay lysis buffer (Millipore) by adding protease and phosphatase inhibitor (PI) cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein samples were separated via electrophoresis in a sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a PVDF membrane. The membranes were blocked with Tris-buffered-saline containing 0.05% Tween® 20 (TBS-T) and 5% non-fat dried milk/TBS-T at room temperature (RT) for 1 hour and were incubated with the primary antibodies (Table 1) overnight at 4 °C. Primary antibodies were discarded, and the blots were washed 5 mins with tris-buffered saline with 0.1% of Tween® 20 (TBS-T) for five times. Membranes were incubated with secondary antibodies (Table 1) at RT for 1 hour. VILBER FUSION FX (Vilber, Collégien, France) was used to quantify the relative protein expression intensity. The band intensity was analysed using Image J software (version 1.53k) with control (GAPDH or β-actin) value normalisation.

Table 1 Primary and secondary antibodies

Type	Antibody	Manufacturer	RRID	Dilution	Host/Isotype
1st antibodies	iNOS	Thermo Fisher Scientific	AB_2538610	1:1000	Mouse / IgG1
	COX-2	Thermo Fisher Scientific	AB_2084941	1:500	Goat / IgG
	p-p38	Thermo Fisher Scientific	AB_2533721	1:1000	Rabbit / IgG
	p-JNK (G-7): sc-6254	Santa Cruz Biotechnology	AB_628232	1:500	Mouse/ IgG

Type	Antibody	Manufacturer	RRID	Dilution	Host/Isotype
	p-NF-κB p65 (ser 535)	Thermo Fisher Scientific	AB_10983078	1:1000	Rabbit / IgG
	β-actin	Thermo Fisher Scientific	AB_2536844	1:1000	Mouse / IgG1
	GAPDH	Thermo Fisher Scientific	AB_568547	1:1000	Mouse / IgG
2nd antibodies	Anti-mouse	Thermo Fisher Scientific	AB_2533933	1:1000	Rabbit / IgG
	Anti-rabbit		AB_228341	1:1000	Goat / IgG
	Anti-goat		AB_228395	1:1000	Rabbit / IgG

Lactate dehydrogenase (LDH) release assay

The protective role of BM or CS or the combination against H_2O_2-induced plasma membrane damage was quantified in terms of the per cent of LDH released by means of CyQUANT™ LDH Cytotoxicity Assay **(**Thermo Fisher Scientific, Bremen, Germany**).** The N2a cells were seeded 2 x 10^4 cells in a 96-well plate for 24 hours; they were preincubated with BM (100 µg/mL) or CS (1 µg/mL) or combination (BM 100 µg/mL+ CS 1 µg/mL) for 3 hours prior the treatment, the cells were exposed to 500 µM and 1 mM of H_2O_2. LDH activity was estimated in the supernatant (100 µL) following centrifugation of cells at 2000 rpm for 5 min at 4 °C. The total LDH activity was measured by LDH Positive Control of untreated cells.

Statistical Analysis

One-way analysis of variance (ANOVA) was used to determine the significance of differences between experimental groups. As a post hoc analysis, Tukey's honest difference was conducted based on the results of a one-way ANOVA. All numerical data were presented in the form of a column (mean ± standard error of measurement) for at least three independent experiments. P-value under 0.05 was deemed statistically significant. Using GraphPad Prism 8.4.2 software, the statistical analysis test was performed (GraphPad Software, San Diego, CA, USA).

Chapter 4

Results

Qualitative LC–MS analysis of *Bacopa monnieri*

An evaluation of a qualitative LC–MS analysis of *Bacopa monnieri* (BM) was carried out in a negative mode. Thirteen different compounds were found among them all. Groups of compounds that are characteristic of the substance include a complex of triterpene saponins (bacopaside I) and flavonoids of derivatives such as isosakuranin, kaempferol, and apigenin. The most important component is called bacopaside I (Figure. 9). The findings of the qualitative investigation into BM are depicted in Figure 8 and Table 2, respectively.

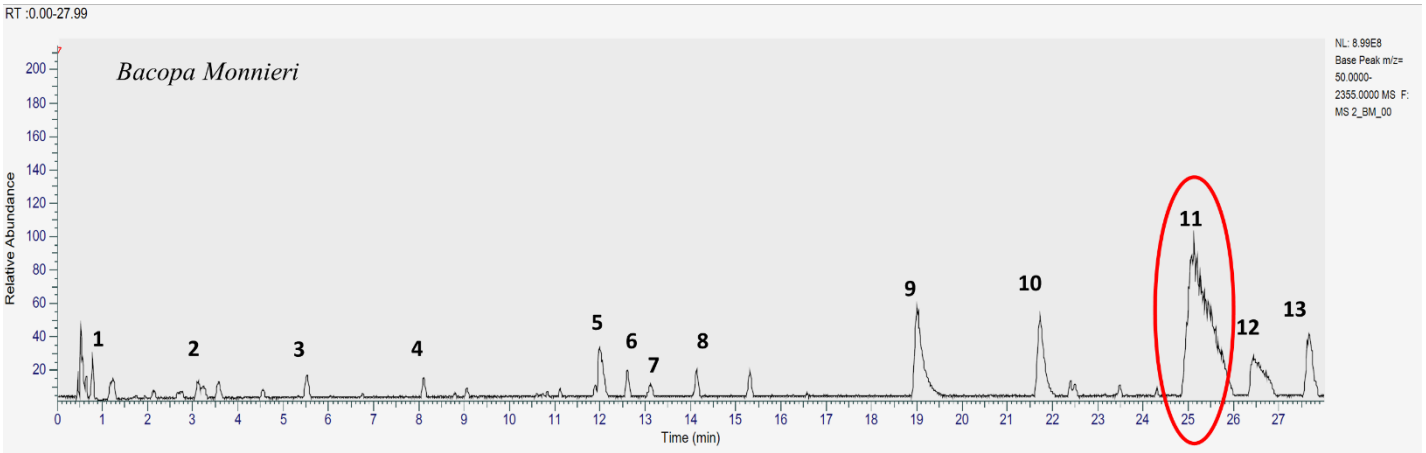


Figure 8. The qualitative assessment of commercial *Bacopa monnieri* (BM) using LC–MS in negative mode ionisation.

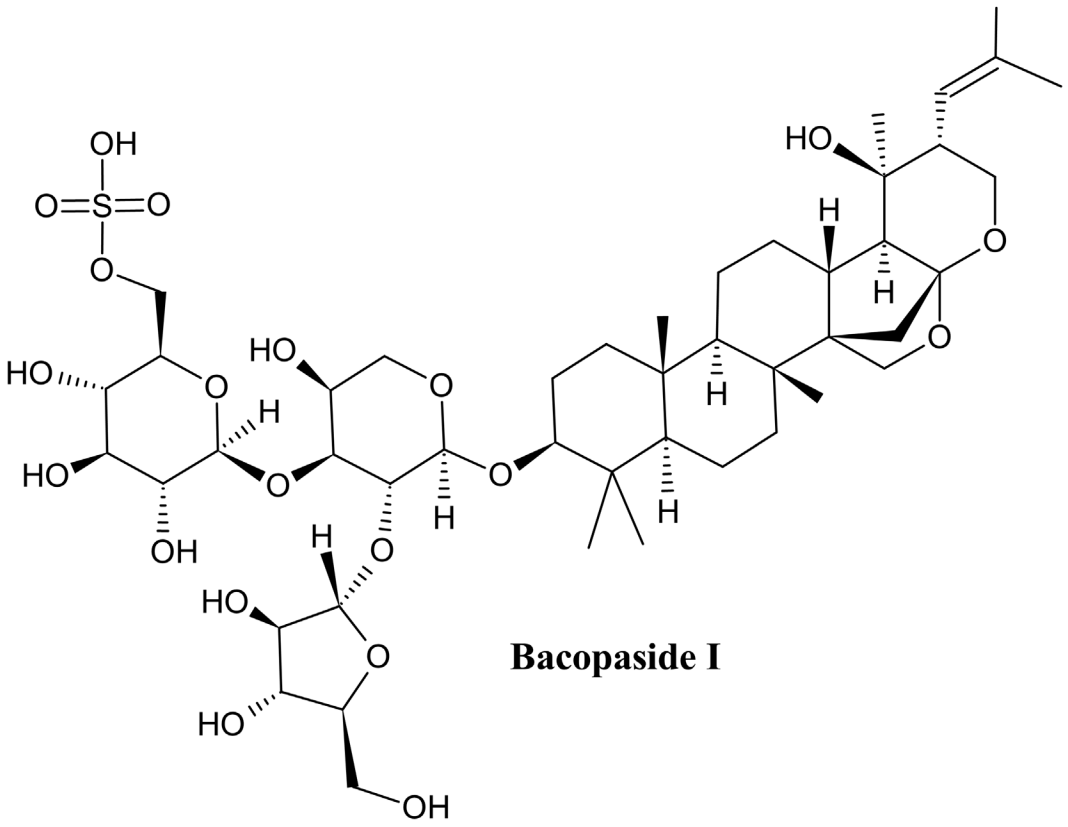


Figure 9. Bacopaside I structure.

Table 2. Qualitative LC–MS analysis of BM

PEAK	RT	[M-H]-	FORMULA	COMPOUND	INTENSITY
1	1.24	345.1192	$C_{15}H_{21}O_9$	ACUBIN	1.10E+08
2	3.12	313.0932	$C_{14}H_{18}O_8$	GLUCOVANILLIN	9.27E+07
3	5.52	121.0284	$C_7H_6O_2$	BENZOIC ACID	1.21E+08

PEAK	RT	[M-H]-	FORMULA	COMPOUND	INTENSITY
4	8.10	493.1540	$C_{23}H_{26}O_{12}$	GLUCOPYRANOSYL DYHYDROXY-DIMETHOXYFLAVONONE	1.55E+08
5	11.98	369.1226	$C_{17}H_{22}O_{9}$	SINAPALDEHYDE HEXOSIDE	2.66E+09
6	12.59	477.1403	$C_{23}H_{26}O_{11}$	UNKNOWN	1.43E+08
7	13.14	447.1301	$C_{22}H_{24}O_{10}$	ISOSAKURANIN	6.59E+07
8	14.14	477.1403	$C_{23}H_{26}O_{11}$	UNKNOWN	1.48E+08
9	19.01	285.0406	$C_{15}H_{10}O_{6}$	KAEMPFEROL	4.76E+08
10	21.73	269.0457	$C_{15}H_{10}O_{6}$	APIGENIN	4.27E+08
11	25.12	977.4430	$C_{60}H_{65}O_{12}$	BACOPASIDE I	7.71E+08
12	26.45	845.4006	$C_{44}H_{62}O_{16}$	UNKNOWN BACOPASIDE	2.10E+08
13	27.60	987.5180	$C_{49}H_{80}O_{20}$	AQUILEGIOSIDE	3.38E+08

Qualitative LC–MS analysis of *Camellia sinensis*

Using negative mode, a qualitative LC–MS analysis of *Camellia sinensis* (CS) was performed and analysed. Twelve different compounds, most of which were catechin derivatives, were found. Epigallocatechin gallate (EGCG) is an important component that must be present. The findings of the qualitative investigation into CS are represented here in Figure 10 and Table 3, respectively.

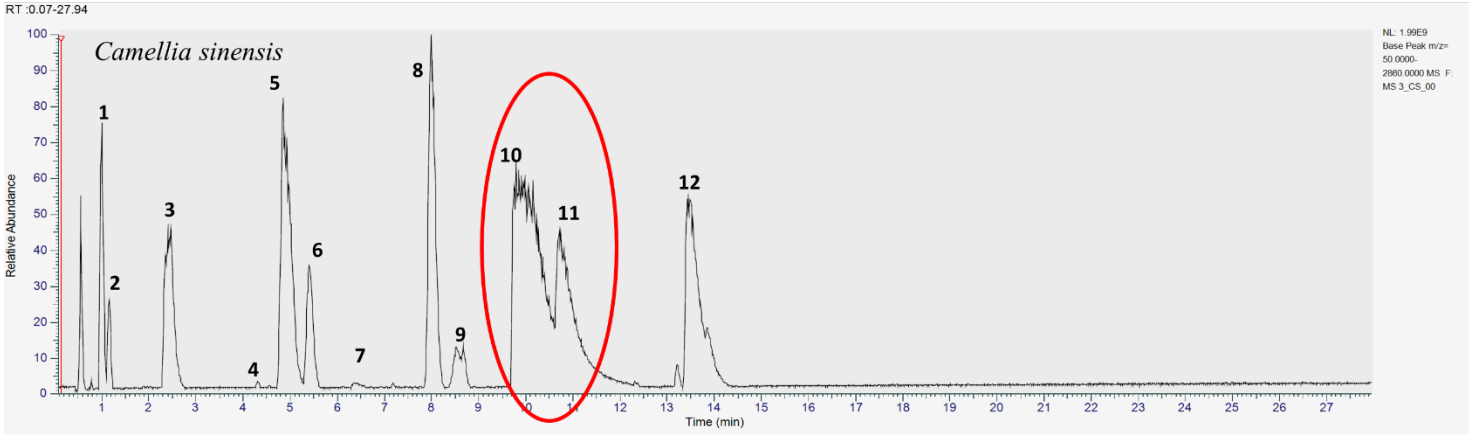
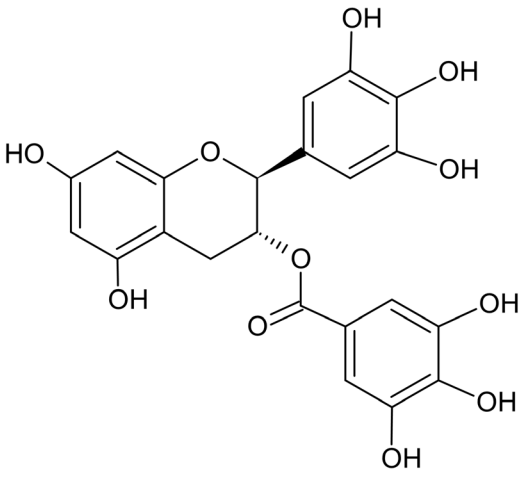
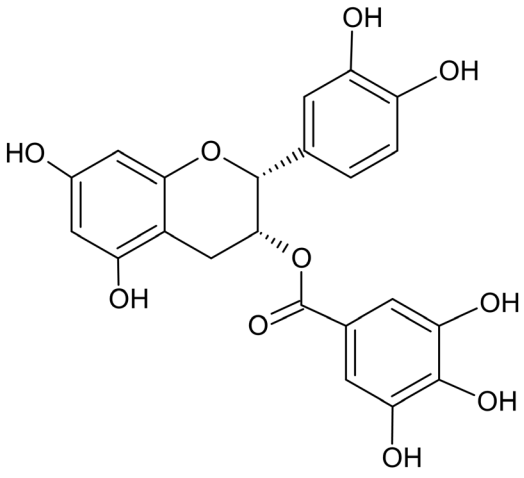


Figure 10. The qualitative assessment of commercial *Camellia sinensis* (CS) using LC–MS in negative mode ionisation.



Epigallocatechin gallate (EGCG)



Epicatechin gallate (ECG)

Figure 11. Epigallocatechin gallate (EGCE) and Epicatechin gallate (ECG) structure.

Table 3. Qualitative LC–MS analysis of CS

PEAK	RT	[M-H]-	FORMULA	COMPOUND	INTENSITY
1	1.00	169.0134	$C_7H_6O_5$	GALLIC ACID	1.45E+09
2	1.16	125.0232	$C_6H_6O_3$	PYROGALLOL	4.39E+08
3	2.40	305.0666	$C_{15}H_{14}O_7$	EPIGALLOCATECHIN	8.84E+08
4	4.31	183.0291	$C_8H_8O_5$	METHYLGALLATE	3.38E+07
5	4.89	305.0666	$C_{15}H_{14}O_7$	EPIGALLOCATECHIN	1.57E+09
6	5.40	289.0717	$C_{15}H_{14}O_6$	CATECHIN	6.57E+08
7	6.39	593.1304	$C_{30}H_{26}O_{13}$	PRODELPHIDIN B3	2.48E+07
8	7.99	289.0717	$C_{15}H_{14}O_6$	EPICATECHIN	1.95E+10
9	8.55	577.1353	$C_{30}H_{26}O_{12}$	PROCYANIDIN DIMER	2.31E+08
10	9.79	457.0774	$C_{22}H_{18}O_{11}$	EPIGALLOCATECHIN GALLATE	1.61E+09
11	10.71	457.0774	$C_{22}H_{18}O_{11}$	EPIGALLOCATECHIN GALLATE	5.64E+08

PEAK	RT	[M-H]-	FORMULA	COMPOUND	INTENSITY
12	13.45	441.0825	$C_{22}H_{18}O_{10}$	EPICATECHIN GALLATE	1.03E+09

Antioxidant activity of the BM or CS

The total phenolic content (TPC) and antioxidant capacities (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) of the sample are presented in Table 4. The phenolic content of the BM is 42.391.42 mg GAE/g, while the phenolic content of the CS is 507.791.78 mg GAE/g. The fact that CS has a higher TPC content than BM may be attributable to the wide range of phytochemicals present in CS, including epigallocatechin gallate, epigallocatechin, epicatechin, catechin, and gallic acid.

When compared to the BM, the ABTS radical scavenging activity of the CS demonstrated significantly higher levels of radical scavenging activity. This suggests that the CS, in comparison to the BM, may have a more potent ability to protect against the damaging effects of free radicals.

Table 4. Total phenolic content and antioxidant properties of BM and CS (each data represents the mean ± SEM)

Extract	**ABTS inhibition (%)**	**ABTS Antioxidant activity (mmol TEAC/g dw)**	**Total Phenolics (mg GAE/g dw)**
Bacopa monnieri	65.77±0.15	22.07±3.02	42.39±1.42
Camellia sinensis	79.73±1.03	564.69±42.17	507.79±1.78

Cell Viability on BV2 microglial cells

Cell viability of BV2 cell was performed by seeding 2 x10^4 cells/well on a 96-well plate and exposure to various concentrations of (100, 50, 10 µg/mL) or CS (1, 0.5, 0.1 µg/mL) or combination (BM 100+CS 1 µg/mL) for 24 h did not alter the viability.

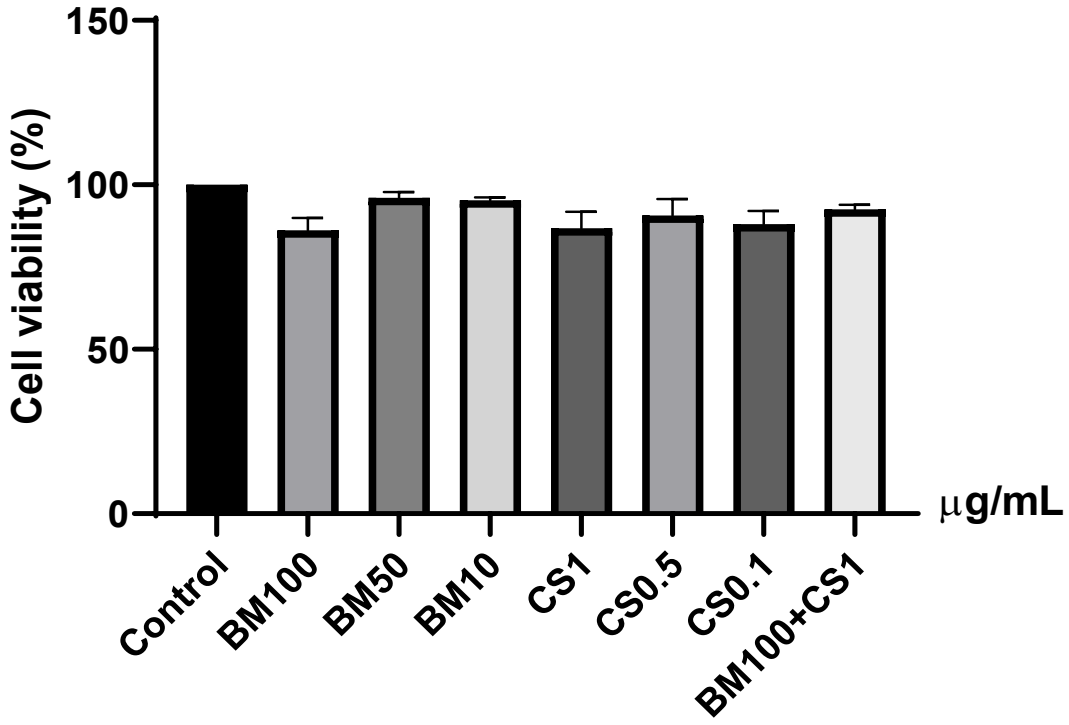


Figure 12. Effect of BM, CS alone and BM+CS combination on BV2 microglial cells viability

BM and CS significantly attenuate the expression of pro-inflammatory cytokines (IL-6 and TNF-α)

Microglia are the primary cell type responsible for producing inflammatory cytokines in the brain. The activation of the TLRs pathway by endotoxin exacerbates neuroinflammation by increasing the expression of pro-inflammatory cytokines such as interleukin (IL)-6 and tumour necrosis factor-alpha (TNF- α). Using an ELISA kit, the level of pro-inflammatory cytokines (IL-6 and TNF-α) in conditioned media of LPS-stimulated BV2 microglial cells pretreated with BM or CS alone and in combination was measured.

In conditioned media, LPS stimulation at 10 ng/mL for 24 hours significantly increases the expression of IL-6 and TNF-α. In a concentration-dependent manner, BM (100, 50 µg/mL) and CS (1, 0.5 µg/mL) significantly decreased the expression of IL-6 and TNF-α (Figure 13). The combination (BM 100+CS 1 µg/mL) also results in a significant decrease in IL-6 and TNFα- levels, but this decrease is not significantly different from that of the single treatment.

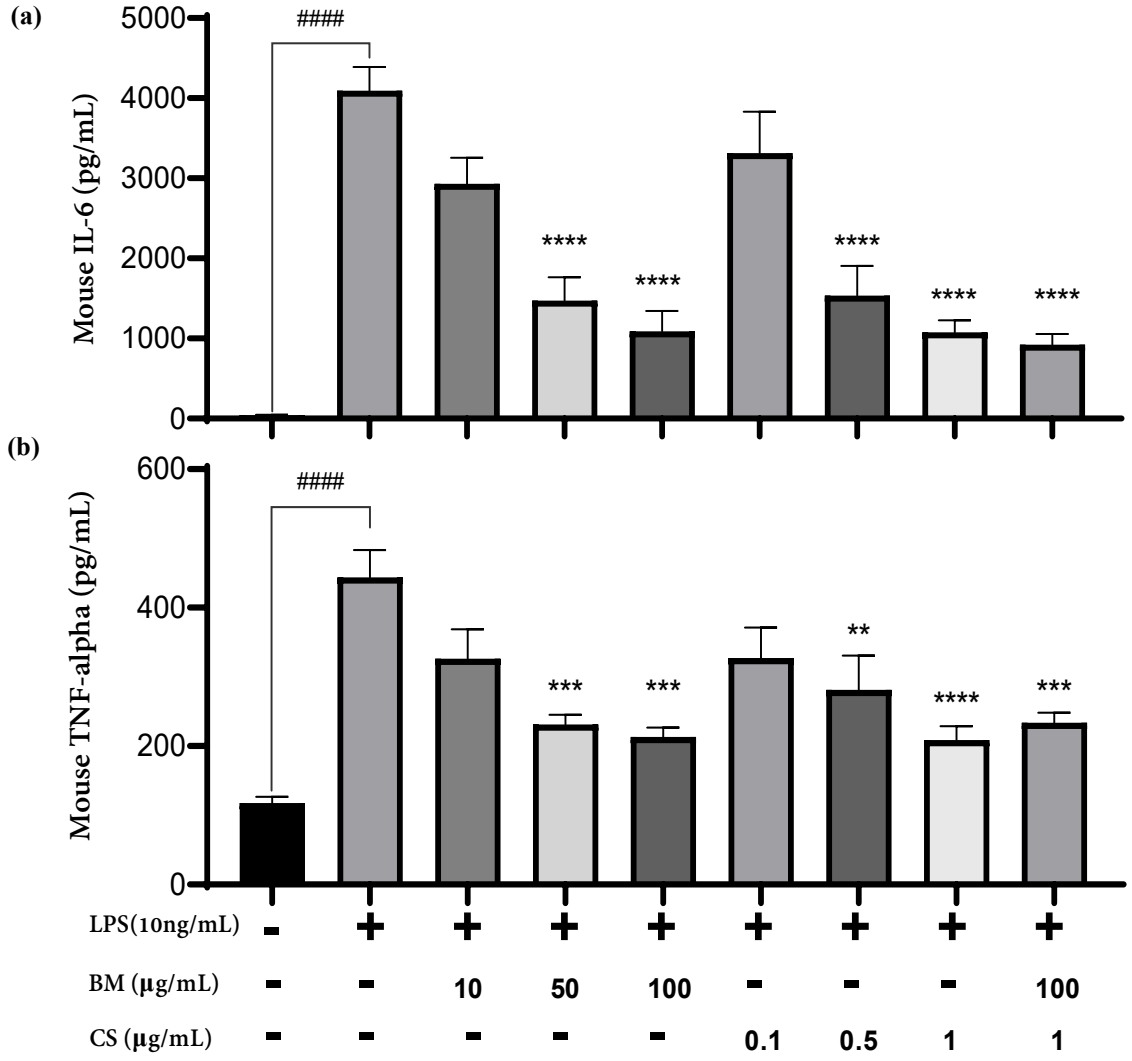


Figure 13. Both extracts can significantly attenuate the expression of pro-inflammatory cytokines (a) Interleukin-6 (IL-6) and (b) TNF-α in a concentration-dependent manner as well as the combination. Each data column represents the mean ± SEM. (n=9 replicates from 3 independent experiments) ####$p < 0.0001$ vs the control, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$ vs. the LPS group.

Inhibition of monocyte chemoattractant protein-1(MCP-1) release by BM and CS supplements

LPS significantly increased the expression of MCP-1 in BV2 microglial cells. However, the levels of MCP-1 were significantly decreased in the presence of BM (100, 50, 10 μg/mL) and CS (1, 0.5, 0.1 μg/mL) in a concentration-dependent manner. Meanwhile, The combination (BM 100+CS 1 μg/mL) significantly decreased the MCP-1 level on LPS-stimulated BV2 cells, which was substantially different from the single treatment. These data indicated that the combination has an additive effect on reducing the chemokine level.

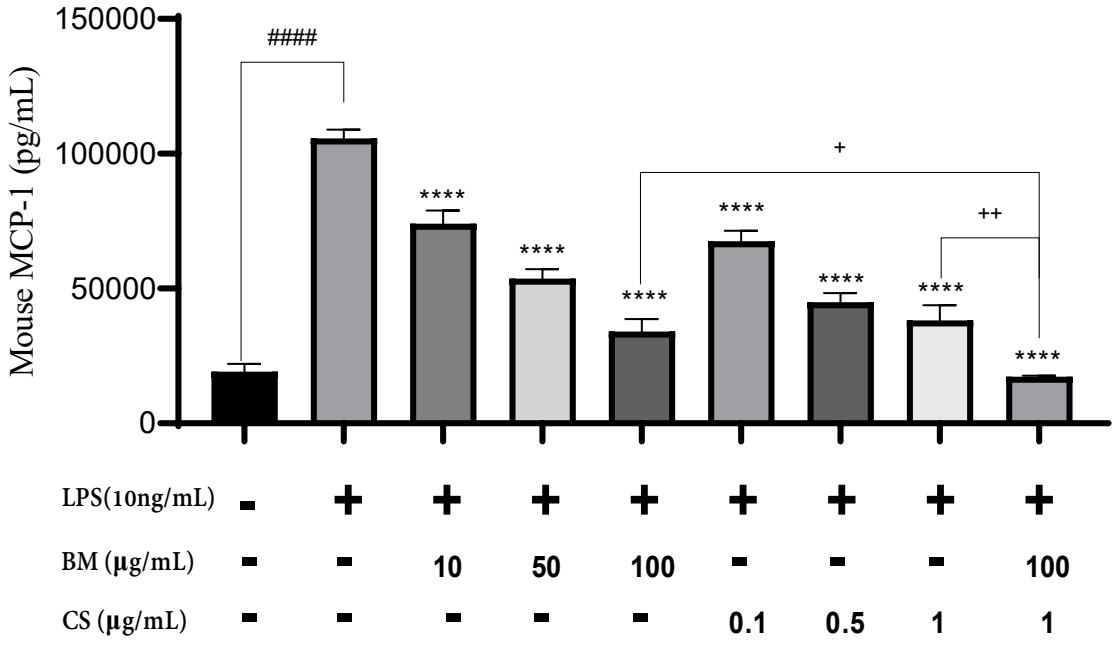
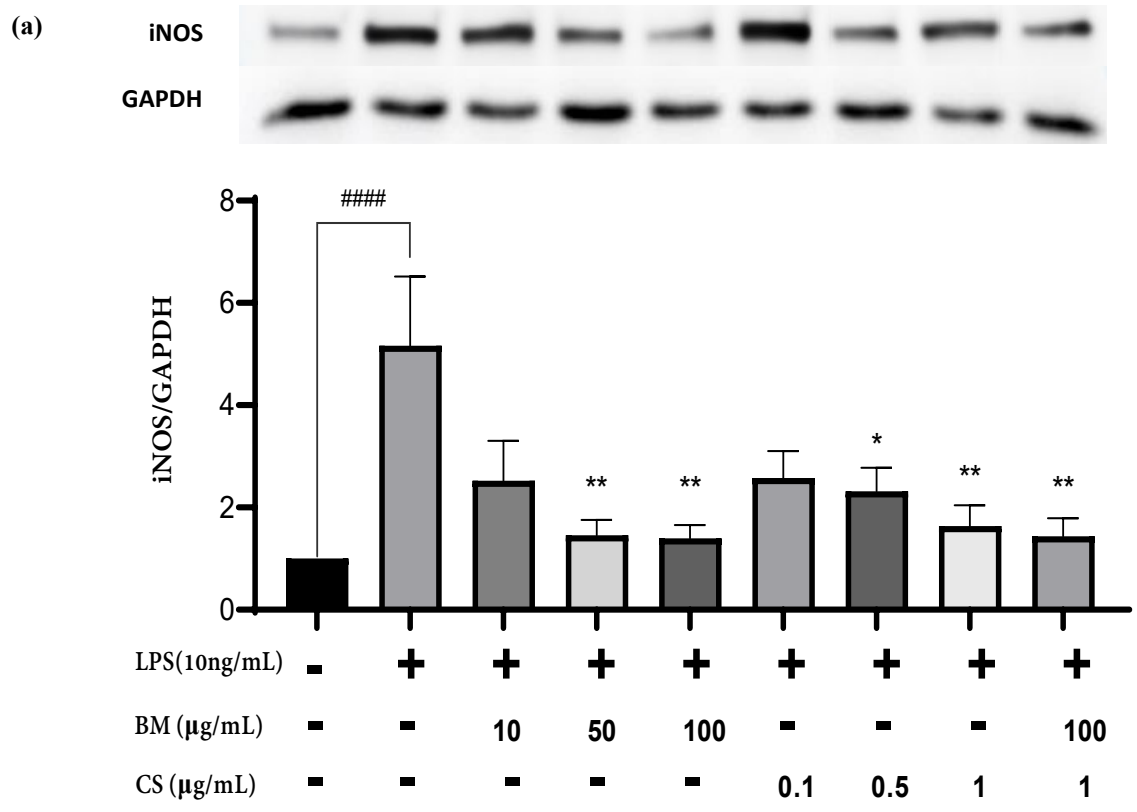


Figure 14. BM, CS alone and BM+CS combination inhibits chemokine (MCP-1) expression in LPS-stimulated BV2 microglial cells in a concentration-dependent manner. Each data column represents the mean ± SEM. (n=9 replicates from 3 independent experiments) ####$p < 0.0001$ vs the control group, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p<0.05$ vs. the LPS group, ++$p < 0.01$, +$p<0.05$ vs. the treatment group.

BM and CS reduced nitric oxide (NO) induction and inducible NO synthase (iNOS) expression by LPS in the BV2 cell

Inducible nitric oxide synthase (iNOS) is an essential enzyme in the microglia's inflammatory response, as it is the source of nitric oxide (NO) that is strongly induced by proinflammatory stimuli (McNeill et al., 2015). Immunoblot analysis was used to determine whether the inhibitory effect of BM and CS on NO production was mediated by iNOS expression. LPS stimulation (10 ng/mL for 24 h) significantly increased the expression of iNOS (Figure 15a.) and the production of NO (Figure 15b.), which was attenuated by the combination of BM and CS. In a concentration-dependent manner, BM (100, 50 μg/mL) and CS (1, 0.5 μg/mL) significantly decreased the expression of iNOS and NO production.



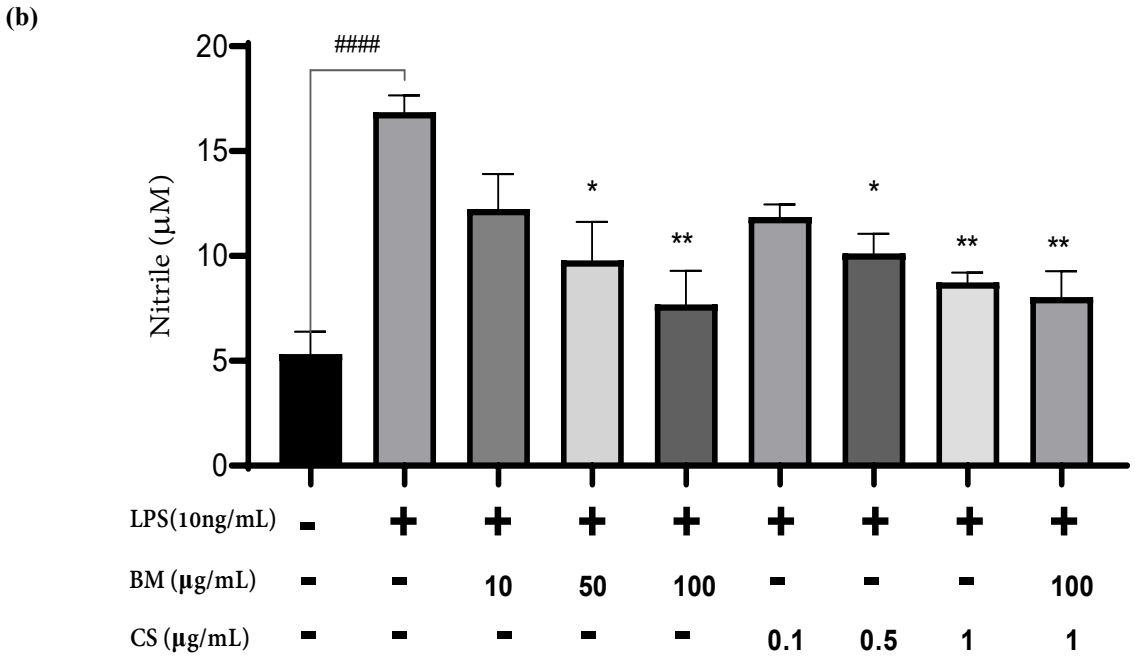


Figure 15. BM and CS alone and in combination can significantly attenuate microglial (a) iNOS expression and (b) NO production in response to LPS. Each data column represents the mean ± SEM. (n=9 replicates from 3 independent experiments) ####$p < 0.0001$ vs the control group, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p<0.05$ vs. the LPS group.

Inhibition of Cyclooxygenase-2 (COX-2) by BM and CS supplements

When microglia are stimulated with LPS, MAPK signalling pathways are involved in the regulation of inflammatory mediators, playing an essential part in this process. According to the findings of (Kim, Quang, Oh, & Kim, 2017), LPS causes microglia to overexpress COX-2 by activating the JNK, ERK, and p38 MAPK pathways. BV2 microglial cells were pretreated with BM (100, 50, 10 μg/mL) or CS (1, 0.5, 0.1 μg/mL) or combination (BM 100 μg/mL+ CS 1 μg/mL) for 3 hours prior to LPS (10 ng/mL) exposure for 24 h. CS reduces COX-2 expression at all concentrations and in combination, whereas only BM at 100 μg/mL has a dramatic effect on COX-2 expression.

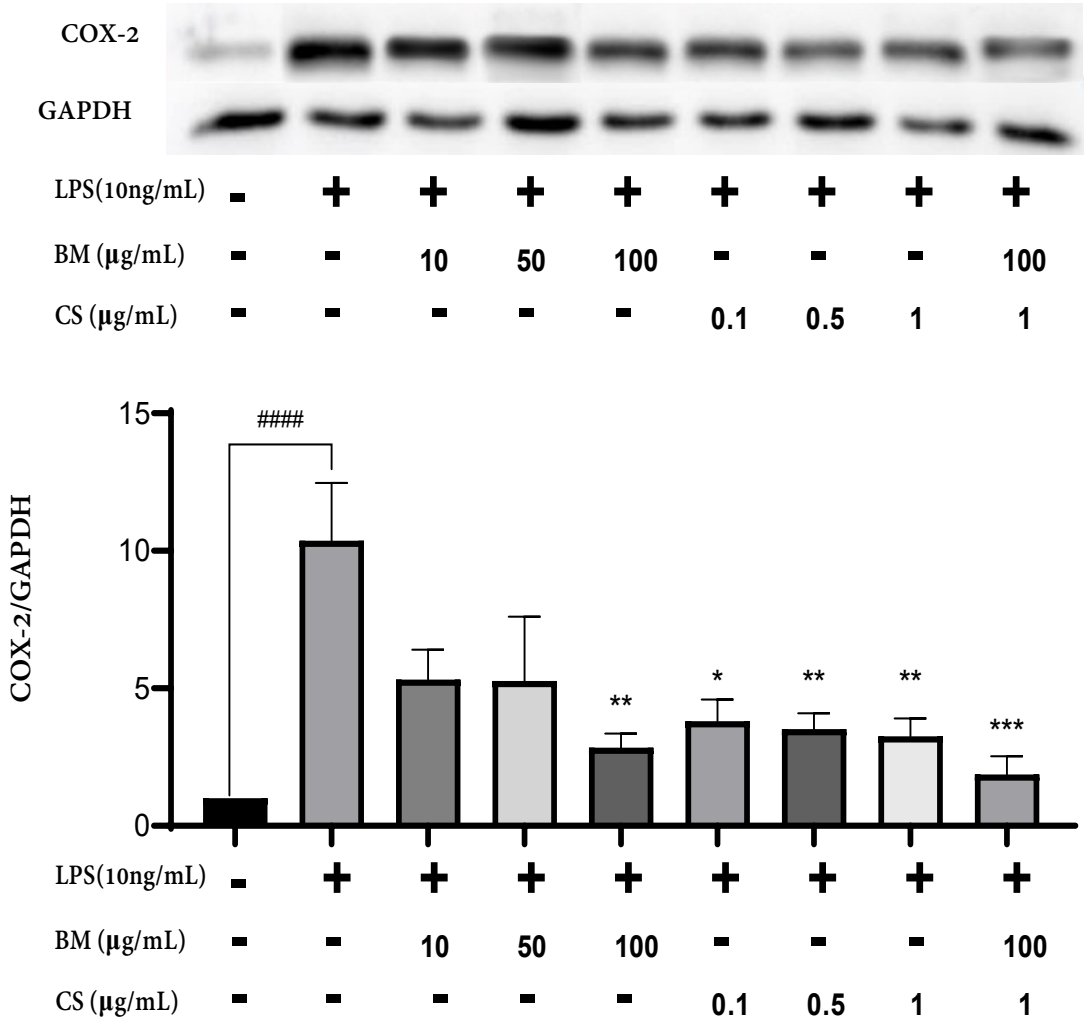
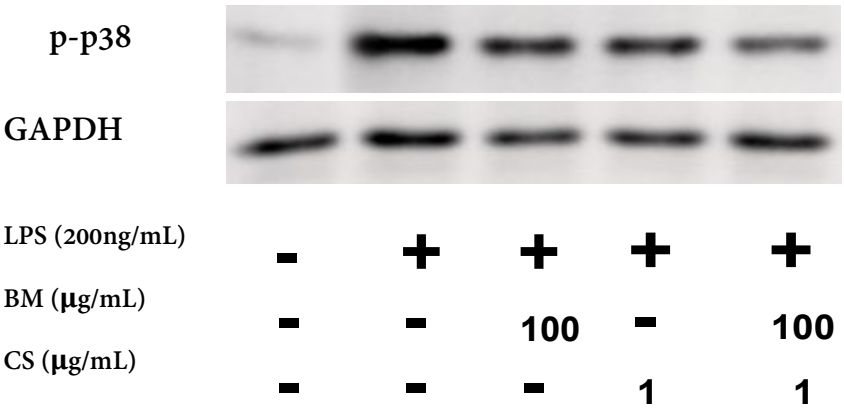


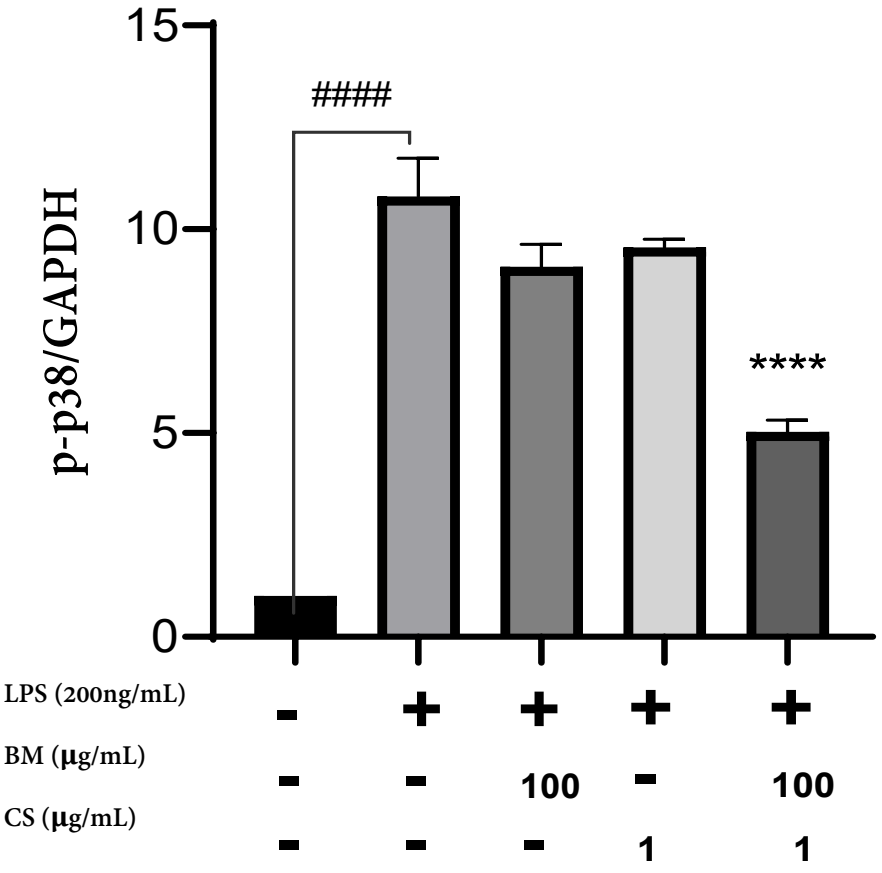
Figure 16. BM and CS alone and, in combination, can significantly attenuate COX-2 expression. Each data column represents the mean ± SEM. (n=9 replicates from 3 independent experiments) ####$p < 0.0001$ vs the control group, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p<0.05$ vs. the LPS group.

The combination of BM and CS significantly inhibits the expression of p38, p-JNK, and p-NFκB p65

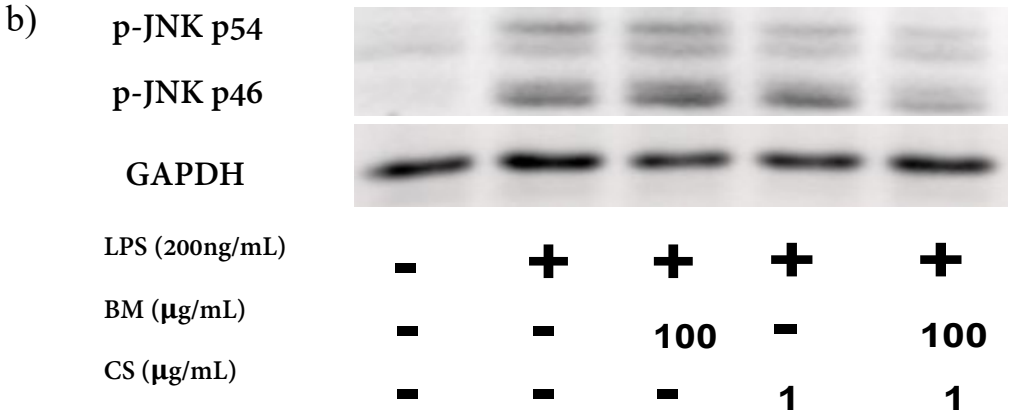
MAPK activation is involved in the modulation of inflammatory response. In Figure 17, we examined the effect of BM, CS, and the combination of BM+CS on MAPK signals in LPS-induced inflammatory response in BV2 cells. The phosphorylation of p38 (a) and JNK (b) in these cells was significantly enhanced after treatment with LPS 200 ng/mL for 30 mins. In contrast, phosphorylation of p38 and JNK were reduced after pretreatment of the combination of BM 100 μg/mL and CS 1 μg/mL for 3 hours. The expression pattern of NF-κB p65 proteins (c) was examined by western blotting to determine whether the NF-κB pathway is involved in the anti-inflammatory capability of the combination of BM 100 μg/mL and CS 1 μg/mL. The expression of phosphorylated NF-κB p65 was upregulated in BV2 cells in response to LPS 200 ng/mL for 30 mins. However, the combination of BM 100 μg/mL and CS 1 μg/mL markedly down-regulated the induced expression of phosphorylated NF-κB p65. These results demonstrated that the combination of BM and CS exerted an anti-inflammatory effect via modulating the NF-κB and MAPK pathways.

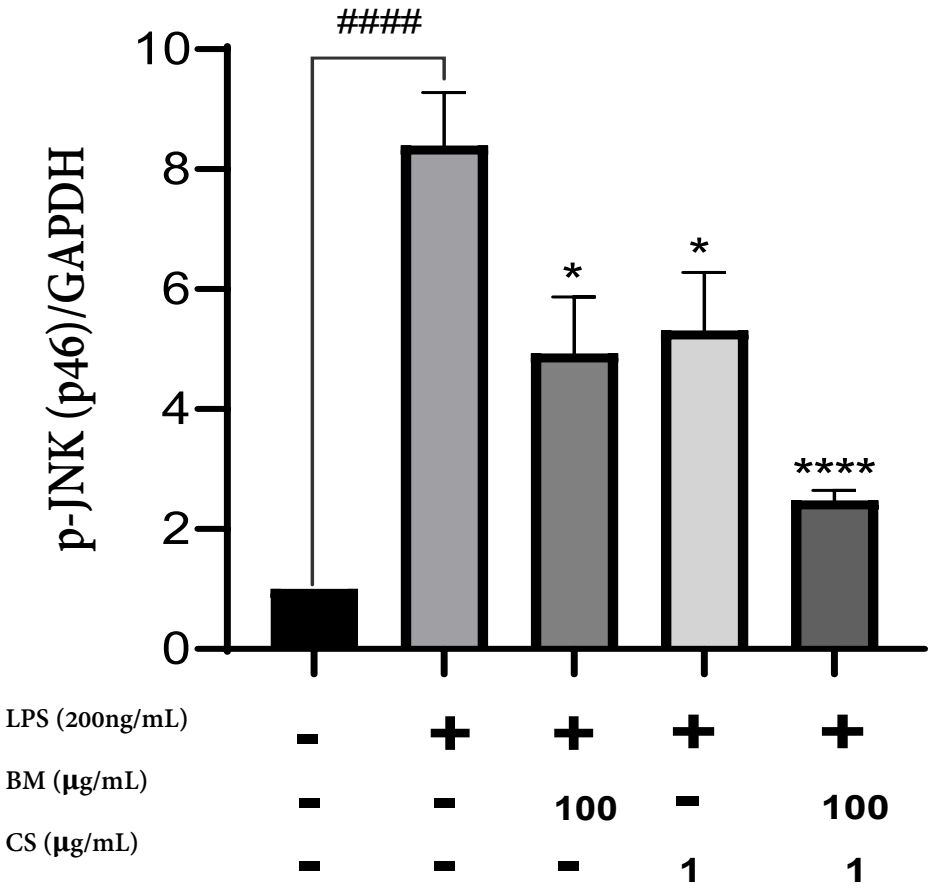
a)


p-p38
GAPDH
LPS (200ng/mL)
BM (µg/mL)
CS (µg/mL)
- + + + +
- - 100 - 100
- - - 1 1


15
10
5
0
p-p38/GAPDH
####
****
LPS (200ng/mL)
BM (µg/mL)
CS (µg/mL)
- + + + +
- - 100 - 100
- - - 1 1

b)


p-JNK p54
p-JNK p46
GAPDH
LPS (200ng/mL) - + + + +
BM (µg/mL) - - 100 - 100
CS (µg/mL) - - - 1 1


####
p-JNK (p46)/GAPDH
10
8
6
4
2
0
*
*
****
LPS (200ng/mL) - + + + +
BM (µg/mL) - - 100 - 100
CS (µg/mL) - - - 1 1

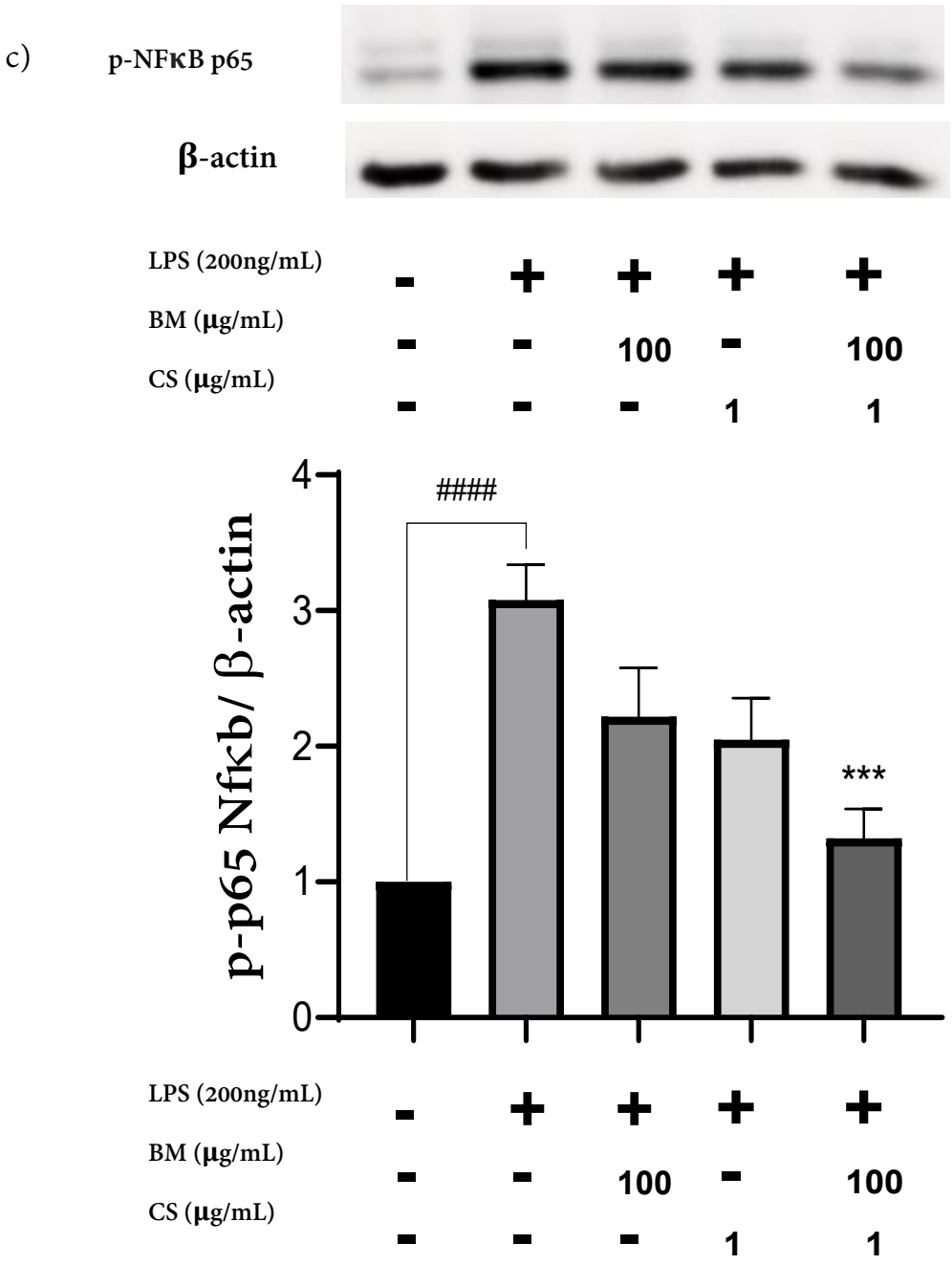


Figure 17. Western blot analysis shows that the phosphorylation of (a) p38 and (b) JNK and (c) NF-κB p65 were significantly suppressed by the combination of BM 100 μg/mL and CS 1 μg/mL. Each data column represents the mean ± SEM. (n=9 replicates from 3 independent experiments) ####$p < 0.0001$ vs the control group, ****$p < 0.0001$, ***$p < 0.001$, **$p < 0.01$,*$p<0.05$ vs. the LPS group.

Cell Viability and % LDH release on N2a cell

The neuroprotective effect of BM and CS on H_2O_2-induced cell death has been investigated. Exposure of cells to high concentrations (500 μM or 1 mM) of H_2O_2 significantly reduced the cell viability compared to the control group, especially 1mM H_2O_2 after 24 h exposure (Figure. 18a). H_2O_2-induced anti-cell proliferation was attenuated by pretreatment of cells with BM 100 μg/mL, CS 1 μg/mL and the combination (BM 100 μg/mL+ CS 1 μg/mL), as shown in Figure. 18a. The combination of BM and CS almost neutralised 500 μM of H_2O_2-induced cell death compared to the control.

The releasing of lactate dehydrogenase (LDH) is an assay to measure cytotoxicity. LDH levels in the cell supernatant were determined using a CyQUANT™ LDH cytotoxicity assay kit. High concentrations (500 μM or 1 mM) of H_2O_2 significantly elevated LDH release, which significantly decreased by pretreatment of the combination (BM 100 μg/mL+ CS 1 μg/mL).

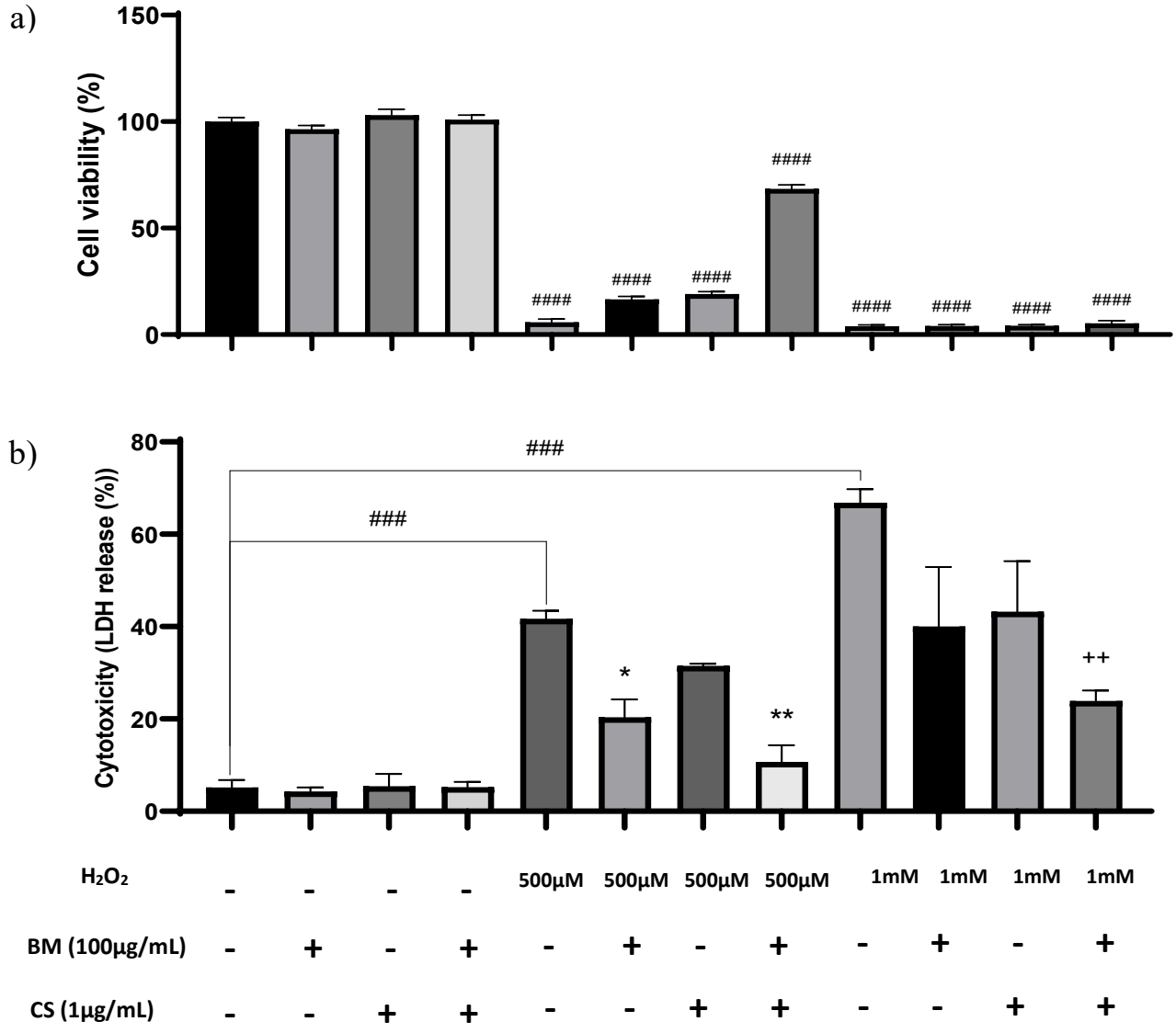


Figure 18. Effect of BM, CS and the combination on (a) H_2O_2 induced N2a cell viability and (b) LDH leakage. Cell proliferation was determined by CCK8 assay. LDH levels in the cell supernatant were determined using a CyQUANT™ LDH cytotoxicity assay kit. Each data column represents the mean ± SEM. (n=9 replicates from 3 independent experiments) ####$p < 0.0001$, ###$p < 0.0005$ vs the control group, **$p < 0.01$, *$p < 0.05$ vs. the 500 µM H_2O_2 group, ++$p < 0.01$, vs. the 1 mM H_2O_2 group.

Chapter 5

Discussion

Microglia is the essential innate immune response in CNS, regulating innate and adaptive immune responses and destroying pathogens. Neurodegeneration induced by viruses is notable because it refers to the interaction between the central nervous system (CNS) and environmental and viral factors and suggests that the immune response plays an important role in neurodegeneration (Czirr & Wyss-Coray, 2012). As well as the onset of neurodegenerative diseases such as trauma, stroke, Alzheimer's disease, Parkinson's disease, and multiple sclerosis, microglial cells become activated and produce proinflammatory cytokines and neurotoxic materials, including TNF-α, IL-6, and NO. Numerous proinflammatory mediators, such as NO, iNOS, and COX-2, as well as proinflammatory cytokines (IL-6 and TNFα-) or chemokine (MCP-1), constitute the majority of the neuroinflammatory conditions in microglia. (Rojo, Fernández, Maccioni, Jimenez, & Maccioni, 2008).

Aside from medicine, nutraceuticals are considered an alternative choice for treating neurological diseases. *Bacopa monnieri* (BM) and *Camellia sinensis* (CS) are candidate compounds due to traditional knowledge and their phytochemical composition. Previous studies have shown that BM increases dendritic pruning to enhance network efficiency (McPhee, Downey, Wesnes, & Stough, 2021) and improve memory (Rajendran Jeyasri et al., 2020). The health benefits of CS or green tea include antioxidant, anti-inflammatory, and neuroprotective properties that have also been reported (Malar et al., 2020). However, the combination may have an additive effect on neurological disease.

The results of our qualitative LC-MS analysis of BM are consistent with the findings of the Christopher group, which identified three elite genotypes of *Bacopa monnieri* with exceptionally high levels of Bacoside A and Bacopaside I (Christopher, Johnson, Mathew, & Baby, 2017). Our results only provide Bacopaside I, which is the only dissimilarity. This may be due to the different origins of herbal medicine sources. Our sample is a commercial ingredient in which the raw extract is combined with a carrier agent that increases the solubility of the phytocomplex, whereas the other study used the extract directly obtained from the plants. Similarly to *Camellia sinensis*, the

primary components were epigallocatechin gallate (EGCG) and epicatechin gallate (ECG), which corresponded with the Wang research group's studies (Y. Wang, Kan, Wan, McGinley, & Thompson, 2019).

Treatment of neuroinflammation could be exerted through the inhibition of proinflammatory cytokines, chemokine, and mediators. In this study, LPS-induced increases in proinflammatory cytokine levels in BV2 microglial cell was used as *in vitro* models of neuroinflammation (Nam et al., 2018) and H_2O_2-induced neuronal cell death in N2a cells was used as a model for test the neuroprotective effect (Ghaffari et al., 2014).

Therefore, the anti-neuroinflammatory effects of BM, CS, and its combination in BV2 microglial cells were also investigated. BM (100, 50 μg/mL) and CS (1, 0.5 μg/mL) significantly decreased the expression of IL-6 and TNF-α (Figure 13). The levels of MCP-1 were significantly decreased in the presence of BM (100, 50, 10 μg/mL) and CS (1, 0.5, 0.1 μg/mL) in a concentration-dependent manner. Our findings for BM and CS on LPS-induced neuroinflammation in microglial cells were consistent with previous findings, which showed that BM (Nemetchek, Stierle, Stierle, & Lurie, 2017) and CS (Cheng-Chung Wei et al., 2016) significantly inhibited the release of TNF-α and IL-6 from activated microglial cells *in vitro*. The combination (BM 100+CS 1 μg/mL) also significantly decreases IL-6 and TNF-α levels, but this decrease is not significantly different from that of the single treatment. Meanwhile, the combination (BM 100+CS 1 μg/mL) significantly decreased the MCP-1 level on LPS-stimulated BV2 cells, which was substantially different from the single treatment. These data indicated that the combination has an additive effect on reducing the chemokine level (Figure. 14). BM (100, 50 μg/mL), CS (1, 0.5 μg/mL) and the combination of BM and CS significantly decreased the production of NO (Figure 15b) and attenuated the protein expression of iNOS (Figure 15a) and COX-2 (Figure 16) in BV2 cells. These findings support the earlier investigation of Jeyasri et al. and Ahn et al., showing that BM and CS inhibit the expression of inflammation-responsible genes such as COX2 and iNOS in LPS-induced macrophages cells (R. Jeyasri et al., 2022) (Ahn & Kim, 2011). This result may be due to the antioxidant activity of significant components in BM and CS.

Activation of the NF-κB and MAPK signalling pathways has been linked to LPS-induced inflammation in BV2 microglial cells. NF-κB is a transcription factor involved in the regulation

of proinflammatory mediators and cytokines' production or expression. MAPKs, including p38 and JNK, are activated by a variety of extracellular or intracellular stimuli, such as LPS. Thus, these proteins are associated with the inflammatory response. This study investigated the effects of BM, CS, and their combination on LPS-induced activation of NF-κB and MAPKs. Pretreatment with the combination of BM and CS appeared to deactivate NF-κB by inhibiting the phosphorylation and translocation of p65 in BV2 cells, as indicated by the obtained results (Figure 17c). Our results are similar to those obtained by Viji et al. (Viji et al., 2010) for BM and Wang et al. for CS (Q. Wang et al., 2022). The combination of BM and CS inhibited the phosphorylation of JNK and p38 MAPK induced by LPS (Figure 17a-b). These findings suggested that the combination of BM and CS exerts anti-neuroinflammatory effects via the additive inactivation of the NF-B, JNK, and p38 MAPK pathways.

Neuronal cell death occurs during normal development, as a result of injury, and neurodegenerative diseases such as Alzheimer's disease. Although developmental neuronal cell loss is necessary for the proper functioning of the nervous system, pathological forms of neuronal cell death can severely impair mental, cognitive, and motor abilities. Considerable attention has been devoted to the pathways that regulate cell death in the nervous system (Savitz & Rosenbaum, 1998). Traditionally, necrosis and apoptosis have been described as two distinct mechanisms of cell death; however, they may represent opposite ends of a continuum. Apoptotic cell death is a tightly regulated, energy-dependent process characterised by a series of morphological and biochemical alterations. Chromatin condensation, chromosomal and nuclear fragmentation, cellular shrinkage, and membrane blebbing characterise apoptosis through the formation of apoptotic bodies. Biochemically, the apoptotic cascade involves the activation of multiple enzymes and proteins, culminating in the activation of DNA-degrading caspase enzymes. Rapid loss of membrane and organelle integrity, resulting in cell swelling and lysis, defines necrotic cell death. In necrosis, the release of cellular contents triggers an inflammatory response, whereas, in apoptosis, this response is typically absent. As indicators of necrosis and apoptosis, respectively, the release of lactate dehydrogenase (LDH) and the accumulation of nucleosomes within cells was used. Both neuronal cell types released significant amounts of LDH; however, the pattern of LDH release was variable and agonist-dependent. In response to the nitric oxide generator sodium nitroprusside (SNP), cortical cells displayed pronounced LDH release and dramatic morphologic changes.

The neuroprotective effect of BM and CS on H_2O_2-induced cell death has been investigated on N2a cells. Exposure of cells to high concentrations (500 μM or 1 mM) of H_2O_2 significantly reduced the cell viability compared to the control group, especially 1mM H_2O_2 after 24 h exposure (Figure. 18a). Unlike the result from Russo et al. which showed that BM extract can prevent the neuronal cell death from H_2O_2 by slow down the releasing of lactate dehydrogenase (LDH) (Russo, Izzo, Borrelli, Renis, & Vanella, 2003). Only the combination of BM and CS almost neutralised 500 μM of H2O2-induced cell death compared to the control. The releasing of LDH is an assay to measure cytotoxicity. LDH levels in the cell supernatant were determined using a CyQUANT™ LDH cytotoxicity assay kit. High concentrations (500 μM or 1 mM) of H_2O_2 significantly elevated LDH release, which significantly decreased by pretreatment of the combination (BM 100 μg/mL+ CS 1 μg/mL).

This study demonstrated an additive anti-neuroinflammatory and neuroprotective effect of BM and CS in microglia cells stimulated with LPS. Furthermore, this study revealed that the combination of BM and CS inhibited inducible NF-kB activation and the subsequent induction of proinflammatory mediators, such as NO, IL-6, TNF-α, and MCP-1, and that it could be an excellent candidate for in vivo studies and clinical trial aimed to investigate the prevention activity of the combination of BM and CS against neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

Chapter 6

Summary and Conclusions

In conclusion, this study found that combining *Bacopa monnieri* and *Camellia sinensis* (BM+CS) reduced H_2O_2-induced cytotoxicity in N2a cells and ameliorated neuroinflammatory reactions in LPS-stimulated microglia BV2 cells via inhibitions of NF-κB and MAPK with an additive effect. This effect may be a direct result of this combination's neuroprotective and anti-neuroinflammatory properties. In addition, Bacopacide I, the primary component of BM, and Epigallocatechin, the primary component of CS, may be closely associated with their neuroprotective and anti-neuroinflammatory effects. The obtained results showed that neurodegenerative diseases linked to neurotoxicity, oxidative stress, and neuroinflammation might be prevented and treated with the combination of BM and CS. It has the potential to prevent cognitive decline, protect the brain, and reduce brain inflammation.

Further *in vivo* studies and clinical trials are necessary to confirm the efficacy of the combination of BM and CS.

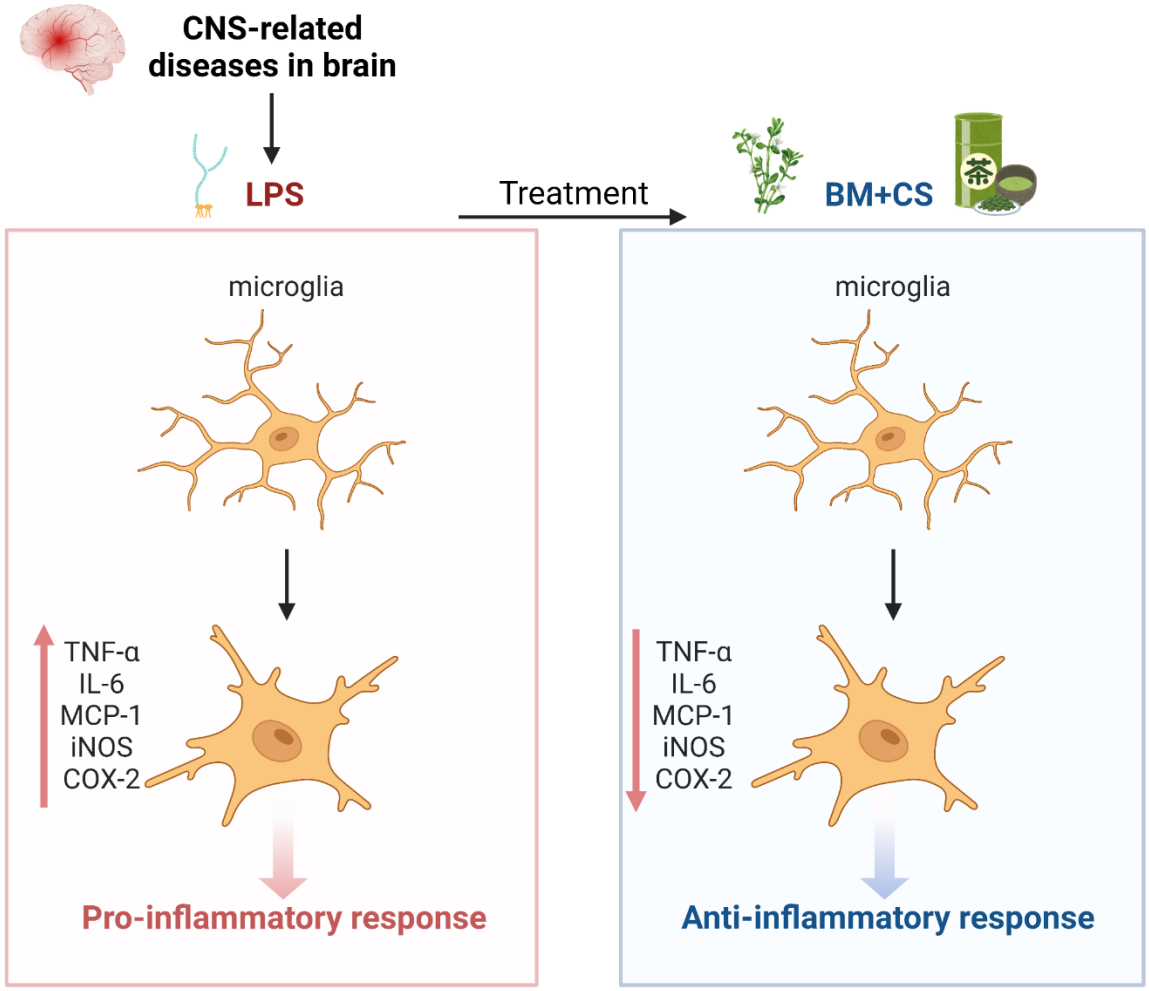


Figure 19. Summary of anti-neuroinflammation and neuroprotective effect of BM or CS and its combination

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Appendix

Poster at the 2022 Young Investigator Symposium, Dublin, Ireland (26-27th October 2022)

Investigation of *Bacopa monnieri* and *Camellia sinensis*, alone and in combination, as candidates for potential neurotherapeutic agents

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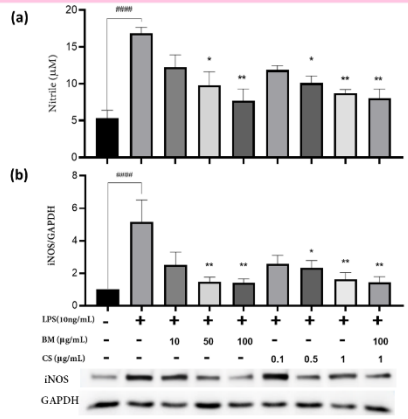
Introduction

Bacopa monnieri

Bacopa monnieri (BM) and *Camellia sinensis* (CS) are natural plants used as nutraceutical products in Asia with neuroprotective properties. BM is a memory and learning enhancer (Brimson et al., 2021), while CS is a supplement with high antioxidant activity (Malar et al., 2020). However, the scientific support is unclear. Several previous studies have investigated the neurotherapeutic effect on a single extract, but there is currently no evidence regarding the combination of BM and CS. This study was a preliminary investigation to explore the effects of BM and CS, both alone and in combination, on microglial activation. Using lipopolysaccharide (LPS)-stimulated BV2 microglia as an *in vitro* model of neuroinflammation.

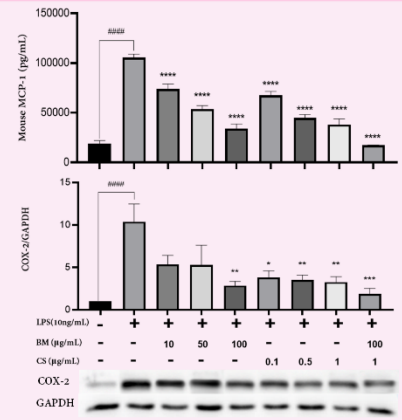
Camellia sinensis (Green tea)

BM and CS reduced nitric oxide (NO) induction and inducible NO synthase (iNOS) expression by LPS in the BV2 cell

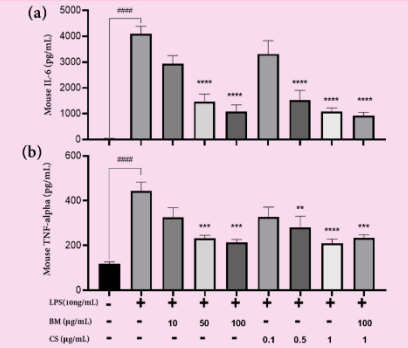


BM and CS alone and in combination, can significantly attenuate microglial (a) NO production and (b) iNOS expression in response to LPS. Each data column represents the mean ± SEM.(n = 3). ####p <0.0001 vs. the control, **p < 0.01, *p < 0.05 vs. the LPS group.

Inhibition of monocyte chemoattractant protein-1(MCP-1) release and Cyclooxygenase-2 (COX-2) by BM and CS supplements

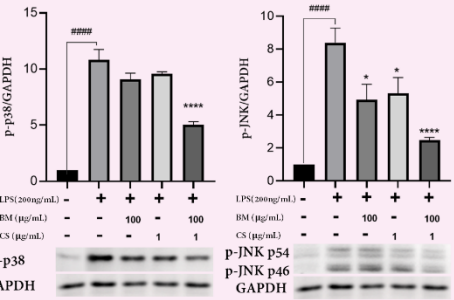


BM and CS significantly attenuate the expression of pro-inflammatory cytokines (IL-6 and TNF-α)



Both extracts can significantly attenuate the expression of pro-inflammatory cytokines (a) Interleukin-6 (IL-6), and (b) TNF-α in a concentration-dependent manner as well as the combination. Each data column represents the mean ± SEM. (n=3) ####p < 0.0001 vs the control, ****p < 0.0001, ***p < 0.001, **p < 0.01 vs. the LPS group.

Combination of BM and CS significantly inhibit the expression of p38 and p-JNK



Each data column represents the mean ± SEM. (n=3) ####p < 0.0001 vs the control group , ****p < 0.0001, ***p < 0.001, **p < 0.01,*p<0.05 vs. the LPS group.

Summary

- BM and CS both reduce microglial activation in response to LPS in a concentration-dependent manner as well as the combination.
- Combination of BM and CS has an additive inhibitory effect on release of MCP-1 only.
- BM and CS likely act on microglia via inhibition of p-p38 and p-JNK.

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SPECIFICA TECNICA
TECHNICAL SPECIFICATION

Prodotto: **E.S. TIT. BACOPA 20% BACOSIDI**
Product *BACOPA D.E. 20.0% BACOSIDES*

SPECIFICA *SPECIFICATION*	**METODO** **METHOD**	**Lim. Inf. - Lim. Sup.** *Lower Lim. - Upper Lim.*	**u.m.**
Numero CAS *CAS number*		93164-89-7 *93164-89-7*	
Nome botanico *Botanical name*		Bacopa monniera *Bacopa monniera*	
Solvente di estrazione *Extraction solvents*		Etanolo *Ethanol*	
Parte della pianta *Botanical part*		Erba *Herb*	
Eccipienti *Excipients*		Maltodestrina (20-40%) *Maltodextrin (20-40%)*	
Rapporto Droga/Estratto *Drug/Extract ratio*		Da 8:1 a 12:1 *8:1 to 12:1*	
Aspetto *Aspect*		Polvere da verdastra a verde chiaro con odore caratteristico *Greenish to green powder with characteristic odor*	
Identificazione HPLC *Identification HPLC*		Conforme *Conform*	
Solubilità *Solubility*			
- In acqua 1% p/v *In water 1% w/v*		>=50,00	%
- In alcool (1% w/v) *In alcohol (1% w/v)*		>=50,00	%
Contenuto Bacosidi *Content Bacosides*		20,00 - 25,00	%
Altri solventi usati nella produzione *Other solvent used in the manufacture*		Acetone *Acetone*	

I metodi di analisi non indicati sono metodi interni del produttore ottenibili su specifica richiesta

The analytical methods not listed are internal methods of the manufacturer obtainable upon specific request

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SPECIFICA TECNICA
TECHNICAL SPECIFICATION

Prodotto: **E.S. TIT. BACOPA 20% BACOSIDI**
Product *BACOPA D.E. 20.0% BACOSIDES*

Idrocarburi Policiclici Aromatici *Polycyclic aromatic hydrocarbons*			
- Benzo(a)pirene *Benzo(a)pyrene*		<=10 *<=10*	ppb
- Somma di Benzo(a)pirene, Benzo(a)antracene,		<=50 *<=50*	ppb
Ceneri solforiche *Sulphated ash*		<=4,00	%
Perdita all'essiccamento *Loss on drying*		<=8,00	%
Solventi Residui *Residual Solvent*		Conforme Dir. 2009/32/EC *Conforms Dir. 2009/32/EC*	
Pesticidi *Pesticicdes*		Conforme EP *Conform EP*	
Metalli Pesanti totali *Heavy Metals*			
- Metalli pesanti *Heavy Metals*		<=20	ppm
- Piombo *Lead*		<=3	ppm
- Cadmio *Cadmium*		<=1	ppm
- Mercurio *Mercury*		<=0,1	ppm
- Arsenico *Arsenic*		<=1	ppm
Conta batterica totale *Total aerobic count*		<=5.000	CFU/g
Lieviti e muffe *Yeasts and mould*		<=100	CFU/g
E.coli *E.coli*		Assente/10g *Absent/10g*	
Salmonella *Salmonella*		Assente/25g *Absent/25g*	

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SPECIFICA TECNICA
TECHNICAL SPECIFICATION

Prodotto: **E.S. TIT. BACOPA 20% BACOSIDI**
Product *BACOPA D.E. 20.0% BACOSIDES*

Staphylococcus aureus *Staphylococcus aureus*		Assente/10g *Absent/10g*	
Pseudomonas aeruginosa *Pseudomonas aeruginosa*		Assente/10g *Absent/10g*	
Batteri Gram negativi bile tolleranti *Bile-tolerant gram-negative bacteria*		Assente/10g *Absent/10g*	
Coliformi *Coliforms*		<=10	CFU/g
Granulometria *Particle size*			
- Attraversa 20 mesh *Through 20 mesh*		>=100	%
- Attraversa 40 mesh *Through 40 mesh*		>=70,00	%
- Attraversa 80 mesh *Through 80 mesh*		>=50,00	%
Densità tappata *Tapped bulk density*		0,40 - 0,70	g/ml
Densità di massa libera *Loose bulk density*		0,30 - 0,60	g/ml
Revisione Capitolato *Specification Revision*		2	
Data Approvazione *Approval Date*		16/05/2019	

I metodi di analisi non indicati sono metodi interni del produttore ottenibili su specifica richiesta
Le informazioni sopra riportate non Vi sollevano dall'obbligo di identificare il prodotto prima dell'impiego. La nostra società non si assume alcuna responsabilità per danni a persone o cose derivanti dall'impiego dei prodotti da noi commercializzati

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SPECIFICA TECNICA
TECHNICAL SPECIFICATION

Prodotto: **E.S. TIT. BACOPA 20% BACOSIDI**
Product *BACOPA D.E. 20.0% BACOSIDES*

I metodi di analisi non indicati sono metodi interni del produttore ottenibili su specifica richiesta
Le informazioni sopra riportate non Vi sollevano dall'obbligo di identificare il prodotto prima dell'impiego. La nostra società non si assume alcuna responsabilità per danni a persone o cose derivanti dall'impiego dei prodotti da noi commercializzati

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The above information will not relieve you to identify the product before use. Our company does not assume any responsibility for damage to persons or property arising by the use of the products we sell

Technical specifications of *Camellia sinensis*

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SPECIFICA TECNICA
TECHNICAL SPECIFICATION

Prodotto: **E.S. TIT. TE' VERDE CATECHINE 50 %**
Product *GREEN TEA D.E. 50 % CATECHIN*

SPECIFICA *SPECIFICATION*	**METODO** **METHOD**	**Lim. Inf. - Lim. Sup.** *Lower Lim. - Upper Lim.*	**u.m.**
Nome botanico *Botanical name*		Camellia sinensis *Camellia sinensis*	
Parte della pianta *Botanical part*		Foglia *Leaf*	
Aspetto *Apperance*		Polvere marrone-giallastra *Yellowish-brown powder*	
Polifenoli (UV) *Polyphenols (UV)*		>=70,0	%
Titolo *Assay*			
- Catechine *Catechins*		>=50,0	%
- EGCG *EGCG*		>=25,0	%
- Caffeina *Caffeine*		<=8,0	%
Perdita all'essiccamento *Loss on drying*		<=5,0	%
METALLI PESANTI *HEAVY METALS*			
- Metalli pesanti *Heavy metals*		<=10	ppm
Granulometria *Particle size*		>= 98% (attraversa 80 mesh) *>= 98% (throught 80 mesh)*	
METALLI PESANTI *HEAVY METALS*			
- Arsenico *Arsenic*		<=1.0	ppm

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SPECIFICA TECNICA
TECHNICAL SPECIFICATION

Prodotto: **E.S. TIT. TE' VERDE CATECHINE 50 %**
Product *GREEN TEA D.E. 50 % CATECHIN*

- Piombo *Lead*		<=2.0	ppm
- Cadmio *Cadmium*		<=1.0	ppm
- Mercurio *Mercury*		<=0.1	ppm
Specifiche microbiologiche *Microbiological specification*			
- Conta batterica totale *Total aerobic count*		<=1.000	CFU/g
- Lieviti e muffe *Yeasts and mould*		<=100	CFU/g
- E.coli *E.coli*		Negativo *Negative*	CFU/g
- Salmonella *Salmonella*		Negativo *Negative*	CFU/g
Aflatossine *Aflatoxins*		<=10	ppb
Revisione Capitolato *Specification Revision*		3	
Data Approvazione *Approval Date*		18/03/2019	

I metodi di analisi non indicati sono metodi interni del produttore ottenibili su specifica richiesta
Le informazioni sopra riportate non Vi sollevano dall'obbligo di identificare il prodotto prima dell'impiego. La nostra società non si assume alcuna responsabilità per danni a persone o cose derivanti dall'impiego dei prodotti da noi commercializzati

The analytical methods not listed are internal methods of the manufacturer obtainable upon specific request
The above information will not relieve you to identify the product before use. Our company does not assume any responsibility for damage to persons or property arising by the use of the products we sell