

T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MASTER OF SCIENCE THESIS

CHEMOPREVENTIVE APPROACHES IN IN VITRO NEUROINFLAMMATION MODELS: THERAPEUTIC EFFECTS AND MOLECULAR MECHANISMS OF NOVEL AND KNOWN BIOACTIVE MOLECULES

Begüm KURT

Biomolecular Sciences

ÇANAKKALE

T.C.

CANAKKALE ONSEKIZ MART UNIVERSITY

GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

MASTER OF SCIENCE THESIS

CHEMOPREVENTIVE APPROACHES IN IN VITRO NEUROINFLAMMATION MODELS: THERAPEUTIC EFFECTS AND MOLECULAR MECHANISMS OF NOVEL AND KNOWN BIOACTIVE MOLECULES

Begüm KURT

Biomolecular Sciences

Date of Defense: **07/31/2019**

Thesis Supervisor:

Assoc. Prof. Dr. Tuğba TÜMER

ÇANAKKALE

The study entitled **"Chemopreventive Approaches in In Vitro Neuroinflammation Models: Therapeutic Effects and Molecular Mechanisms of Novel and Known Bioactive Molecules"**, submitted by **Begüm KURT** under supervision of Assoc. Prof. Dr. Tuğba TÜMER was defended on **07/31/2019.** This thesis has been approved as **Master of Science** in **Biomolecular Sciences** of Çanakkale Onsekiz Mart University Graduate School of Natural and Applied Sciences by the Examining Committee Members.

EXAMINING COMMITTEE

Associate Prof. Dr. Tuğba TÜMER ……

Supervisor

Prof. Dr. Kemal Melih TAŞKIN ……

Member

Assistant Prof. Dr. İlknur DURSUN ……

Member

Prof. Dr. Levent GENÇ
Director
Gratude School of Natural
and Applied Sciences

Order No:……

This study has been supported by Çanakkale Onsekiz Mart University Scientific Research Project Coordination Unit. Project Number: (FYL-2017-1339).

PLAGIARISM DECLARATION PAGE

I declare that all the information and results presented in visual, audio and written form are obtained by myself observing the academic and ethical rules and, all other results and information indicated in the thesis but not specific to this study are cited.

Begüm KURT

ACKNOWLEDGEMENTS

I would like to express my sincerest gratitude to my supervisor Assoc. Prof. Dr. Tuğba TÜMER for all her time and support. I came to her with a new idea for a thesis project, and she graciously allowed me to pursue my interests. All my work would not be possible without her guidance.

My sincere thanks also goes to Dr. Seda KULABAŞ for her precious support, coaching and endless patient. She shared her expertise with me very generously and I have learned a lot from her.

I would like to thank Dr. Volkan ESKİZEYBEK for always letting me come to his office to ask questions or for guidance during my education. His courses were some of my favorites, where I learned a great deal about what it meant to do research.

Thank you as well to my lab friends, Adem, Berkay, Ece, Elif, Gizem, Gözde, Kübra and Raşit, who made getting my degree incredibly fun and informative we have had in the last three years. And thank you as well for their everlasting support and faith in me.

I would also like to say a heartfelt thank you to İhsan GÜNDÜZ, who always there for me not only during my M.Sc. but all the moment we shared, thank you for supporting me, encouraging me and believing in me all the time.

Finally, I must express my profound gratitute to my parents for providing me with constant support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

Begüm KURT

Çanakkale, July 2019

ABBREVIATIONS

BCA	Bicinchoninic acid
CO2	Carbondioxyde
COX-2	Cyclooxygenase-2
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxyde
FBS	Fetal Bovine Serum
HO-1	Heme Oxygenase-1
IL-1B	Interleukine 1 Beta
iNOS	Inducible Nitric Oxide Synthase
LPS	Lipopolysaccharide
MTT	[3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide]
NO	Nitric Oxide
NQO1	NAD(P)H dehydrogenase [quinone] 1
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PPARγ	Peroxysome Proliferator-Activated Receptor
RNA	Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate

ÖZET

IN VITRO NÖROİNFLAMASYON MODELLERİNDE KEMOPREVENTİF YAKLAŞIMLAR: ÖZGÜN VE BİLİNEN BİYOAKTİF MOLEKÜLLERİN TERAPÖTİK ETKİLERİ VE MOLEKÜLER MEKANİZMALARI

Begüm KURT
Çanakkale Onsekiz Mart Üniversitesi
Fen Bilimleri Enstitüsü
Biyomoleküler Bilimler Anabilim Dalı Yüksek Lisans Tezi
Danışman : Doç. Dr. Tuğba TÜMER
07/31/2019, 89

Kronik nöroinflamasyon tüm dünyada Alzheimer, Parkinson, Multipl Skleroz, depresyon gibi en yaygın görülen nörolojik bozuklukların başlıca sebeplerinden biridir. Günümüzde nöroinflamasyon kaynaklı nörodejeneratif hastalıkların tedavisinde kullanılabilecek etkin bir yöntem hala bulunamamıştır. Bu hastalıkların tedavi edilebilmesi ve önlenebilmesi için nöroinflamasyonun mekanizmaları araştırılarak anti-inflamatuar ve sitoprotektif odaklı bitkisel kökenli yaklaşımların geliştirilmesi gerekmektedir.

Bu çalışma kapsamında bilinen bazı maddelerden apigenin, luteolin, kafeik asit, rosmarinik asit ve GR24 ile yeni bir bileşik olan Kaffeoyl-rosmarinik asidin nörodejeneratif hastalıkların gelişiminde merkezi bir rolü olan kronik nöroflamasyona karşı düşük dozlarda koruyucuetkisi taranarak en etken maddenin tespit edilmesi ve daha sonra bu maddenin ileri anti-neuroinflamatuar etkilerini araştırmak ve moleküler etki mekanizmalarını tespit etmek planlanmıştır. Bu amaçla, tarama sonuçlarına göre öncül olarak tespit edilen ve bir çeşit yeni nesil bitki hormonu olan Strigolakton analoğu GR24'ün nörovasküler birimin üyelerinden beyin endotel hücreleri ve mikrogliada oluşturulan nöroinflamasyon modellerine karşı test edilmesi yenilikçi bir yaklaşımdır. Son çalışmalar, kronik nöroinflamasyonun oluşumunda merkezi sinir sistemi (MSS) elemanları ile sistemik inflamasyon arasındaki yakın ilişkiyi ortaya koymaktadır. Bu tez kapsamında, KBB ve mikroglial hücrelerin inflamasyon modelindeki nöroinflamatuvar süreçleri incelenmiş ve inflamasyon sadece merkezi sinir sistemi ile sınırlı kalmayan bir hastalık olarak değerlendirilmiştir.

Çalışma kapsamında mikroglia ve beyin endotel hücreleri üzerinde nöroinflamasyonun gelişiminde rol oynayan pek çok farklı parametre gözlemlenmiştir.

GR24'ün beyin endotel ve mikroglia hücrelerinde LPS uyarımı ile eksprese olan proinflamatuvar moleküller üzerinde belirgin ölçüde baskılayıcı ve sitoprotektif molekülleri önemli ölçüde indükleyici etki gösterdiği görülmüştür. Buna göre GR24'ün düşük dozlarda kronik nöroinflamasyona karşı etkin, nöroprotektif bir bileşik olduğu belirlenmiştir. Bu çalışma sonucu GR24'ün nörodejeneratif hastalıkların tedavisinde potansiyel bir terapötik fitokimyasal olabileceği öne sürülmektedir.

Anahtar sözcükler: Nöroinflamasyon, LPS, Mikroglia, Beyin Endotel, GR24, KBB.

ABSTRACT

CHEMOPREVENTIVE APPROACHES IN IN VITRO NEUROINFLAMMATION MODELS: THERAPEUTIC EFFECTS AND MOLECULAR MECHANISMS OF NOVEL AND KNOWN BIOACTIVE MOLECULES

Begüm KURT

Çanakkale Onsekiz Mart University

Graduate School of Natural and Applied Sciences

Master of Science Thesis in Biomolecular Sciences

Advisor : Assoc. Prof. Dr. Tuğba TÜMER

07/31/2019, 89

Chronic neuroinflammation is one of the major causes of the most common neurological disorders in the worldwide such as Alzheimer's, Parkinson's, Amyloid Sclerosis, and depression. Currently, there is still no effective treatment for neuroinflammation-related neurodegenerative diseases. In order to treat and prevent these diseases, the mechanisms of neuroinflammation should be investigated and anti-inflammatory and cytoprotective-oriented plant-based approaches should be developed.

The purpose of this study was to screen protective roles of apigenin, luteolin, caffeic acid, rosmarinic acid and GR24, and a novel compound, caffeoyl-rosmarinic acid at low doses against chronic neuroinflammation, which has a pivotal role in the progression of neurodegenerative diseases. As result of screening studies among tested compounds GR24 was found to be effective against LPS induced NO production at relatively lower doses. Therefore, this new generation plant hormone Strigolactone analog was tested on neuroinflammation modelled-brain endothelial cells and microglia which are members of the neurovascular unit as an innovative approach.

Recent studies demonstrated a close dynamics between systemic inflammation and the central nervous system (CNS) units in the formation of chronic neuroinflammation. In this study, LPS induced neuroinflammation models in microglial and endothelial cells were investigated and it was considered as a whole-system disease.

Several parameters that play a role in the development of neuroinflammation have been observed in LPS stimulated brain endothelial and microglia cells. Subsequently, cells were treated with the lead compound GR24and the possible anti-neuroinflammatory and

cytoprotective effects were examined. As a result, it was observed that GR24 had a significant inhibitory effect on proinflammatory molecules expressed by LPS stimulated brain endothelial and microglia cells and significantly induced cytoprotective molecules among all substances. Accordingly, GR24 was found to be an effective neuroprotective compound against chronic neuroinflammation at low doses. As a result of this study, GR24 may considered and further developed as a therapeutic phytochemical for the treatment and also prevention of neurodegenerative diseases.

Keywords: Neuroinflammation, LPS, Microglia, Brain Endothelial, GR24, BBB.

TABLE of CONTENTS

Page No

THESIS DEFENSE EXAM RESULT FORM ii
PLAGIARISM DECLARATION PAGE iii
ACKNOWNLEDGEMENTS iv
ABBREVIATIONS v
ÖZET vi
ABSTRACT viii
LIST of FIGURES xiii
LIST of TABLES xv
CHAPTER 1 1
INTRODUCTION 1
1.1. Endogenous and Exogenous Immunity 2
1.2. Microglia 3
1.3. The Blood-Brain Barrier 6
1.4. The Neurovascular Unit 9
1.5. Introduction to Neuroinflammation 11
1.6. Major links between Neuroinflammation and Neurodegenerative Diseases in the Scope of CNS Immunity and BBB 13
1.7. Natural Products 15
1.7.1. Luteolin&Apigenin 15
1.7.2. Caffeic Acid&Rosmarinic Acid 16
1.7.3. Caffeoly-Rosmarinic Acid 17
1.7.4. Strigolactones 18
1.8. Aim of the Study 19
CHAPTER 2 21
PREVIOUS STUDIES 21
CHAPTER 3 23
MATERIALS AND METHODS 23
3.1. Materials 23
3.1.1. Chemicals 23
3.1.2. Equipments 24
3.1.3. Cell Lines 24
3.2. Methods 25

3.2.1. Nitric Oxide Inhibitory Assay for Screening Compounds and for the Identification of IC50 of the Lead Compounds 25
3.2.2. Cell Viability Assays 26
3.2.3. Enzyme-Linked ImmunoSorbent Assay (ELISA) 26
3.2.4. Gene Expression Analysis 27
3.2.4.1. RNA Isolation 28
3.2.4.2. Spectrophotometric Analysis of RNA 28
3.2.4.3. Imaging of Total RNA by Agarose Gel Electrophoresis 28
3.2.4.4. cDNA synthesis 28
3.2.4.5. Quantitative Polymerase Chain Reaction: qPCR 29
3.2.5. SDS-PAGE and Western Blot Analysis 29
3.2.5.1. Protein Collection for SDS-PAGE and Western Blot Analysis 29
3.2.5.2. Determination of Total Protein Concentrations, SDS-PAGE and Western Blot Analysis 30
3.2.6. Permeability assay on bEnd.3 cells 32
3.2.7. Statistical Analysis 33
CHAPTER 4 34
RESULTS AND DISCUSSION 34
4.1. Results 34
4.1.1. Time optimization for the Effects of LPS on NO Released from SIM-A9 Cells 34
4.1.2. The Effects of Positive Control, 1400W on NO Production in SIM-A9 cell line: IC50 value determination 35
4.1.3. Screening for the Effects of Compounds on NO Production in LPS-induced SIM-A9 cell line: Identification of the lead compound(s) 36
4.1.4. The Effect of Lead Compound on NO production in SIM-A9 Cells Following LPS Treatment: IC50 Determination 37
4.1.5. Cell Viability in SIM-A9 Cells Following GR24 Treatment 37
4.1.6. GR24 Reduced Cytokine Release in SIM-A9 Cells Following LPS Treatment 38
4.1.7. Time Optimization for the Gene Expression Levels in SIM-A9 cells Following LPS Treatment 39
4.1.8. GR24 Reduced the Proinflammatory and Induced the Cytoprotective Gene Expression Levels in SIM-A9 Cells Following LPS Treatment 40
4.1.8.1. GR24 Reduced the Proinflammatory Gene Expression Levels in SIM-A9 Cells Following LPS Treatment 40

4.1.8.2. GR24 Induced the Cytoprotective Gene Expression Levels in SIM-A9 Cells Following LPS Treatment....43
4.1.9. Time Optimization for the Protein Expression Levels in SIM-A9 cells Following LPS Treatment....44
4.1.10. Protein Expression Levels of Proinflammatory and Cytoprotective Genes in LPS-Induced SIM-A9 Cells Following the Treatment of GR24....45
4.1.10.1. GR24 Reduced the Proinflammatory Protein Expression Levels in SIM-A9 Cells Following LPS Treatment....45
4.1.10.2. GR24 Induced the Cytoprotective Protein Expression Levels in SIM-A9 Cells Following LPS Treatment....51
4.2.1. Cell Viability in Brain Endothelial Cells Following GR24 Treatment....53
4.2.2. The Proinflammatory and Cytoprotective Gene Expression Levels in Brain Endothelial Cells Following LPS and GR24 Treatment....53
4.2.2.1. GR24 Reduced the Proinflammatory Gene Expression Levels in Brain Endothelial Cells Following LPS Treatment....54
4.2.2.2 GR24 Induced the Cytoprotective Gene Expression Levels in Brain Endothelial Cells Following LPS Treatment....56
4.2.3. Permeability assay on Brain Endothelial Cells....58
4.3. Discussion....59
CHAPTER 5....73
CONCLUSION....73
REFERENCES....75
BIOGRAPHY....I

LIST of FIGURES

Page No

Figure 1.1. Leukocyte infiltration from blood to brain....3
Figure 1.2. The development and primary roles of microglia in CNS....4
Figure 1.3. Systemic injection with intravital dyes to the mouse. Brain remains unstained unlike other organs....7
Figure 1.4. Intraventricular injection with intravital dyes. Brain and spinal cord stained unlike the systemic part of the mouse....7
Figure 1.5. Development of BBB and the neurovascular unit....8
Figure 1.6. The neurovascular unit of BBB....9
Figure 1.7. The tight junction and kissing points between brain endothelium....10
Figure 1.8. The onset of inflammation/neurodenegeration....12
Figure 1.9. BBB destruction in the process of inflammation....13
Figure 1.10. The chemical structures of luteolin (a.) and apigenin (b.)....16
Figure 1.11. The chemical structures of CA (a.) and RA (b.)....17
Figure 1.12. The chemical structure of Caffeoly-rosmarinic acid....17
Figure 1.13. The chemical structure of GR24....18
Figure 3.1. Immunological analysis of released cytokines using specific antibodies....27
Figure 3.2. Techniques and immunologic basis of Western Blot....32
Figure 4.1. NO production in SIM-A9 cells at different incubation times following LPS treatment.....35
Figure 4.2. NO production in SIM-A9 microglia cells at different incubation times following treatment of LPS and iNOS inhibitor, 1400W....36
Figure 4.3. NO production in SIM-A9 cells at different incubation times following the treatment of LPS and test compounds at 10 μM doses.....37
Figure 4.4. NO production in SIM-A9 cells at different incubation times following treatment of LPS and iNOS inhibitor, 1400W....37
Figure 4.5. Relative cell viability in SIM-A9 cells at different incubation times following treatment of LPS and iNOS inhibitor, 1400W....38
Figure 4.6. Cytokine secretion of TNF-α and IL-1β in SIM-A9 cells at different incubation times following treatment of LPS and GR24....39
Figure 4.7. iNOS gene expression levels in SIM-A9 cells at different incubation times following treatment of LPS....40
Figure 4.8. iNOS mRNA expression in SIM-A9 cells at different incubation times following treatment of LPS and GR24....41
Figure 4.9. GR24 reduced COX-2 mRNA expression levels in SIM-A9 cells following LPS treatment....41
Figure 4.10. GR24 reduced IL-1β mRNA expression levels in SIM-A9 cells following LPS treatment.....42
Figure 4.11. GR24 reduced TNF-α mRNA expression levels in SIM-A9 cells following LPS treatment....42
Figure 4.12. GR24 induced Nrf2 mRNA expression levels in SIM-A9 cells following LPS treatment....43
Figure 4.13. GR24 induced NQO1 mRNA expression levels in SIM-A9 cells following LPS treatment....43
Figure 4.14. GR24 induced HO-1 mRNA expression levels in SIM-A9 cells following LPS treatment.....44
Figure 4.15. Time optimization for iNOS protein expression levels in SIM-A9 cells following LPS treatment (1μg/mL) at 4, 6, 12 and 24 hours....45

Figure 4.16. GR24 reduced iNOS protein expression levels in SIM-A9 cells following LPS treatment 46
Figure 4.17. GR24 reduced COX-2 protein expression levels in SIM-A9 cells following LPS treatment..... 47
Figure 4.18. GR24 reduced TNF-α protein expression levels in SIM-A9 cells following LPS treatment..... 48
Figure 4.19. GR24 induced PPARγ protein expression levels in SIM-A9 cells following LPS treatment..... 49
Figure 4.20. GR24 induced AMPK protein expression levels in SIM-A9 cells following LPS treatment..... 50
Figure 4.21. GR24 reduced nuclear acuumulation of NF-κB in SIM-A9 cells following LPS treatment..... 51
Figure 4.22. GR24 induced nuclear accumulation of Nrf2 in SIM-A9 cells following LPS treatment 52
Figure 4.23. GR24 induced Nrf2 protein expression levels in SIM-A9 cells following LPS treatment 53
Figure 4.24. Effect of GR24 on cell viability in brain endothelial cell line 53
Figure 4.25. GR24 reduced iNOS mRNA expression levels in brain endothelial cells following LPS treatment 54
Figure 4.26. GR24 reduced IL-1β mRNA expression levels in brain endothelial cells following LPS treatment 55
Figure 4.27. GR24 reduced TNF-α mRNA expression levels in brain endothelial cells following LPS treatment. 55
Figure 4.28. COX-2 mRNA expression levels in brain endothelial cells following LPS and GR24 treatment 56
Figure 4.29. Nrf2 mRNA expression levels in brain endothelial cells following the LPS and GR24 treatment 56
Figure 4.30. HO-1 mRNA expression in brain endothelial cells following LPS and GR24 treatment 57
Figure 4.31. GR24 induced NQO1 mRNA expression levels in brain endothelial cells following LPS treatment. 58
Figure 4.32. GR24 reduced the permeability in brain endothelial cells following LPS treatment 59
Figure 5.1. Multitarget potential of GR24 in LPS activated components of NVU 74

LIST of TABLES

Page No

Table 3.1. List of Chemicals.....23
Table 3.2. List of Equipments.....24
Table 3.3. Medium Conditions of SIM-A9 and bEnd.3 Cell Lines.....25
Table 3.4. The amount of reagents per packed cell volume (μL) in NE-PER method.....30
Table 3.5. Gel components in SDS-PAGE method.....31

CHAPTER 1
INTRODUCTION

The immune response is a fundamental process of both peripheral and central nervous system (CNS) for body's homeostasis and health. (Dendrou, McVean, & Fugger, 2016). In CNS, microglia are the primary component of immune response and messengers to communicate with other CNS members. In case of brain injury or infections, microglia are "answering the call" and switching their morphology to fight against foreign dangers in the brain. In the meantime, microglia are also defending the brain with secretion of cytokines, which are proinflammatory molecules that initiate the inflammation process. This event causes stimulation of other cells in CNS, such as glial cells or/and brain endothelial cells, the latter is the primary structure of the blood-brain barrier (BBB).

The differences among acute and chronic inflammation have been well established. In acute inflammation, the response is self-limiting. However, inflammatory impulse may be persistent in some cases. Such that the proinflammatory cytokines may be expressed from microglia without self-limit and inflammation will become "chronic", which means continuously (Dinarello, 2000). The longterm effects of chronic inflammation eventually may result in cognitive disorders including Alzheimer's Disease (AD), Parkinson's disease (PD), depression, etc.

It is well documented that CNS inflammation has a great role in systemic neurodegeneration, but the mechanisms are remain unclear (Cunningham, 2013). This study aims to evaluate chemopreventive and therapeutic effects of novel and known bioactive phytochemicals and their molecular mechanism of action against neuroinflammation. With this aim, neuroinflammation model were established in brain microglia and BBB regulating brain endothelial cell lines by using lipopolysaccharide (LPS), a stimulant purified from the outer membrane of gram-negative bacteria.

In this study, the identification of some phytochemicals and their possible potential ameliorative effects and molecular mechanisms against neuroinflammation related neurodegenerative diseases is intended. These compounds are apigenin, luteolin, caffeic acid (CA) and rosmarinic acid (RA), which is commonly used for traditional treatment against antioxidant, anti-inflammatory and anticarcinogen in worldwide. On the other hand, novel compounds can be listed as caffeoyl-rosmarinic acid, which was originally isolated in the scope of a project leaded by our laboratory, it is a hybrid molecule formed by the ester bond

between caffeic and rosmarinic acid. Another compound is a phytohormone; strigolactone analog GR24. We hypothesize that these compounds can be potential candidates as chemopreventive/neuroprotective and therapeutic agents against neuroinflammatory and neurodegenerative diseases and can be predicted as effective drugs that may capable of passing through the BBB.

Before explanining inflammatory processes and their influences on the CNS, it is good to understand how immune cells contribute to neurodegenerative physiology in the CNS.

1.1. Endogenous and Exogenous Immunity

Neuroinflammation can be driven by both endogenous (microglia, astrocytes) or exogenous (systemic macrophages). In case of a stimuli, they can be activated into proinflammatory state and can remain in "chronic" conditions. Endogenous and exogenous immunity are tightly related and they have a dynamic interaction for the CNS homeostasis. In the organism, immune cell migration into other organs is a typical process against a pathogen signal. However, in the CNS, situations became more complicated owing to the BBB structure (Bechmann, Galea, & Perry, 2007). A strong inflammatory impulses such as pathogen-related (e.g. LPS) invasions cause infiltration of leukocytes into the CNS by passing through BBB from the periphery by neuroinflammation (Noh, Jeon, & Seo, 2014). After the activation of glial cells, proinflammatory mediators released throughout the bloodstream and attracted peripheral leukocytes to the area of inflammation. These interactions resulted in upregulation of cellular adhesion molecules expression, which is essential for cell migration across the capillary venules (Figure 1.1.)(Engelhardt, 2008).

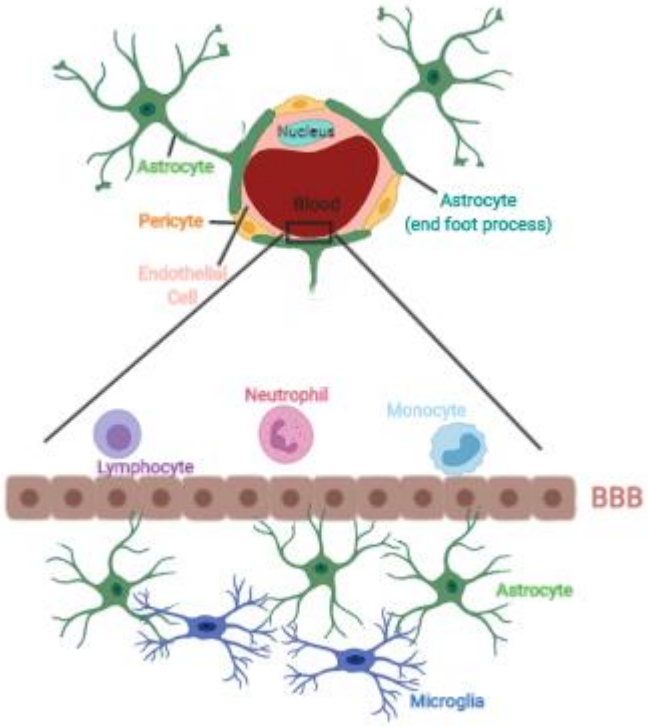

Figure 1.1. Leukocyte infiltration from blood to brain

However, in the CNS, some mechanisms are very fragile. Leukocyte traffic can be deleterious for the CNS when it becomes persistent and may lead to many different neurodegenerative diseases. For instance, it was reported that auto-aggressive T-cells infiltrated from periphery collaborate with antigen-presenting cells and promote demyelination in multiple sclerosis (Rezai-Zadeh, Gate, & Town, 2009). Another study revealed that auto-aggressive T-cells were recruited with the "substantia nigra pars compacta (SNpc)", the main brain region that affected from pathogenesis of the PD (Brochard et al., 2009).

In summary, today the peripheral immune cells are well-established for their role in the etiology and related with several brain disorders, such as AD, PD, MS and brain cancers (Lenz & Nelson, 2018). Recently, it has became clear that peripheral cells, such as microglia are the master regulator of CNS-related pathologies of the brain.

1.2. Microglia

Microglia are the most abundant cells in the CNS and among the first line defense members of the brain against pathogens or injury (Perry, Nicoll, & Holmes, 2010). During early embryonic days in mice, microglia derived from myeloid progenitors in the yolk sac

expressing the transcription factor “RUNX1” and “the receptor tyrosine kinase c-Kit”, also known as CD117. Microglia then enter the CNS,and in CNS, the precursor forms of microglia change, grow well and invade the brain parenchyma to a certain level. In parenchyma, microglial density varies greatly from one region to the other, nevertheless, the main reasons of these regional differences are not known clearly (de Haas, Boddeke, & Biber, 2008). Microglia have fundamental roles in CNS homeostasis, such as brain development, promoting apoptosis and clearance of apoptotic neonatal neurons and also the axon and synapse developing as pruning (Figure 1.2.). After the transition from development to adulthood, microglial processes become highly mobile and are constantly in contact with neurons, axons, and dendritic spines to continually explore their local environment. (Ransohoff & Perry, 2009). Microglia have an enormous life span compared to other macrophages or even other cells; 28% of microglia renew slowly per year and some microglia are maintained by self-renewal more than two decades (Réu et al., 2017). With the continuous slow turnover of the microglial population, microglia maintained in the human brain along with adult life (Réu et al., 2017).

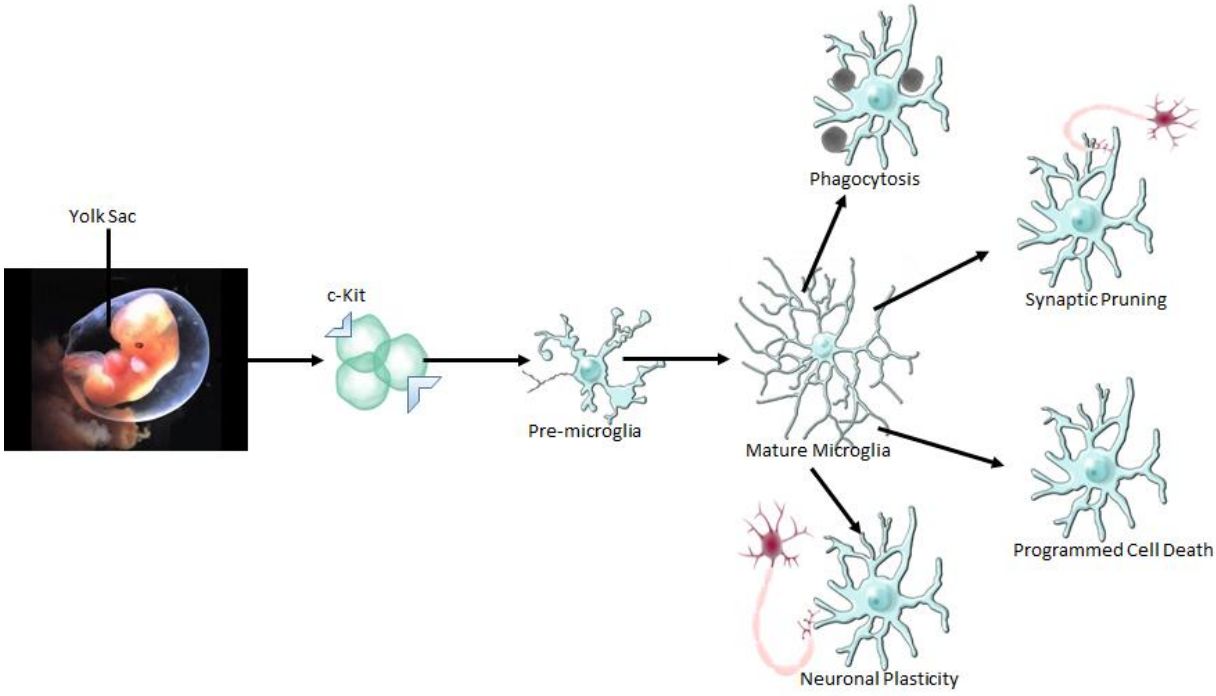

Figure 1.2. The development and primary roles of microglia in CNS

Microglia are called as “sensors” of the CNS and they are very sensitive to almost every stimulation in the brain. In return, these cells can change their morphology and/or “de novo” synthesis of cell surface receptors (Kreutzberg, 1996). Microglia have unique features

and abilities, for instance, local self-renewal, different origins and different expression profiles compared to macrophages (Butovsky et al., 2014). It is now clear that the term "resting state" does not reflect the actual meaning and causes some misunderstanding (Cherry, Olschowka, & O'Banion, 2014). Recent studies have shown that even in so-called "resting state", microglia are highly dinamic and constantly explore their local micro-environments (Nimmerjahn, Kirchhoff, & Helmchen, 2005). Also, microglia can secrete neuroprotective factors such as "brain-derived neurotrophic factor (BDNF)" and "nerve-growth factor (NGF)" in "resting state", although hypothesized only activated (M2) state can be neuroprotective&neurosupportive. The terms, M1 and characterize the different states of microglia very limitedly. In general, M1 refers the activation of microglia through TLRs or interferon- γ and M2 refers the activation of microglia by interleukin family. In reality, microglia have a diverse range of forms and secrete several different cytokines and synthesize receptors probably disease-specific (Perry et al., 2010). Another data showed has shown in mice microglia; the phenomenon of activation fails to conform to M1/M2 patterns according to the modern transcriptome profiling of microglia (Salter & Stevens, 2017).

Microglial activation (Hanisch, 2013) refers to activation of the cell by a noxious stimulus via "pathogen recognition receptors (PRRs)" such as "Toll-Like Receptors (TLRs)", "NOD-like receptors (NLRs)" and "RIG-I-like receptors (RLRs)". This stimulation is a response to damaged or dying cells which is called "pathogen-associated molecular patterns" (PAMPs) or "damage-associated molecular patterns" (DAMPs) and cause the self or non-self recognition in the CNS, as a result of activated microglia by an insult (Varnum & Ikezu, 2012). In the brain, microglia express TLRs, and this signaling has a pivotal role to initiate the innate immune response not only to the infectious activity but also inflammatory conditions and inflammation-related disorders (Kofler & Wiley, 2011). TLRs have specific leucine-rich binding regions able to detect PAMPs and DAMPs, and all these mechanisms have an important role to initiate the host response against CNS damage (Kofler & Wiley, 2011).

Briefly, several of impulses can activate microglia and diverse range of cytokine secretion from these cells via different mechanisms. These impulses include ROS and nitrogen species, arachidonic acid metabolites, histamine and glutamate (Nakajima & Kohsaka, 2001). However, the classification of microglia only with the "activated" or "rested" terms may not be enough to define these unique cells and their broad range of mechanisms. Besides, it is not be understood what microglia are capable of doing in their microenvironment affecting the whole system of the organism.

With these high level of sensitivity, microglia interact with every unit of CNS. The initiation of chronic inflammation by microglia leads to disrupt the integrity of their microenvironment. This can lead to the destruction of BBB, the main unit that provides the integrity of the CNS. As a results of inflammation, microglial activation disrupts BBB integrity through the release of several of mediators that lead to an excessive permeability, which have same pathology in some cognitive disorders including AD, PD or MS (Perry et al., 2010). Also, triggered by a pathogen stimulation, "Activated" microglia can secrete several of chemokines may result in serious alterations in BBB (Obermeier, Daneman, & Ransohoff, 2013). Sharief et al. (1992) discovered that patients with pathogen-related meningitis following brain edema showed vigorous BBB disorder during the disease associated with high levels of TNF-α, one of the proinflammatory molecule that is secreted by microglia.

In summary, the features and role of BBB physiology should be evaluated to understand the microglial stimulation and molecular mechanisms of inflammation and its effect on BBB.

1.3. The Blood-Brain Barrier

Cerebral circulation has a critical role in providing oxygen and nutrients to organs in the body. It maintains also normal brain function and stabilizes ionic balance in the brain (da Fonseca., 2014). Cerebral circulation has a specific type of cells called brain endothelial cells (ECs), these cells build up a highly selective structure called "the blood-brain barrier", a unique specialized composition of the brain and semi-permeable system (Pardridge, 2015).

The discovery of BBB dates back more than 100 years with Paul Ehrlich's experimental design to compare oxygen consumptions of different organs by intravenous administration of certain dyes (eg trypan blue). After the injection, the dye has stained all organs strongly except the brain and spinal cord, these specific areas were stained weakly or not stained at all (Figure 1.3.) (Hüntelmann & Hüntelmann, 2019). He concluded that the dyes had a lower affinity for binding to the nervous system than other tissues.

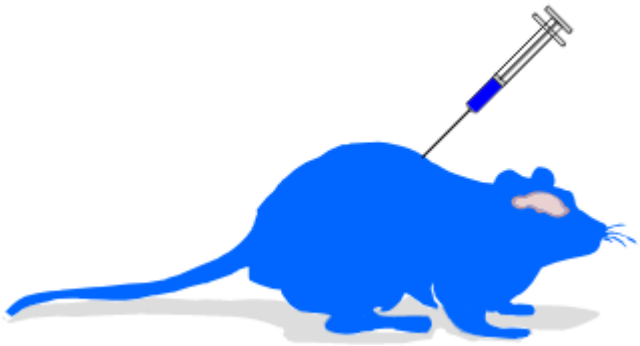

Figure 1.3. Systemic injection with intravital dyes to the mouse. Brain remains unstained unlike other organs

After that, the Berlin physician Lewandowski claimed that “Bluthirnschranke”, is a “barrier” among blood and the brain, and these capillaries in the barrier prevent the passage of hydrophilic molecules in 1900. Another remarkable study was followed Lewandowski in 1913 by Edwin Goldman. He demonstrated the same experiment with a different idea; when he directly injected the same dye that Ehrlich’s used into the “cerebrospinal fluid (CSF)”, he observed that the nervous tissue was all stained but not other tissues (Figure 1.4.) (Smith, 1951). All these studies had a one vital point; the brain has a tissue barrier that made the brain an immunologically privileged site (I. Galea, Bechmann, & Perry, 2007).

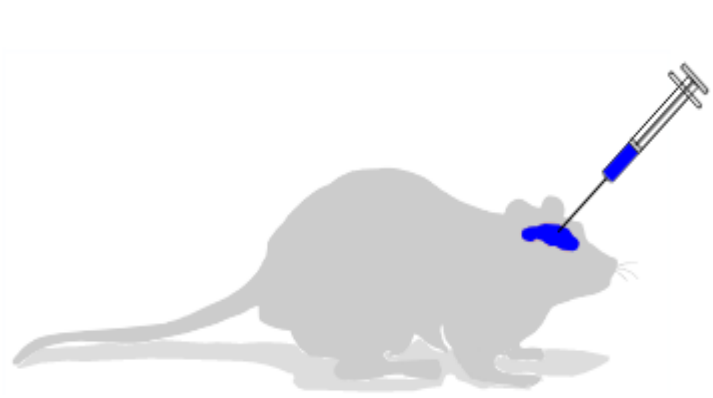

Figure 1.4. Intraventricular injection with intravital dyes. Brain and spinal cord stained unlike the systemic part of the mouse

Organisms with a complex networks and specilized nervous system require to a “control room” for the transportation of brain’s essential components among the blood and the brain. This supports the homeostasis of the parenchymal microenvironment and also is a vital step for the maintenance of cognitive functions (Bauer, Krizbai, Bauer, & Traweger,

2014). The formation of barrier is a gradual process; during embryogenesis, BBB starts to spread, originates new branches from the perineural vascular plexus. CNS-specific angiogenesis initiates with the release of Wnt ligands from the neural progenitor. At this point, the endothelial cells express tight junction proteins. During angiogenesis, PDGF-β released from brain endothelial cells, and connect pericytes. Pericytes secrete different neural factors such as TGF-β and it will repress the expression of "leaky" properties of the endothelial cells and vesicle trafficking is decreased by TJs. Astrocytic endfeet are connected with brain vessels and secrete multiple mediators for increasing BBB properties as angiotensinogen (Agt), Angiopoietin (Ang-1) and apolipoprotein E (ApoE) and P-glycoprotein (PGP). This leads to vascular sprouting by integrating the endothelial growth factors such as "PDGF-β", "VEGF", and "TGF-β", and provides a physical barrier. (Fantin, Plein, & Ruhrberg, 2012) (Figure 1.5.).

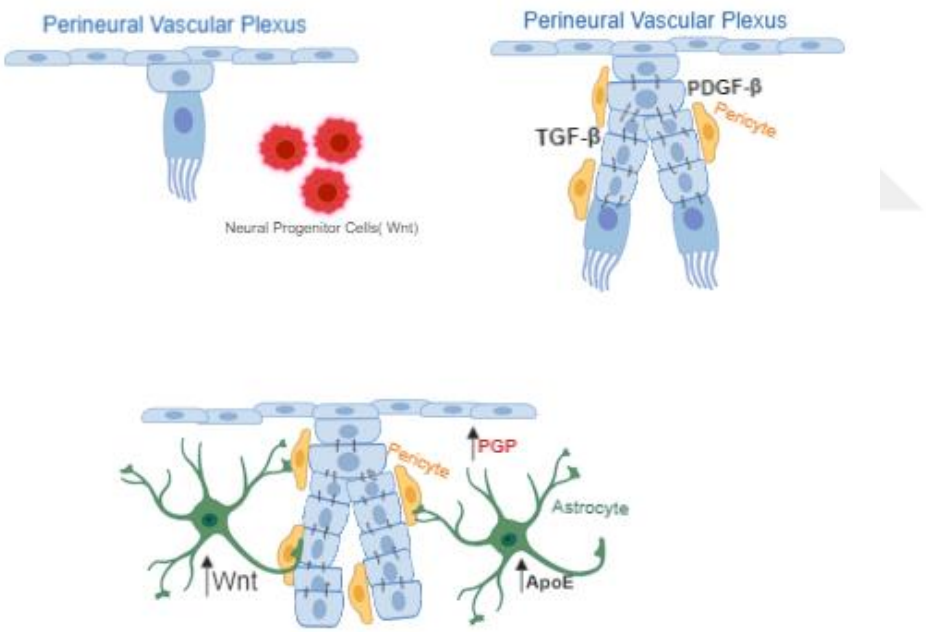

Figure 1.5. Development of BBB and the neurovascular unit

In the CNS, cerebral blood vessels has been covered under the term "neurovascular unit" (NVU). The endothelial cell remains central to the NVU, but its function is regulated by input from adjacent endothelial cells, pericytes, astrocytes, microglia and neurons.

1.4. The Neurovascular Unit

CNS functions are maintained with the help of multiple highly coordinated cell types, called as neurovascular unit (NVU). The NVU contains specialized cells for the maintenance of CNS, including endothelial cells, pericytes, microglia, astrocytes and neurons (Figure 1.6.) (Zhao, Nelson, Betsholtz, & Zlokovic, 2015). Each component is closely and mutually connected to one another which results in a highly efficient regulation system of blood circulation by composing an anatomical and functional unity (Abbott & Friedman, 2012). The interactions between NVU are formed with gap junctions, adhesion molecules and ion channels which are maintained neuromodulators such as ATP and as well as efflux and influx of Ca^{++} and Na^{+} through the blood and the brain (Muoio, Persson, & Sendeski, 2014).

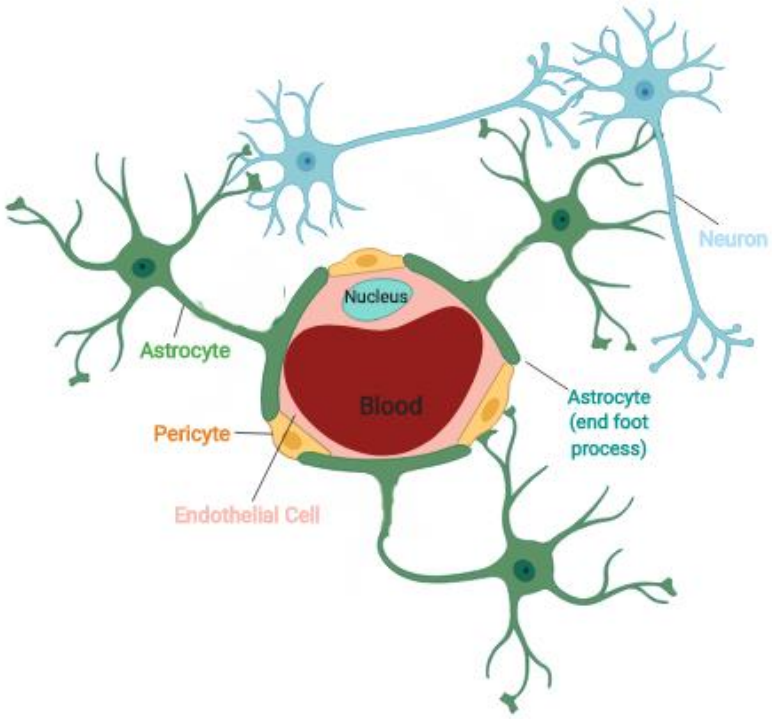

Figure 1.6. The neurovascular unit of BBB

The physical barrier of BBB is composed of different factors including endothelial TJs, adherens junction (AJ) proteins and different layers of barriers. The endothelium which forms BBB is composed of the epithelium of both the choroid plexus and the arachnoid mater (Abbott et al., 2010).

The primary function of the endothelium to allow free diffusion of carbon dioxide from the brain to blood and oxygen from the blood to the brain. For the maintenance of

cognitive balance in CNS and stabilize pH of the NVU, this process is essential for the organism. Also, the endothelium helps to regulate the uptake and entry of leukocytes as well as the innate immune response and therefore play a role in the surveillance and reactive functions (see chapter 1.2. Endogenous Immunity) (Abbott & Friedman, 2012). In endothelium, the TJ proteins are core structure which seal the barrier. TJs are the most apical cell-cell junction complex, and it has focal areas generally known as “kissing points”. In kissing points, adjacent cell membranes come into close position and it can be imaged by ultrathin-section electron microscopy (Figure 1.7).

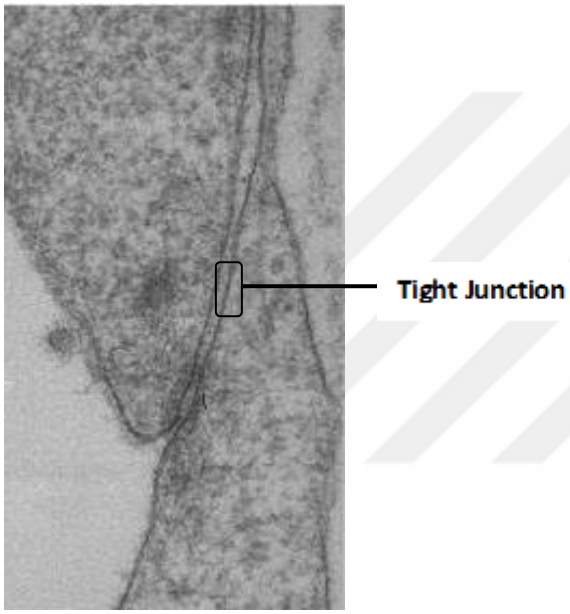

Figure 1.7**.** The tight junction and kissing points between brain endothelium

There are se<<veral TJ proteins to construct barrier on the endothelium. Occludin is the first protein that have been found to localize at the TJ areas. The integral membrane proteins of the TJ are bound to the cytoskeleton by the scaffold proteins “ZO-1, ZO-2 and ZO-3” (Furuse et al., 1993). Previous researches have assumed that occludin is the transmembrane protein which main structure of TJs. Although, mice carrying null mutation of occludin showed that there was no significant change of TJ morphology, postnatal retardation have been observed. Also in this mice, several histologic deficiencies found for instance gastric epithelium hyperplasia and calcification in the brain (Saitou et al., 2000). Another protein, claudin have been shown to be essential for the TJ morphology. A total of 27 different claudins have been known in human and mice. Claudin-1 and Claudin-2 were identified for the first time by Furuse et al. (1993). Several in vivo experiments have shown

that claudins are the main transmembrane proteins for the construction of TJ. For example, mice with claudin-1 deficiency leads enormous water loss from cells and consequently mice die shortly after birth.

In 2006, Enerson and Drewes concluded that BBB expressed mainly claudin family; especially claudin-5 is the most common form in the BBB due to SAGE analysis of murine brain. Claudin-5 keeps TJ “tight” literally. In mammals, it is very important to "seal" TJs in BBB because TJ deficiency results in a size-dependent opening of BBB, leading diffusion of molecules lower than 1000 kDa but not larger molecules. The importance of these researches is that the deficiency of claudin-5 may be the potential target of the efficient drug delivery processes to CNS through the BBB in the future (Matter & Balda, 2003).

1.5. Introduction to Neuroinflammation

Although the microenvironment of the CNS is highly conserved and privileged by immune cells and BBB, the homeostasis of the CNS is very sensitive to almost any impulse and give response immediately. The degeneration of the CNS is defined by loss of the neuronal integrity and function followed by cognitive disorders. While the progression of the CNS degeneration is poorly understood, there is mounting evidence that the degeneration is triggered by many factors such as age, environment, genetics or pathogen invasion (Chen, Zhang, & Huang, 2016). Neuroinflammation is one of the factors that causes neuronal dysfunction and mental impairment by producing inflammatory cytokines, chemocines. Glial cells produce these mediators at both basal and disease conditions. By this way, they provide the protection to the environment and carry out some functions including phagocytosis, steroid release, tissue and cellular repair via immunity (Schain & Kreisl, 2017). These mechanisms work until the danger in CNS is ended and inflammatory conditions eventually will be terminated. In chronic situation, however, the production of the inflammatory mediators triggered by glial cells within the endless loop create a persistent impulse that eventually transformed mediators into neurotoxic factors (DiSabato, Quan, & Godbout, 2016).

In many inflammatory diseases, stimulation of microglia and the associated production of proinflammatory mediators including “nitric oxide (NO)”, “prostoglandin (PGE_2)” and cytokines including “tumor necrosis factor-alpha (TNF-α)” and “interleukin-1βeta (IL-1β)” are primary stages. In this process, the increase in NO production in microglia is one of the main mechanism by increasing the expression of “inducible nitric oxide synthase (iNOS)” enzyme (Li et al., 1995). The iNOS enzyme catalyzes an oxidation reaction where the amino

acid of L-arginine is converted to L-citrulline and NO. This enzyme is induced at the level of mRNA and protein through proinflammatory mediators such as interferon-alpha (IL-α), TNF-α, IL-1β and interleukin-6 (IL-6) in microglia (Li et al., 1995). This situation causes an increase in enzyme activity and results in a significant increase in NO level, which is the final product of the enzyme (Figure 1.8.).

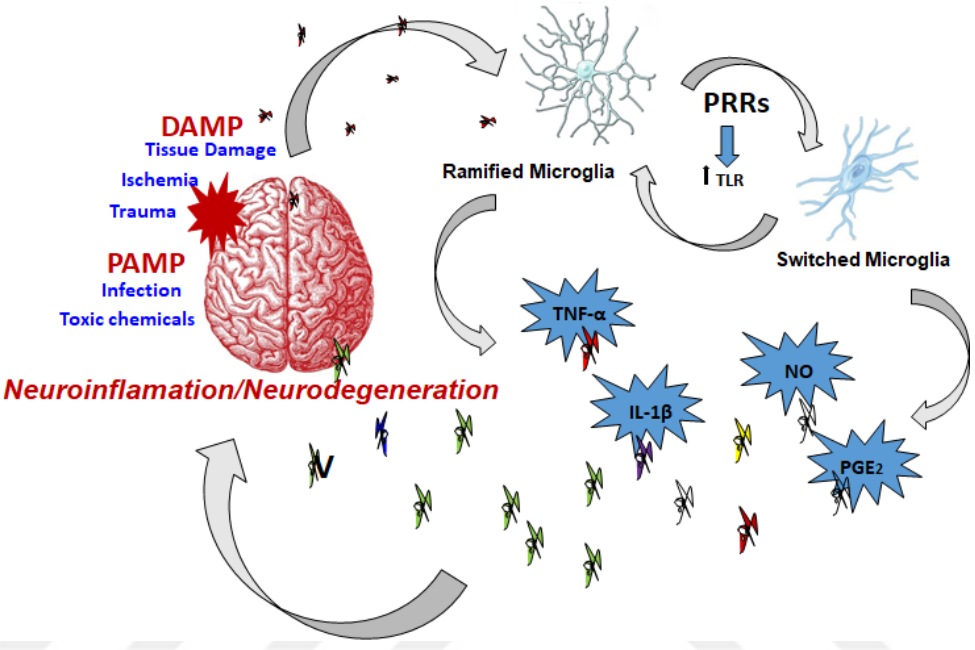

Figure 1.8. The onset of inflammation/neurodenegeration

iNOS-derived NO is one of the major sources of toxic free radicals in the brain. NO has high lipid content and according to this, oxygen consumption capacity and presence of redox active metal ions such as Cu^{+} and Fe^{+}, the brain is a very sensitive organ to oxidative and nitrosative stress. In an in vitro study, it was reported that LPS increased the production of NO in microglia cells via inducible iNOS and caused death in co-cultured neuronal cells within 48 h (Bal-Price & Brown, 2001). Due to the important role of NO in neurotoxicity, inhibition of the production of NO could be an alternative approach of therapeutic intervention in neuropathogenesis.

Inflammation induced by LPS has long been known to break down the BBB (Abbott, Rönnbäck, & Hansson, 2006). Damage in endothelial cells or TJ changes are the results of changes in the histological level of CNS due to the deficits in the BBB, which can be catastrophic in a long term. Treatment with LPS has also damaged BBB composition and induced an intense increase in blood and brain levels of many cytokines (Figure 1.9.).

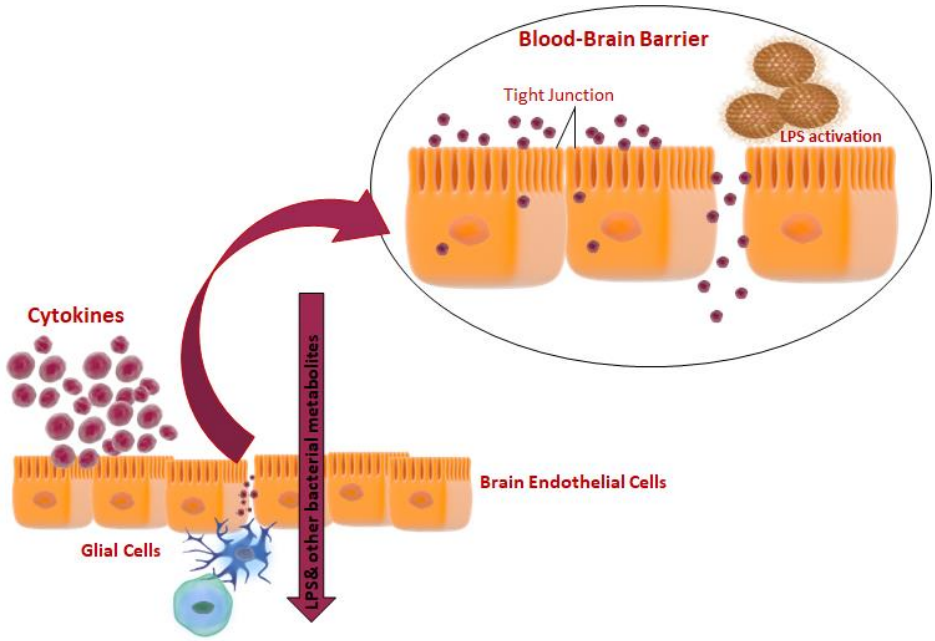

Figure 1.9. BBB destruction in the process of inflammation

Circulating cytokines can influence the CNS by the cytokine transportation through BBB. For example, the transport of IL-1 primarily mediates cognitive disorders associated with sickness behavior (Johnson et al., 2019). Most likely, IL-1 passes from the blood to the brain stimulates directly the release of cytokines from brain sources (da Fonseca et al., 2014). TNF-transports mediates neurotoxicity in SNpc area, providing a way for these cytokines to play a role in promoting PD.

1.6. Major links between Neuroinflammation and Neurodegenerative Diseases in the Scope of CNS Immunity and BBB

Neurodegenerative disorders can be described as a functional deficit that leads many different brain disorders such as AD, PD, MS or even mood disorders for instance depression (Scuderi et al., 2009). In recent years, neuroinflammation considered as one of the underlying causes of the mechanisms of neurodegeneration in the CNS. Suppressing inflammation is now considered as one of the main therapeutic approach in chronic neurodegenerative diseases. This approach is based on the positive effects of “non-steroidal anti-inflammatory drugs (NSAIDs)” against the development of many common cognitive diseases such as AD and PD (Esposito et al., 2007).

Microglia with activated morphology are present in CNS tissue of the patients with AD, PD, ALS and prion disease. Basically, NSAIDs are the inhibitors of cyclooxygenase activity. In inflammatory conditions, microglia secrete a large number of proinflammatory

prostoglandins which are produced by COX-2, and this is a strong link between neurodegenerative diseases and neuroinflammation (Vlad, Miller, Kowall, & Felson, 2008).

Roberts et al. (2014) reported that damaged astrocytes secrete misfolded proteins such as mSOD1, the main cause of familial ALS, which activates microglia through toll-like receptor 2 (TLR2) and TLR4. Also, PET scan showed that excessive microglial activity in motor cortex of both with ALS patients and SOD1G93A mice (Turner et al., 2004). Another study revealed that P2 receptors which are responsible for an alarm signal from neuron to microglia were upregulated in mutant SOD1 microglia purified from mice. As a result, expression of TNF-alpha and cyclooxygenase-2 were upregulated, and finally mutant SOD1 were expressed as a result in microglial activation-related toxicity on neuronal cell lines (D'Ambrosi et al., 2009).

Recent study showed that under brain ischemic conditions, TLR4 was activated and TLR4 dependent agonists such as HMGB1, Hsp60, and Hsp70 was increased (Yu, Wang, & Chen, 2010) resulting in excessive proinflammatory mediators in the environment by activated microglia and neuronal cell death. Parada et al. (2019) revealed that blockage of TLR4 may lead to neuroprotection in case of post-stroke and promising therapeutic approach for future research.

MS is an autoimmune disease of the CNS related with demyelination and axonal loss. During the inflammatory conditions, the peripheral immune cells migrate through the BBB and accumulate the specific region, Virchow- Robin space and trigger a local immune response which targets myelins and axons. LPS stimulations can induce cytotoxic factors including pro-inflammatory cytokines, and ROS accumulation which may contribute to disease pathogenesis (Ortiz et al., 2014).

Increasing evidences have shown that the pathogen-associated BBB disruption leads to an inflammatory response resulting in neonatal cerebral white matter injury (Hu et al., 2017).

Another microglia-BBB interaction has been shown that VEGF expressed by endothelial cells which promotes microglial chemotaxis. As a result, activated microglia accumulate amyloid-β composition in AD (Ryu, Cho, Choi, Wang, & McLarnon, 2009). Bain et al., suggested that the same mechanism has been related to cerebral ischemia (Bain, Moore, Ren, Simonishvili, & Levison, 2013).

Taken together, neuroinflammation can be considered and targeted in the new treatment strategies against neurodegeneration through glial cells and endothelium, which are closely related and manipulated with each other. In this aspect, natural products can serve

as a good starting point. Increasing evidences show the potential use of natural products both in prevention and treatment of neurological disorders (Zhao et al., 2017). To prevent chronic inflammation and the progression of neurological disorders, evaluating the molecular mechanisms of these natural compounds associated with microglial activation-related BBB disruption will provide an important point of view for drug discovery studies.

1.7. Natural Products

The usage of natural products on CNS disease has been gained popularity over the last years. The neuroprotective effects of plant extracts and pure phytochemicals have been studied for a while and the results are promising for the disease treatment and new strategies are being developed for drug design. In this chapter, the focal points are some of natural flavones, phenolic compounds and some novel compounds that have great potential against neuroinflammation.

1.7.1. Luteolin&Apigenin

Flavonoids are a phytochemical-based phenolic group which are found in vascular plants. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is common flavonoid found in a number of plants such as broccoli, pepper, thyme and celery (Figure 1.9a). Luteolin-rich plants are used in traditional Chinese medicine for the treatment of various diseases such as cancer, hypertension, and inflammatory disorders. Apigenin (4′,5,7-trihydroxyflavone) is another compound presented in a large number of natural sources including in parsley, chamomile, celery, basil and oregano (Shields, 2016) (Figure 1.9b). Apigenin has various biological functions, such as antiviral, antibacterial, antioxidant, strong anti-inflammatory activities and anti-hypertensive effect. Both compounds are well recognized for their anticancer activity through the induction and inhibition of cancer metabolism and promotes angiogenesis (Khan & Mukhtar, 2010, Shamsizadeh, Roohbakhsh, Ayoobi, & Moghaddamahmadi, 2017).

a. b.

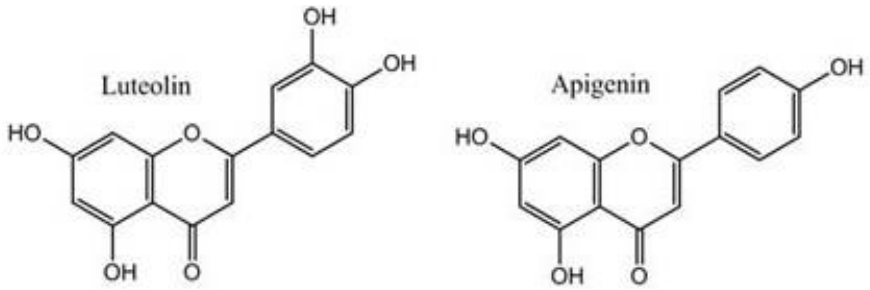

Figure 1.10. The chemical structures of luteolin (a.) and apigenin (b.)

There are also some in vitro and in vivo examples demonstrating their effectiveness against neuroinflammation found in the literature. The examples were given in Chapter 2.

1.7.2. Caffeic Acid&Rosmarinic Acid

Caffeic acid (CA) a nonflavonoid group containing polyphenol, found in all plants and is an important element in the biosynthesis of lignin, one of the essential components of woody plant biomass (Figure 1.10a)(Brahmachari, 2017). It is also used commonly as a consumable products found in coffee beans (Coffea arabica) and tea (Camellia sinensis). Another compound Rosmarinic acid (RA) an ester of CA, naturally found in several plants of Boraginaceae and Lamiaceae family, including rosemary (Rosmarinus officinalis), sage (Salvia officinalis), basil (Ocimum tenuiflorum) and oregano (Origanum vulgare) (figure 1.10.) (Alfieri & Mann, 2014)). These phenolic compounds exhibit antioxidant and anti-inflammatory effects. An in vivo study in Tg2576 transgenic AD mice that expressed human amyloid precursor protein (APP) at levels more than 5-fold showed that the oral administration of RA inhibited both amyloid beta (Aβ) oligomerization and deposition (Hamaguchi, Ono, Murase, & Yamada, 2009). Pre-treatment of CA in the PC12 rat adrenal gland cells resulted in a dramatic decrease in tau phosphorylation by inducing AB (Sul et al., 2009).

a. b.

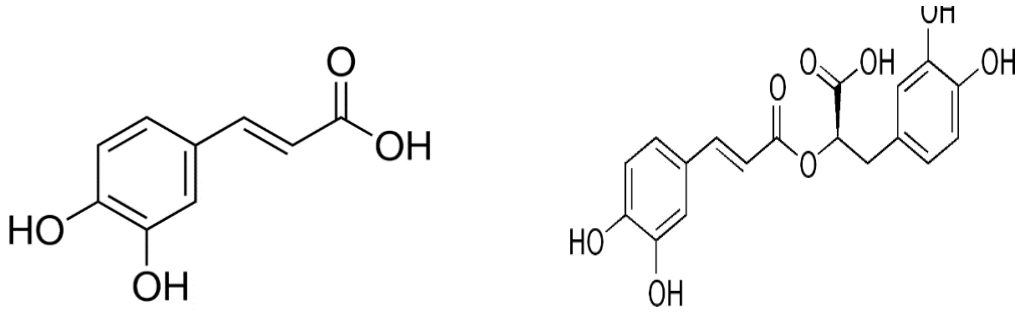

Figure 1.11. The chemical structures of CA (a.) and RA (b.)

1.7.3. Caffeoly-Rosmarinic Acid

The novel phytochemical is caffeoyl-rosmarinic acid (Figure 1.11.), which was isolated from Lavandula stoechas for the first time as a part of a TUBITAK project 112T442 (completed by our laboratory in 2017) and tested on RAW 264.7 macrophage cells for their systemic antiinflammatory effects. This molecule is a hybrid molecule formed by the ester bond between caffeic and rosmarinic acid. There have been no published study on the bioactivity of this molecule yet. However, it was a possible anti-inflammatory effect according to our previous study, which is not published yet.

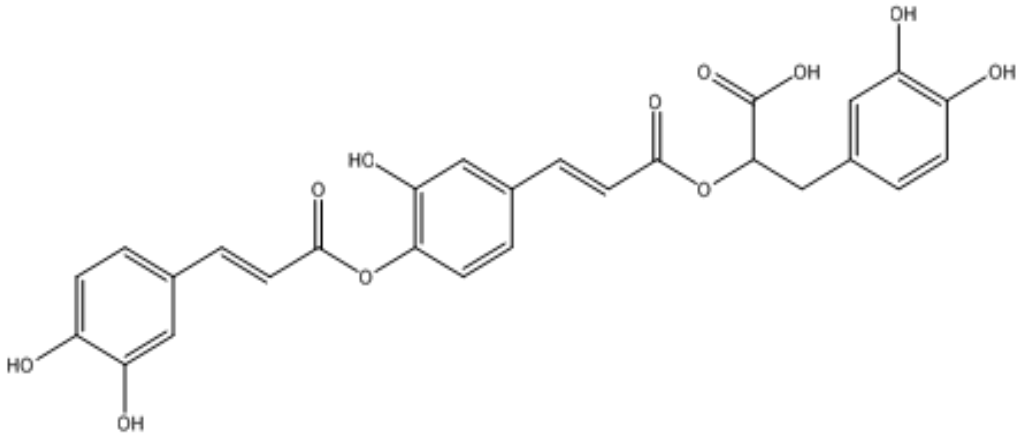

Figure 1.12. The chemical structure of Caffeoly-rosmarinic acid

1.7.4. Strigolactones

Strigolactones were discovered in 1966 as a phytochemical and the germination released agent of Striga plants and recently identified as new phytohormones (Cook, Whichard, Turner, Wall, & Egley, 1966). In 2008, SLs proved to play a fundamental role in the branching of mycorrhizal fungi, flourishing fruit, secondary growth, aging and response

to stress (Zwanenburg et al., 2016). These recent biological properties have led to the discovery of SLs as new plant hormones and the several of natural SLs and synthetic compounds of similar character (Waters, Gutjahr,F Bennett, & Nelson, 2017). At least 20 different SL derivatives are currently defined in nature. GR24 is an analog of SLs and available commercially with the similar activity as natural strigolactones (Figure 1.12) (Smith, Barker, & Zhu, 2006).

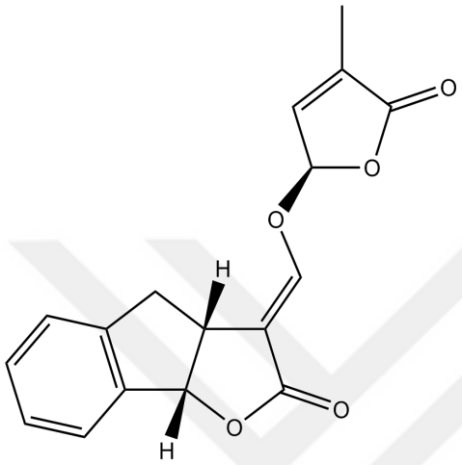

Figure 1.13. The chemical structure of GR24

For a long time, plant hormones have been known as unique chemicals to plants in the regulation of physiological traits. However, very recently it has been shown that mammals also produce several types of hormones that are usually found in plants, and nowadays, these are extensively investigated as potential treatments for viral infections, neurodegenerative diseases, and cancer (Seegobin et al., 2018).

The first studies of GR24 focused on the elucidation of anticancer activities of SL analogs. Pollock et al. (2014) have shown that GR24 significantly inhibits the cell cycle through MAPKs and suppresses proliferation of colon, melanoma, prostate, lung breast, osteosarcoma, and leukemia cell lines and these effects have been associated with the activation of apoptosis. In breast tumor xenograft models, different SLs also inhibited growth of cells (Croglio et al., 2016, Mayzlish-Gati et al., 2015).

Some other researches on mammalian cells demonstrated that SLs are also effective in insulin sensitivity. Modi et al. (2017) revealed that GLUT4 and SIRT1 activation was enhanced by GR24 treatment.

In addition, Tumer et al. (2018) showed that GR24 promoted the activation of AKT by controling the gluconeogenesis in the liver through reducing PEPCK and G6Pase

expression levels in muscle cells. There are no studies showing effects of SLs on neuroinflammation in literature.

1.8. Aim of the Study

The aim of this study is to elucidate the therapeutic/protective effect and also to identify molecular action mechanisms of some known and novel phytochemicals at physiologically attainable lower doses against chronic neuroinflammation which has a fundamental role in the progression of many neurodegenerative diseases. The proposed work has novelty in three aspects,

1) Natural compounds include some novel molecules such as caffeoyl-rosmarinic acid. It is an innovative approach to test these compounds against neuroinflammation models on NVU members brain endothelial cells and microglia.

2) Recent studies have been revealed the close relationship between BBB and microglial activation during chronic neuroinflammation. This thesis aims to investigate the neuroinflammatory processes on inflammation-model of BBB and microglial cells, and how these could be targeted with some phytochemicals. In this aspect, known and novel phytochemical compounds were tested on brain endothelial and microglial cells and this approach will be planned to provide new perspectives and unravel potential mechanisms of CNS immunity by microglial and BBB mediated neurodegeneration.

3) SIM-A9 cell line has been recently isolated glial culture from murine cerebral cortices (Nagamoto-Combs, Kulas, & Combs, 2014). It is the first spontaneously immortalized microglial cell clone behaves comparable to primary microglia and represents a valuable in vitro model for neuroinflammation when challenged with LPS or amyloid β. Besides, there is limited amount of study on this cell line. In the scope of this thesis study, all compounds were tested for the first time in SIM-A9 cell and as the same as in brain endothelial cells.

With these aims, all compounds were applied to LPS-induced SIM-A9 cells and the level of NO and as well as released rate in the brain were measured. The compounds which resulted in 50% inhibition in the LPS induced NO production were choosen as bioactive lead compound. Cell viability assay was applied in both cell lines to determine the non-toxic concentrations of selected compounds. The effects of the selected compound on the proinflammatory proteins, transcription factors and neuroprotective/antioxidant molecules in LPS-induced SIM-A9 and bEnd.3 cell lines were investigated at the gene and protein expression levels. Cytokine secretion levels in microglial cells were also investigated.

In the meantime, LPS-stimulated bEnd.3 cells were examined for the effects of the selected compound on BBB permeability.

CHAPTER 2
PREVIOUS STUDIES

There are some studies that have shown a significant involvement of apigenin, luteolin, caffeic acid and rosmarinic acid in the inflammatory processes with molecular and cellular perspectives and their therapeutic effects in the treatment of cognitive disorders. Zhu et al. (2011) have showed that luteolin manifested anti-inflammatory effects that inhibits the overproduction of proinflammatory mediators such as NO and PGE2, and cytokines such as TNF-α and IL-1β in the LPS induced BV2 microglia. Burton et al. (2015) have shown that the dietary luteolin (6g/kg) reduced stimulated microglia in the brain of LPS injected mice before isolating microglia. After the isolation, cells were stained for IL-1β and IL-6 for flow cytometry. Luteolin-fed mice have shown that the microglial rate stained for IL-1β and IL-6 was decreased by approximately half as compared to controls.

Balez et al. (2016) have showed that apigenin is strong candidate molecule that may prevent AD by down-regulated proinflammatory mediators including NO with IC50 values among 10 and 100 μM in the murine macrophage cell line RAW264.7, microglial cell line C8-B4, and primary microglia. The possible neuroprotective effects of apigenin could prevent PD with suppressing the ROS damage to the dopaminergic neurons by promoting the activation of Nrf2 and ERK1/2 (Wruck et al., 2007). Another study by Zanella et al. (2012) has shown that apigenin induced suppression of inflammatory factor, COX-2 enzyme. Rezai-Zadeh et al. (2009) demonstrated that apigenin and luteolin reduce TNF-α and interleukin production and suppress CD40 protein expression which is the main immunoregulatory molecule in microglial cells.

It is well known that caffeic acid is a promising natural compound against neurodegeneration and as well as certain organ damage like lung, kidney, and liver (Armutcu et al., 2004). A recent study (2019) have demonstrated that the caffeic acid reduced motor deficits, microglial expression and inflammatory mediators in PD mouse models (Zaitone et al., 2019). Another study have suggested that 0.1 μM dose of caffeic acid effectively inhibited the expression levels of iNOS, COX-2, as well as cytokines including IL-6, and IL-1β in BV-2 murine microglia. Additionally, caffeic acid significantly upregulated cytoprotective molecules. In the same study, caffeic acid increased HO-1 protein levels at 0.3 μM dose and in a time dependent manner (0, 2, 4, 8, 16, 24 h) (Tsai et al., 2015).

As an aromatherapy compound, rosmarinic acid was included in the list of European

herbs as a cognitive-improving compound (Kennedy & Scholey, 2006). It has been proved in patients who suffered from cognitive deficits by Jimbo et al., and after oral consumption, all patients showed remarkable recovery in personal orientation. Also, AD patients showed dramatic improvement in total Touch Panel-type Dementia Assessment Scale (TDAS) (Jimbo, Kimura, Taniguchi, Inoue, & Urakami, 2009).

Pretreatment with rosmarinic acid in LPS stimulated N9 microglia reduced microglial stimulation by decreasing the levels of iNOS expression and increasing arginase-1 (Arg-1) at 10 µM dose (Coelho et al., 2017).

As a novel compound, RA-CA which is isolated by our laboratory,have not been associated with any neuroinflammation studies. Although the effects of GR24 on mammalian cells have been investigated extensively, there is no any study in the literature for its possible anti-neuroinflammatory activities. Tumer et al. (2018) reported that GR24 induces Nrf2-activated HO-1 and NQO1 under inflammatory conditions in H4IIE hepatic and RAW 264.7 macrophage cells. This study which was carried out by our laboratory also showed that GR24 suppressed the release of NO in LPS induced RAW 264.7 cells.

Herein we investigated its involvement in neuroinflammatory processes and its possible protective role in inflammation induced microglia BBB endothelial cell lines, which have not been addressed before.

CHAPTER 3
MATERIALS AND METHODS

3.1. Materials

3.1.1. Chemicals

The list of chemicals were given in Table 3.1.

Table 3.1. List of Chemicals

Sigma-Aldrich Chemical	Thermo Fisher Scientific	Santa Cruz Biotechnology	Novus Biologicals
Dulbecco's Modified Eagle's Medium (DMEM) high glucose (D6429)	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (11320074)	Donor Horse Serum (DHL01)	ECL Western Blotting luminol solution (sc-2048)
MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide] (M5655)	PureLink RNA mini kit (12183018A)	Dulbecco's PBS (PBP01)	dimethyl sulfoxide (DMSO; sc-358801)
bovine serum albumin (A6003)	TRIzol (15596018)		1400 W (N-[3 Aminomethyl) benzylacetamidine Dihydrochloride, (sc-3564A)
methanol (24229)	trypsin-EDTA (25200056)		non-fat dry milk powder (sc-2324)
trypan blue solution (T8154)	High Capacity cDNA Reverse Transcription kit (4368814)		anti-peroxisome proliferator-activated receptor-γ (PPAR-γ; sc-7196)
dimethyl sulfoxide (DMSO; D2650)	DNase I (AM2222)		anti-NOS2 (M-19, sc-650)
lipopolysaccharide (LPS from E. coli O55:B5)	TaqMan™ Fast Advanced Master Mix (4444557)		anti-β-actin (C4, sc-47778)
	TaqMan Probes iNOS, COX-2, IL-1β, TNF-α, HO-1, NQO1, Nrf2, β-actin		Anti-Nrf2 antibody (NBP1-32822)
	Microamp Optical Adhesive Film (4360954)		anti-TNF-α (NB600-587)
	bicinchoninic acid protein assay kit (BCA; 23227)		anti-lamin B1 (NBP1-42594)
	NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (78835)		anti-NF-kB (NB100-56712)
	IL-1β mouse ELISA kit (BMS6002)		

GR24 was purchased from Strigolab (Torino, Italy) as in the form of racemic mixture containing R and S isomers (*rac*GR24). For the simplicity, it was mentioned as GR24 throught the thesis.

3.1.2. Equipments

The list of equipments were given in Table 3.2.

Table 3.2. List of Equipments

Vertical electrophoresis system (BioRad)
electroblotter (Thermo Fisher Scientific)
Esco cell culture CO_2 incubator
Nordicsafe Esco sentirel Gold (Class II BSC)
BioSan TS-100 Thermal Shaker
cold sentrifuge (Hettich Zentrifugen Mikro 200R, Hettich Zentrifugen Universal 320)
Applied Biosystems™ 7500 Real-Time PCR (Applied Biosystems, Thermo Fisher Scientific)
C-DiGit Blot Scanner-LI-COR Biosciences
Major-Science (MS) Waver Shaker
Vortex (Scientific Industries)
pH meter (Hanna Instruments HI 2211)
Thermal Cycler (Bio-Rad)
Microplate reader with NanoQuant (Tecan Infinite® M200 PRO)
i-control ™ micro plate reader software (Tecan Infinite® M200 PRO)
Ice machine (İzmak)

3.1.3. Cell Lines

The cell lines were used in the study were given in Table 3.3.

Table 3.3. Medium Conditions of SIM-A9 and bEnd.3 Cell Lines

SIM-A9 Mouse Microglia Cell (CRL-3265, ATCC)	**bEnd.3 Mouse Brain Endothelial Cell (CRL-2299, ATCC)**
DMEM-F12	DMEM
10% FBS	20% FBS
5% Horse Serum	-
100 U/mL penicillin, 100 µg/mL streptomycin	100 U/mL penicillin, 100 µg/mL streptomycin

Cells were optimized in laboratory conditions and created stocks for future experimetns. Cells were incubated at 37ºC in humidified air containing and 5% CO_2. Passage processes were made by 0.25% Trypsin-EDTA until cells were reached 90% confluency. Then, cells were seeded on flasks and plates according to experimental protocol.

3.2. Methods

After gathered all equipments and materials for the experiments, MTT tests were done on bEnd.3 and SIM-A9 cells to evaluate the cell viability following GR24 treatments. All cell lines were adaptated in laboratory conditions, passaged and stocked in liquid nitrogen tank for future experiments. All compounds including apigenin, luteolin, RA, CA, Caffeoly-rosmarinic acid and GR24 were screened for their capacity as an inhibitor of NO production in SIM-A9 microglia cells in order to identify lead compounds for future experiments. All experiments were carried out at three times in triplicate.

3.2.1. Nitric Oxide Inhibitory Assay for Screening Compounds and for the Identification of IC50 of the Lead Compounds

Nitric oxide (NO) which is released to media in LPS induced SIM-A9 cells was assayed with Griess reaction method (Griess, 1879). Griess is an analytical method which is used for the detection of nitrite amount in a solution through react with sulfanilic acid. As a result, the acidic mixture will be produced a red-violet color and measurable with colorimetric analysis. Cells were plated at density of $5x10^4$. Cells were treated in the absence and presence of LPS (1 µg/ml) and test compounds at 10 µM and then different doses of lead compounds for 24 h in phenol red-free media. Griess method was applied as descibed before (Tumer et al., 2018).

3.2.2. Cell Viability Assays

"MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyltetrazolium bromide]" assay was performed to investigate the effects of lead compunds in both SIM-A9 and bEnd.3 cells. MTT is a very sensitive indicator of cell proliferation and used for calculating the reduction of a tetrazolium (MTT) to the formazan crystals, a product of mitochondrial activation of living cells (Mosmann, 1983). Cells at density of $5x10^4$ were plated on 24-well plates and incubated with GR24 for 24 h in a dose dependent pattern (5, 10, 20, 50 and 100 µM). After 24 h, media was discarded, and MTT solution mixed with phenol-red media were pipetted in every well. Media was discarded and formazan were dissolved with DMSO. After 10 min of incubation, cell viability ratio was measured (Tecan Infinite® 200 PRO, Switzerland) at 570 and 690 nm wavelength.

3.2.3. Enzyme-Linked ImmunoSorbent Assay (ELISA)

ELISA is one of the immunological assay commonly used for detecting the level of proteins, antigens, antibodies in biological samples (Voller, Bartlett, Bidwell, Clark, & Adams, 1976). For this experiment sandwich ELISA method was used; basically, primary antibody or capture antibody is used to detect and interact with the target sample. After that, wells are washed and unbound antibodies are removed from the area. The secondary antibody is linked with horse radish peroxidase (HRP), an enzyme which is used for binding with substrate solution and can be measured by colorimetric methods as represented in Figure 3.1.

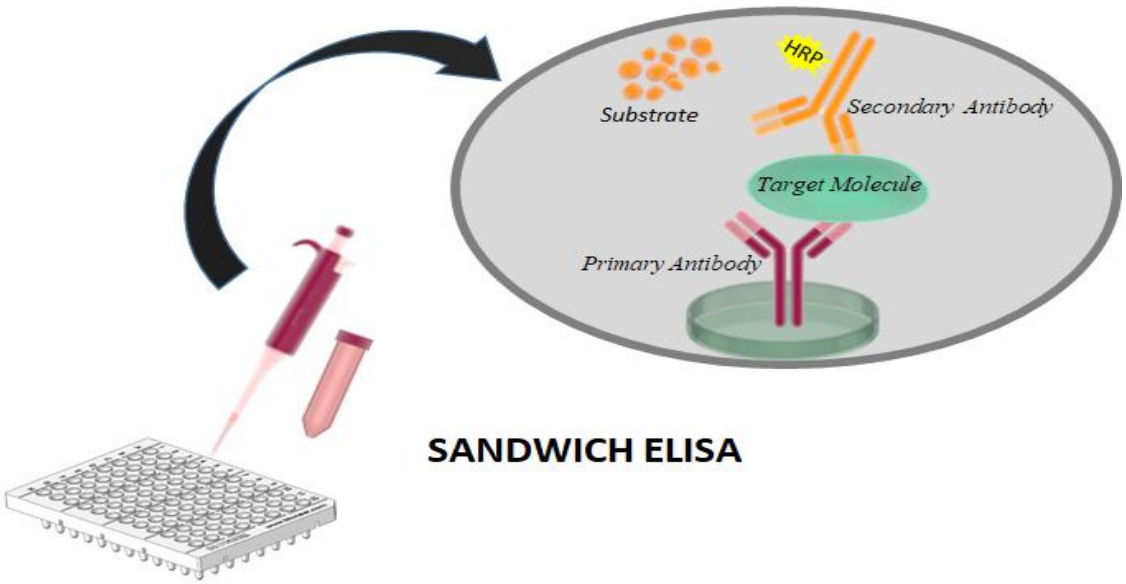

Figure 3.1. Immunological analysis of released cytokines using specific antibodies

Microglia cells were seeded approximately $9x10^5$ cells on 6-well plates in serum-free media, then incubated with DMSO as control and treated with LPS and GR24. Then, medium was collected and performed in duplicate with enzyme-specific mouse ELISA kit. The top concentration for the standard curve was created to be 50 pg/mL for IL-1β and 6000 pg/mL for TNF-α. The capture antibody for IL-1β and TNF-α was added to the ELISA plate. Plate was incubated at RT on orbital shaker adjusted 400 rpm for IL-1β quantification for 2 h, and at 250 rpm for determination of TNF-α for 1 h. After that, plate was rinsed 4 times and incubated with HRP-conjugated secondary antibody on orbital shaker for 1 h at RT for 1 h on microplate shaker set at 400 rpm for IL-1β, 45 min on microplate shaker set at 250 rpm for TNF-α. Wells were rinsed and incubated with substrate solution for 30 min to react with HRP enzyme. At the end, stop solution was pipetted to each well and measured micro plate reader at 450 nm. For each well, measurement of secreted cytokine concentrations were described before (Tumer et al., 2018). The details of the protein quantification were given in 3.2.5.2.

3.2.4. Gene Expression Analysis

RNA isolation was carried out with the PureLink® RNA mini kit with TRIzol® solution from SIM-A9 and bEnd.3 cells. The amount and purity of collected RNAs were checked by using a NanoQuant microplate reader. cDNA was synthesized as described

before (Tumer et al., 2018) The resulting cDNAs were diluted and quantitative polymerase chain reactions (qPCR) were assayed using TaqMan ® probes specific for target genes. PCR cycle, primary concentrations and cDNA amounts were optimized and β-actin was used as an endogenous control.

3.2.4.1. RNA Isolation

bEnd.3 and SIM-A9 cells were plated about $9x10^5$ and $2x10^5$ number of cells, respectively. When cells cover the surface of 24-well culture plate, cells were treated with DMSO as control, LPS and GR24 as triplicate and were incubated for 12 h in SIM-A9 cells and 24 h in bEnd.3 cells. The RNA isolation were carried out as described before (Tumer et al., 2017). The dried RNA pellets were dissolved in RNAase-free water stored at -80°C until use.

3.2.4.2. Spectrophotometric Analysis of RNA

RNA amounts were measured at 260 nm, the wavelength of which nucleic acids gave maximum absorbance. Wavelength of 260, 280 and 230 nm are the maximum wavelengths of the nucleic acids, protein and phenol, respectively. The values of A260 / A280 and A260 / 230 were taken into account when evaluating the purity of RNA. These ratios are ~ 1.9 and 1.8-2.2, respectively, indicating that RNA is pure.

3.2.4.3. Imaging of Total RNA by Agarose Gel Electrophoresis

The preparation of 1% agarose gel was carried out by utilizing the Tris Boric acid-EDTA (TBE)(1X) buffer for the analysis of isolated RNAs. Prepared agarose gel was heated and boiled via microwave oven. After boiling, the agarose solution was allowed to cool immediately before adding 3 µL of SafeView ™ in the solution. The prepared solution was transferred into the gel plate and waited until polymerized. The polymerized agarose gel was put in the electrophoresis tank carefully and the tank was filled with enough 1X TBE buffer. The wells of the gel were loaded with the prepared mixture containing 2 µL of RNA sample and 2 µL of loading dye. Then, the samples were electrophoresed at 100 volts for 45 min. The quality of the RNAs was visualized with the aid of the gel imaging system (VILBER LOURMAT).

3.2.4.4. cDNA synthesis

The cDNA synthesis from RNA was carried out as stated in the manufacturer's

instructions using the 18 nucleotide long oligo d (T) primer and the ABI-High-Capacity cDNA reverse transcriptase kit. For further experiments, the synthesized cDNAs were stored at -20 ° C.

3.2.4.5. Quantitative Polymerase Chain Reaction: qPCR

Quantitative PCR(qPCR) is very useful technique for investigating gene expression levels by the assistance of fluorescent reporters in real-time (Mullis., 1983). qPCR reactions were performed as described in previous studies (Tumer et al., 2017). TaqMan ® probes specific for target genes used for the determine the effects of GR24 on targeted gene expression levels. For each gene, a standard calibration curve was prepared by amplification analysis and the melting curve analysis was performed. β-Actin was used as an endogenous control. The calculated mRNA expression levels by using the comparative ddCt were normalized with β-Actin Ct (threshold). The LPS induced control ddCt value was used as a calibrator and accepted as 1.

3.2.5. SDS-PAGE and Western Blot Analysis

To identify target proteins in protein mixture purified from cells, SDS-PAGE and Western Blot techniques are widely used. With this way, proteins are separated by their size and transferred from gel to a membrane. The target protein visualized by using primary and secondary antibodies. Proteins were isolated from cytoplasmic (iNOS, COX-2, PPARγ, TNF-α), nuclear (NF-kB, Nrf2) or whole lysate (AMPK, p-AMPK) fractions according to their location inside the cell.

3.2.5.1. Protein Collection for SDS-PAGE and Western Blot Analysis

SIM-A9 cells were seeded approximately $7.5x10^5$ cells on 6-well plates and incubated with DMSO as control, with LPS (1 μg/ml) and GR24 (5, 10, 20 μM). Cells were rinsed with PBS and NE-PER™ kit was used for protein extraction assay according to procedure given in protocol. The amount of reagents were given in Table 3.1.

Table 3.4. The amount of reagents per packed cell volume (µL) in NE-PER method

Packed Cell Volume (µL)	CER I (µL)	CER II (µL)	NER (µL)
10	100	5.5	50
20	200	11	100
50	500	27.5	250
100	1000	55	500

Both cytoplasmic and nuclear extracts were stored at -80ºC until use. For whole cell lysate extraction RIPA buffer was used. The details of the procedure were given in Kapche et al. (2017).

3.2.5.2. Determination of Total Protein Concentrations, SDS-PAGE and Western Blot Analysis

For total protein measurement in cell lysates, BCA method was used as stated in the procedure of manufacturer and described before (Tumer et al., 2018). Proteins were separated on 4% stacking and 8.5% seperating gel by using a polyacrylamide-based discontinuous buffer system previously defined by Laemmli (1970). The formula of gels that used on the experiments were given in Table 3.2.

Table 3.5. Gel components in SDS-PAGE method

Stock Solution	Separating Gel (8,5%) (mL)	Separating Gel (12%) (mL)	Stacking Gel (4%) (mL)
Gel Solution	8.5	12	1.3
Distilled Water	13.55	10.04	6.1
Seperating Gel Buffer Tris-HCl, pH: 8.8, 1.5 M	7.5	7.5	-
Stacking Gel Buffer Tris-HCl, Ph: 6.8, 0.5 M	-	-	2.5
10% SDS	0.3	0.3	0.1
10% APS	0.15	0.15	0.050
TEMED	0.015	0.015	0.010

Proteins from cells were electrophoretically separated using the BioRad vertical electrophoresis system. After electrophoretic separation, the gel was incubated in a constant-speed shaker for 10 min in transfer buffer (10% methanol, 25 mM Tris, 192 mM glycine) (Tovey, Ford, & Baldo, 1987).

Electroblotter's electrophoretic transfer was performed with Whatman paper moistened with transfer buffer, and it was placed to the platinum base. Then, the gel and the nitrocellulose membrane were carefully placed on the Whatman paper and place another pre-moistened Whatman paper on the pre-placed gel and membrane to create “sandwich” model. A test tube is gently rolled over while keeping the membrane in its position to ensure complete removal of air bubbles. The electrophoretic transfer tank was filled with transfer buffer so that the sandwiches remain completely in the buffer and connected to the power supply. By adjusting the voltage to 90 V, proteins were transferred electrophoretically for 2 h. Blocking solution containing 5% skimmed milk powder in TBST or 5% BSA was used for 1 h. Then, membrane was incubated with primary antibody overnight. This procedure was followed by secondary antibody incubation (Figure 3.2.).

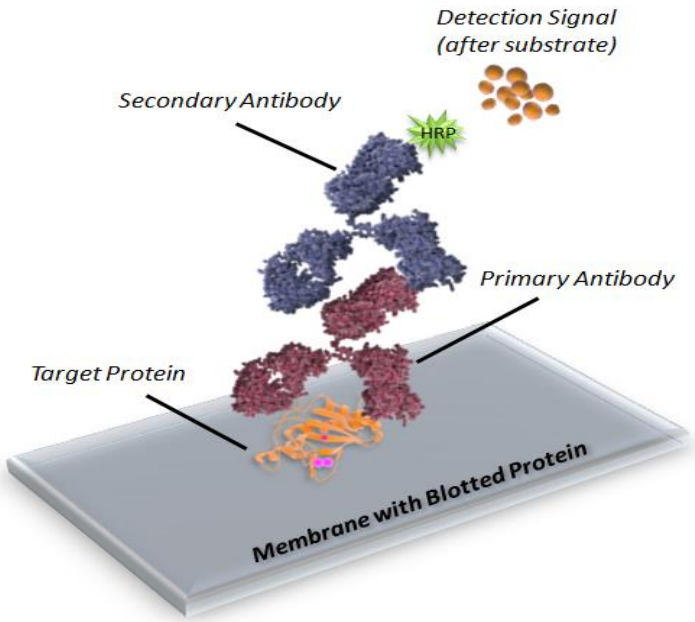

Figure 3.2. Techniques and immunologic basis of Western Blot

The membrane was treated with Western Blotting Luminol Solution to display protein bands on the C-DiGit® Blot Scanner. Protein bands were analyzed densitometrically with Image Studio Digits Software 5.0.

3.2.6. Permeability assay on bEnd.3 cells

In vitro permeability assay is a technique that represents BBB integrity during neurodegenerative conditions. With the vascular permeability modeling, endothelial leakage between blood and the brain can be determined. To examine endothelial cell permeabilization, Evans Blue is used for assessing the permeability of the BBB to macromolecules via bounding serum albumin and albumin-bound Evans Blue (Evans & Schulemann, 1914). bEnd.3 cells were plated at $5x10^4$ of cells onto 24-well Transwell inserts (luminal side) with a 0,4-µM pore. In the outside of the insert (abluminal side), 600 µl of DMEM with 20% FBS was added. After 72 h of incubation, the medium in both abluminal and luminal side was removed and washed with DMEM without phenol red. Cells were incubated DMSO as control as well as with LPS (1 µg/mL) and GR24 in the DMEM without phenol red media for 24 h. To initiate the permeability experiment, the medium in both abluminal and luminal side was removed. 600 µL of assay buffer was added to the abluminal side. Then Evans Blue Albumin (EBA) mixture was prepared (4% Bovine Serum Albumin, 0.67 mg/mL Evans Blue Dye) and diluted in assay buffer, then added to the luminal side in

100 µl for 2 h. The assay buffer in abluminal side was collected seperately and each group was measured at 650 nm.

3.2.7. Statistical Analysis

GraphPad Prism 5 software was used for the all analyses and values were showed as mean ± (SEM), *p<0.1, **p<0.01, ***p<0.001, ****p<0.0001. The details were given in Tumer et al. (2015), Kapche et al. (2017) and Tumer et al. (2018).

CHAPTER 4
RESULTS AND DISCUSSION

4.1. Results

First of all, time optimization by using LPS was performed for time dependent manner to model inflammatory conditions in SIM-A9 microglia cells and NO levels were determined. After the optimum time for LPS treatment was detected, dose optimization for positive control, 1400W was performed by IC50 calculation. Then, NO was screened in LPS induced SIM-A9 cells for investigating the effects of test compounds to determine lead compounds. MTT test was performed to identify non-toxic doses of effective compounds. The lead compound(s) was applied in LPS induced microglia cells and cytokine release was observed by ELISA assay. Time optimization studies were performed to determine LPS induction in microglia cells for both gene expression and protein expression levels and thus optimum time was selected by qPCR and western blot analysis. Experiments in bEnd.3 cells were started with MTT assay to determine the viability of cells when LPS (1 µg/mL) and lead compound(s) treatments were done. The expression levels of proinflammatory and cytoprotective genes and proteins on LPS-induced cells which treated with the test compounds were determined with the selected doses according to MTT results. Finally, the permeability test was performed and the effects of lead compound(s) on endothelial cell permeabilization in LPS induced bEnd.3 cells were examined.

4.1.1. Time optimization for the Effects of LPS on NO Released from SIM-A9 Cells

To determine the optimum time for the maximum level of NO production in microglia by LPS, cells were incubated with LPS for time dependent manner (4, 6, 12, 24 h). Media was collected and incubated with Griess reagent in a dark environment at RT. Then, absorbance was measured with ELISA plate at 550 nm (Tecan Infinite® 200 PRO, Switzerland). Results were showed that LPS-induced SIM-A9 cells gave the highest NO level in 24 h (Figure 4.1.).

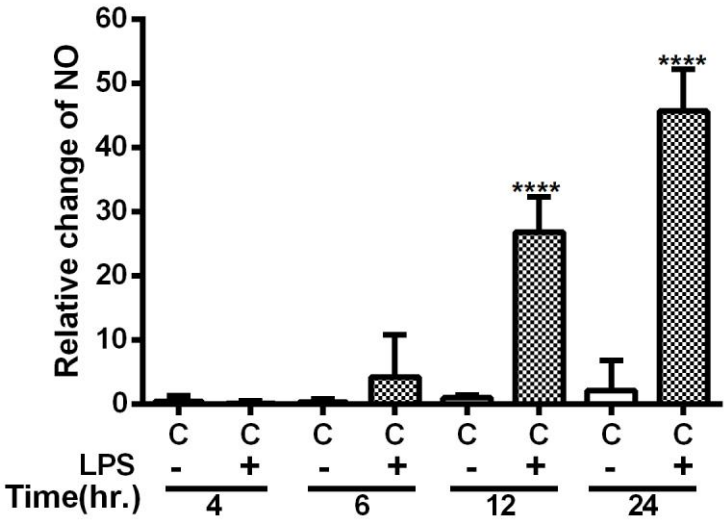

Figure 4.1. NO production in SIM-A9 cells at different incubation times following LPS treatment. C: Control.

4.1.2. The Effects of Positive Control, 1400W on NO Production in SIM-A9 cell line: IC50 value determination

"N-[[3-(aminomethyl) phenyl] methyl]-ethanimidamide, dihydrochloride (1400W)" is a highly selective inhibitor of inducible nitric oxide (iNOS) (Garvey et al., 1997). To determine the best concentration of positive control for iNOS, cells were incubated with LPS and and 1400W with a range of doses. After 24 h, media was collected and incubated with Griess reagent in a dark environment at RT. Then, absorbance was measured with ELISA plate at 550 nm (Tecan Infinite® 200 PRO, Switzerland)(Figure 4.2a.). To determine the 50% inhibition doses (IC50), 1400W was administered at 5 different doses ranging from 1 to 50 μM in LPS-induced microglia cells. As shown in Figure 4.2, the IC50 value for NO inhibition of 1400W was calculated as 2.229 μM.

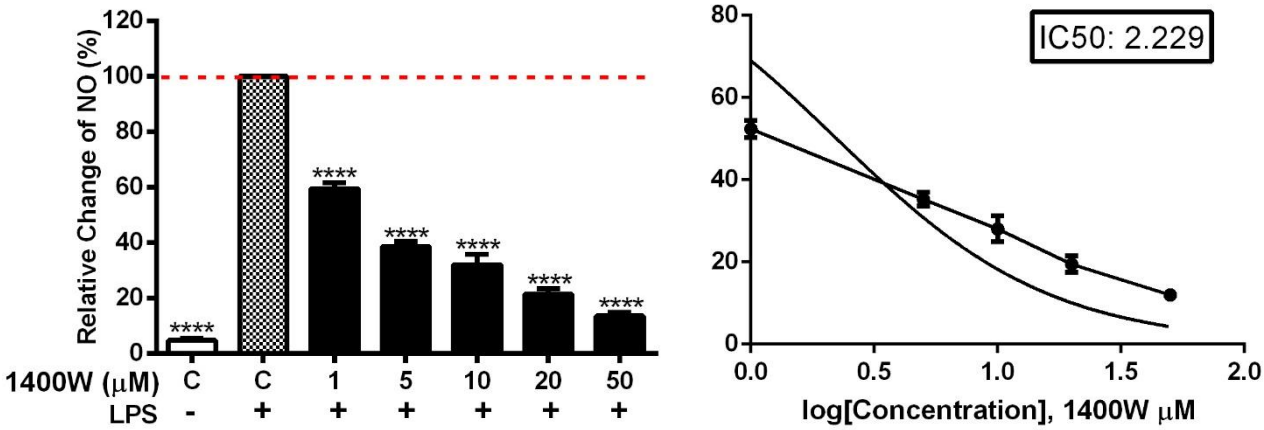

Figure 4.2. NO production in SIM-A9 microglia cells at different incubation times following treatment of LPS and iNOS inhibitor, 1400W. C: Control.

4.1.3. Screening for the Effects of Compounds on NO Production in LPS-induced SIM-A9 cell line: Identification of the lead compound(s)

Test compounds, apigenin, luteolin, rosmarinic acid, caffeic acid, Caffeoyl-Rosmarinic acid and GR24 were tested in LPS-induced microglia cells. Test compounds and positive control at 10 μM were applied on LPS-induced (1 μg/ml) SIM-A9 cells for 24 h. After incubation, media was collected and Griess reagent in a dark environment at RT. Then, absorbance was measured at 550 nm (Tecan Infinite® 200 PRO, Switzerland). (Figure 4.3.).

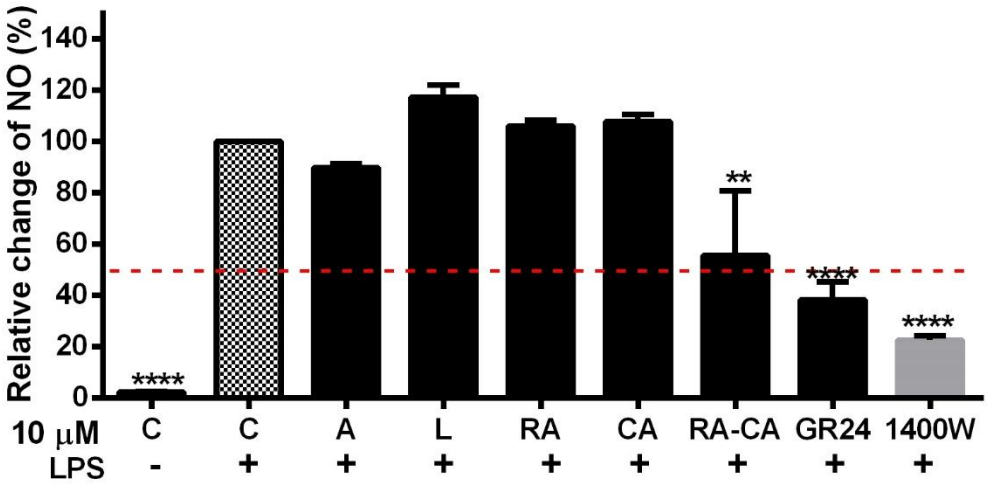

Figure 4.3. NO production in SIM-A9 cells at different incubation times following the treatment of LPS and test compounds at 10 μM doses. C: Control, A: Apigenin, L: Luteolin,

RA: Rosmarinic acid, CA: Caffeic acid, RA-CA: Caffeoyl-Rosmarinic acid, GR24: Strigolactone analog, 1400W: Positive control.

Among the test compounds only GR24 inhibited LPS induced NO production (62%) more than 50% as compared to LPS-stimulated control cells.

4.1.4. The Effect of Lead Compound on NO production in SIM-A9 Cells Following LPS Treatment: IC50 Determination

Studies conducted to determine the dose of GR24 that causes 50% inhibition in LPS induced NO level. Accordingly, GR24 was administered at 8 different doses ranging from 5 to 50 μM on microglial cells. As shown in Figure 4.4, GR24 exhibited a dose-dependent inhibition profile and IC50 value was determined as 6.2 μM.

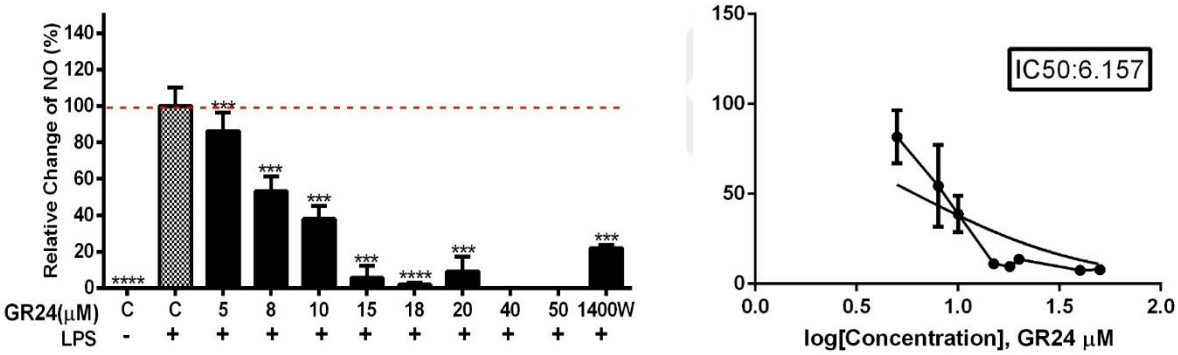

Figure 4.4. NO production in SIM-A9 cells at different incubation times following treatment of LPS and iNOS inhibitor, 1400W. C: Control.

4.1.5. Cell Viability in SIM-A9 Cells Following GR24 Treatment

Cell viability was measured in LPS-induced SIM-A9 cells to determine nontoxic doses of GR24 for further analysis. GR24 was applied to the microglia at 5, 10, 20, 50 and 100 μM. The effects of GR24 on cell viability were given in Figure 4.5.

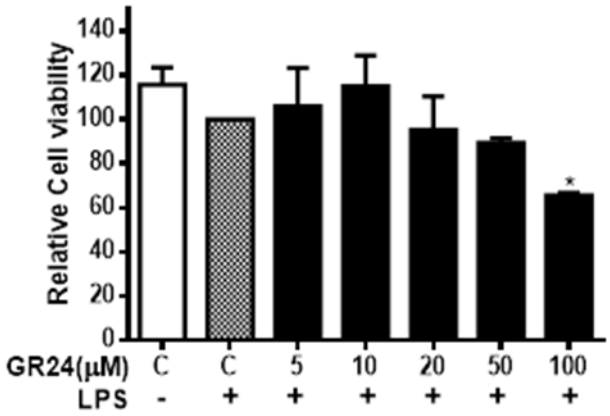

Figure 4.5. Relative cell viability in SIM-A9 cells at different incubation times following treatment of LPS and iNOS inhibitor, 1400W. C: Control.

GR24 did not result in a significant difference in cell viability in SIM-A9 cells up to 100 μM. At 100 μM, the cell viability decreased to 65% as compared to control cells. The experiments were continued with non-toxic doses of GR24 for determining its effects on cytokine secretions in LPS-induced SIM-A9 cells.

4.1.6. GR24 Reduced Cytokine Release in SIM-A9 Cells Following LPS Treatment

To determine the effects of GR24 on cytokine secretion in SIM-A9 cells, GR24 at 10 and 20 μM were applied. Results were referred that GR24 dramatically suppressed the release of TNF-α 83% and 84% for both 10 and 20 μM doses, respectively. For IL-1β, GR24 significantly reduced LPS induced level of IL-1β 30% for 10 μM dose and 40% for 20 μM dose (Figure 4.6.).

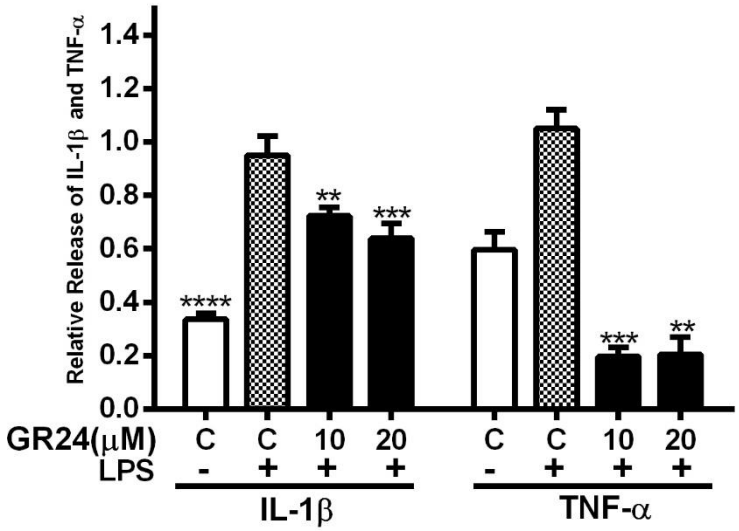

Figure 4.6. TNF-α and IL-1β release in SIM-A9 cells at different incubation times following treatment of LPS and GR24. C: Control.

4.1.7. Time Optimization for the Gene Expression Levels in SIM-A9 cells Following LPS Treatment

iNOS transcription level was used as target in optimization studies. The effects of LPS on iNOS mRNA expressions were determined with qPCR analysis for different incubation times. Cells were incubated with LPS for 4,6,12 and 24 h for time optimization. According to expression levels of iNOS, SIM-A9 cells were strongly induced with LPS at 12 h. The iNOS mRNA level at 12 h was significantly higher than 4, 6 and 24 h (Figure 4.7.).

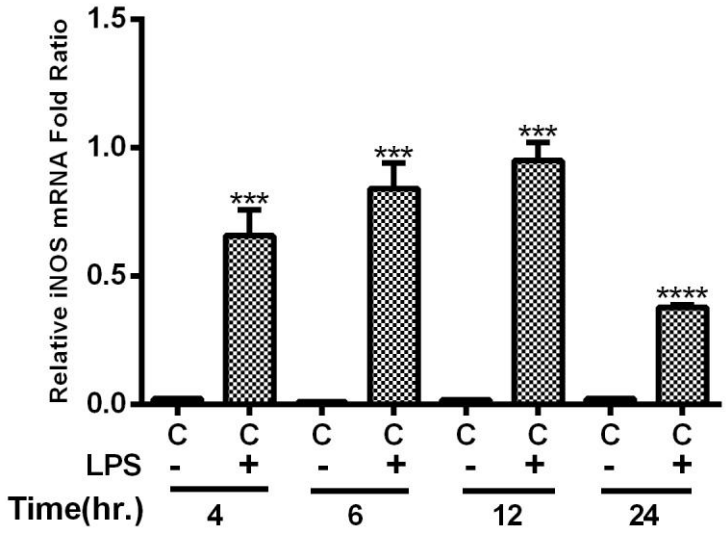

Figure 4.7. iNOS gene expression levels in SIM-A9 cells at different incubation times following treatment of LPS. C: Control.

4.1.8. GR24 Reduced the Proinflammatory and Induced the Cytoprotective Gene Expression Levels in SIM-A9 Cells Following LPS Treatment

GR24 was applied in LPS induced microglia cells at 5, 10 and 20 μM doses at 12 h. After RNA isolation, cDNA synthesis and qPCR analysis, mRNA expression levels of proinflammatory and cytoprotective genes were determined. Gene expression levels were normalized to β-actin. Every experiments were analysed by GraphPad Prism 6 software program ($p<0.05$).

4.1.8.1. GR24 Reduced the Proinflammatory Gene Expression Levels in SIM-A9 Cells Following LPS Treatment

The expression levels of proinflammatory genes treated with GR24 were determined with qPCR analysis. Compared to LPS induced control group, GR24 strongly suppressed the mRNA transcription levels of iNOS in LPS-induced microglia cells 87%, 77% and 79% at 5, 10 and 20 μM doses, relatively (Figure 4.8.). At 10 μM, 1400W downregulated iNOS expression only 37%.

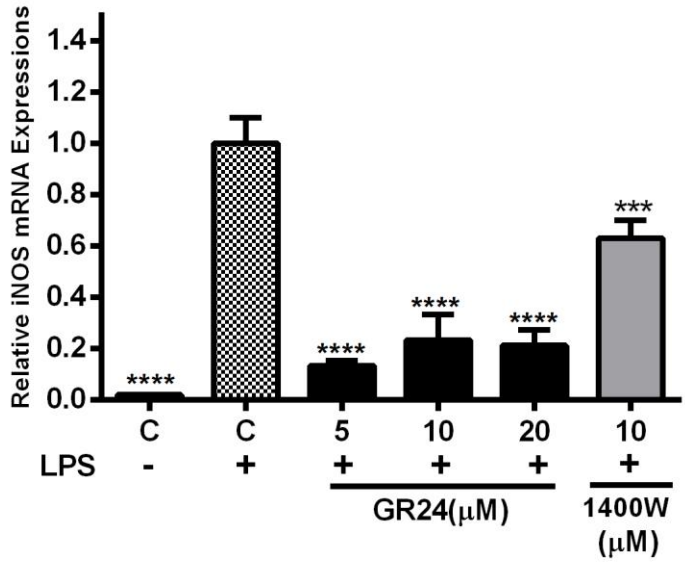

Figure 4.8. iNOS mRNA expression in SIM-A9 cells at different incubation times following treatment of LPS and GR24. C: Control.

As seen in Figure 4.9, Compared to the LPS treated control group, GR24 also resulted in a potent and significant down-regulation in the mRNA expression of COX-2 gene by 58, 72 and 78% at 5, 10 and 20 μM doses, relatively.

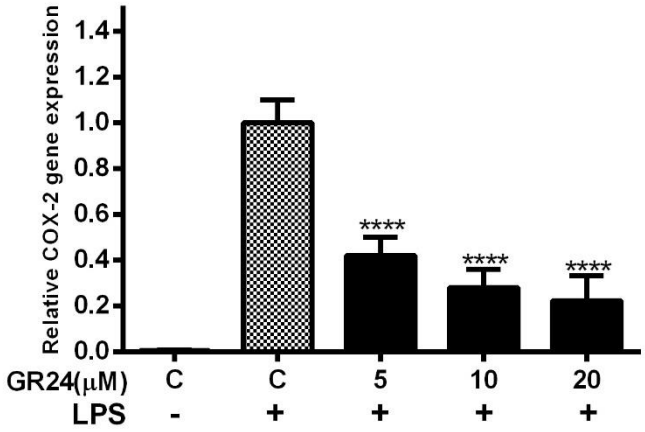

Figure 4.9. GR24 reduced COX-2 mRNA expression levels in SIM-A9 cells following LPS treatment.

As shown in Figure 4.10, GR24 significantly suppressed IL-1β mRNA expression levels at 5, 10 and 20 μM doses by 40%, 86%, 99%, relavitely compared to the LPS-induced control group in microglia cells.

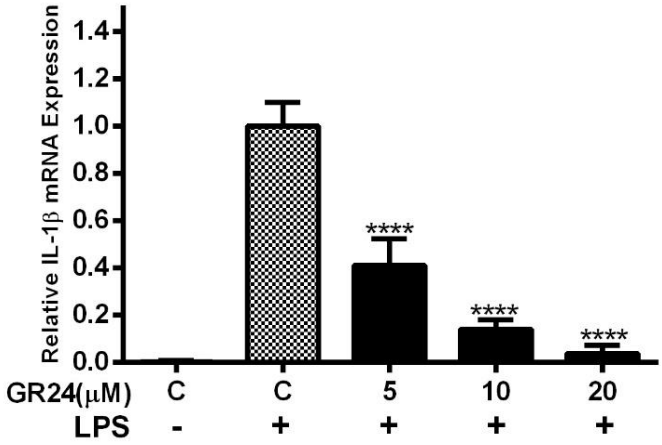

Figure 4.10. GR24 reduced IL-1β mRNA expression levels in SIM-A9 cells following LPS treatment. C: Control.

As represented in Figure 4.11, GR24 at 5, 10 and 20 μM doses resulted in 32%, 62% and 57% downregulation in the mRNA expression of the TNF-α from activated microglia cells in contrast to control group in a dose-related manner.

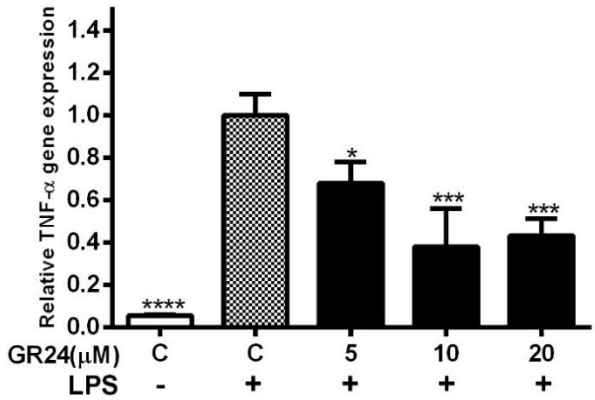

Figure 4.11. GR24 reduced TNF-α mRNA expression levels in SIM-A9 cells following LPS treatment. C: Control.

4.1.8.2. GR24 Induced the Cytoprotective Gene Expression Levels in SIM-A9 Cells Following LPS Treatment

The mRNA expression levels of cytoprotective genes, Nrf2, and its downstream

enzymes HO-1 and NQO1 were investigated after GR24 treatment. Accordingly, GR24 treatments demonstrated a remarkable increase in LPS treated SIM-A9 cells at 20 µM dose in the Nrf2 expression. However, there was no significant difference in other doses (5 and 10 µM) in contrast to LPS induced control (Figure 4.12.).

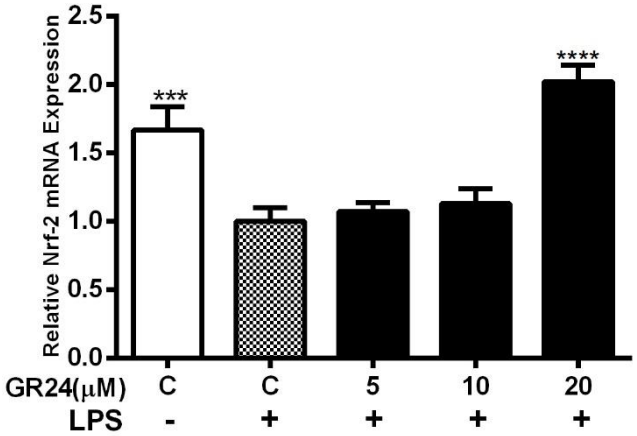

Figure 4.12. GR24 induced Nrf2 mRNA expression levels in SIM-A9 cells following LPS treatment. C: Control.

GR24 showed a dose-related increase in the NQO1 expression levels which were 4.5, 9.5 and 13 fold elevated for 5, 10 and 20 µM of GR24 in LPS treated SIM-A9 cells in contrast to control as represented in Figure 4.13.

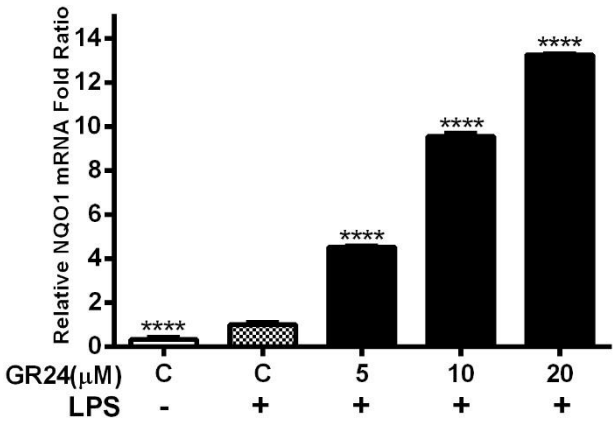

Figure 4.13. GR24 induced NQO1 mRNA expression levels in SIM-A9 cells following LPS treatment. C: Control.

The transcriptional expression of HO-1 was also upregulated by GR24 treatment 1.5 and 2.4 fold at 10 and 20 µM in contrast to control. There was no significant change at 5 µM doses of GR24 on the mRNA transcription level of HO-1 gene in contrast to control value (Figure 4.14).

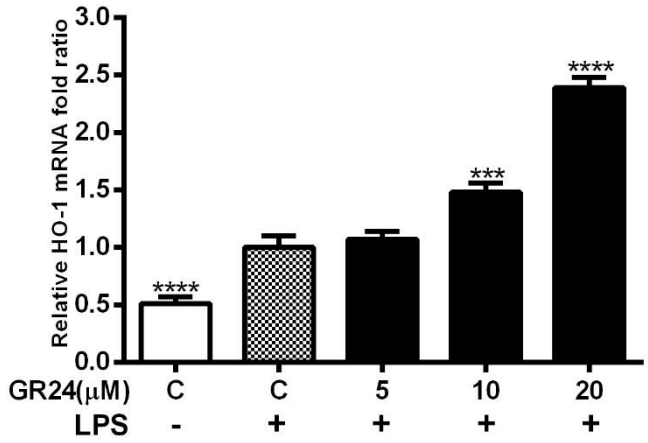

Figure 4.14. GR24 induced HO-1 mRNA expression levels in SIM-A9 cells following LPS treatment. C: Control.

4.1.9. Time Optimization for the Protein Expression Levels in SIM-A9 cells Following LPS Treatment

iNOS protein was used as target in time optimization studies to evaluate the effects of GR24 of related proteins. Western Blot analysis were performed to determine the effects of LPS on iNOS protein expression levels. Cells were incubated with LPS (1 µg/mL) for 4,6,12, 18 and 24 h to determine maximum iNOS protein transcription time by LPS induction. As seen in the Western Blot analysis, SIM-A9 cells were strongly induced with LPS at 12 h. iNOS expression levels at 12 h was clearly higher than 4, 6 and 24 h incubation period (Figure 4.15.).

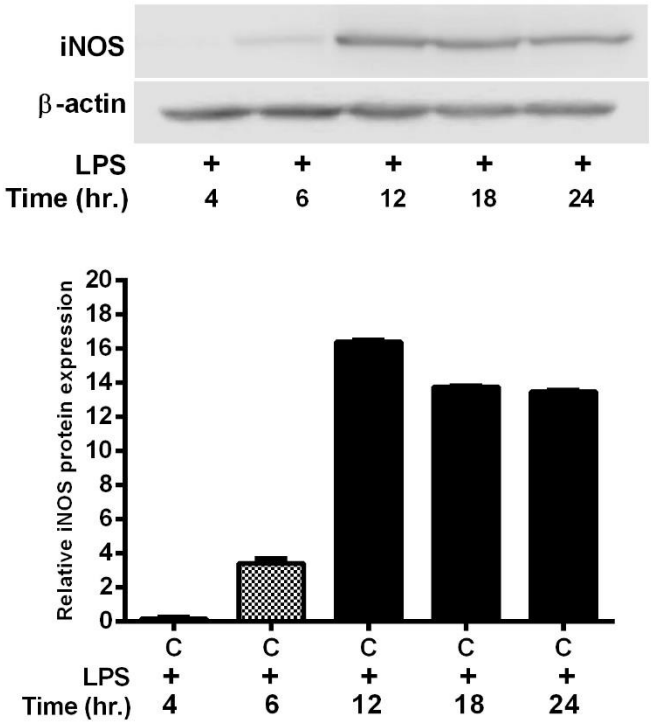

Figure 4.15. Time optimization for iNOS protein expression levels in SIM-A9 cells following LPS treatment (1µg/mL) at 4, 6, 12 and 24 h. C: Control.

4.1.10. Protein Expression Levels of Proinflammatory and Cytoprotective Genes in LPS-Induced SIM-A9 Cells Following the Treatment of GR24

After time optimization, incubation of cells with LPS (1 µg/mL) and GR24 for 12 h were carried out to determine protein expression levels in microglia cells. Therefore, protein isolation, SDS-PAGE and Western Blot analyses were performed. Expression levels of some key proteins related to inflammatory (iNOS, COX-2, NF-kB, TNF-α, PPARγ, AMPK), also neuroprotective (Nrf2) pathways were investigated. The protein bands obtained after Western blotting were analyzed densitometrically by Image Studio Digits software program. β-Actin or Lamin B -depending on the cytoplasmic or nuclear source- were used as endogeneous control in Western Blot analyses. Every experiments were analyzed by using GraphPad Prism 6 software program ($p < 0.05$).

4.1.10.1. GR24 Reduced the Proinflammatory Protein Expression Levels in SIM-A9 Cells Following LPS Treatment

Effect of GR24 on the protein level of the iNOS enzyme in LPS-induced SIM-A9 cells were determined with SDS-PAGE and Western Blot analyses. GR24 was administered at 5,

10 and 20 μM doses which were determined in NO screening for 12 h with LPS treatment. As shown in Figure 4.16, GR24 at 5 μM dose did not significantly suppress the level of iNOS. However, at 10 and 20 μM doses of iNOS protein levels decreased by 31% and 32%, respectively in contrast to LPS control group.

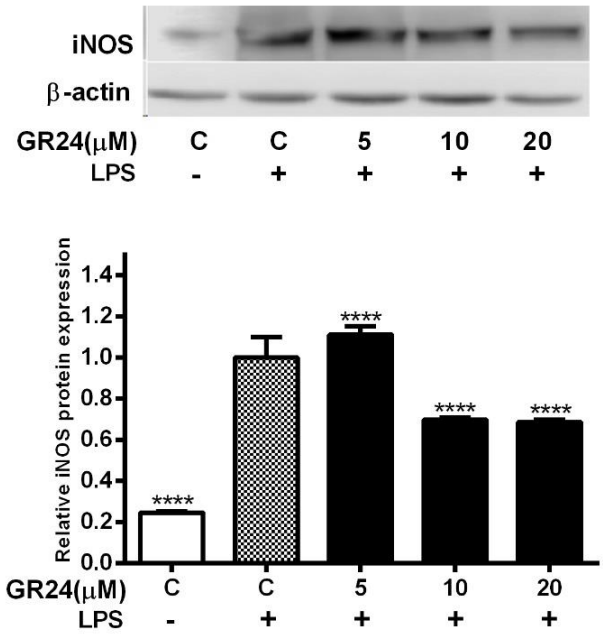

Figure 4.16. GR24 reduced iNOS protein expression levels in SIM-A9 cells following LPS treatment. C: Control.

GR24 was applied at 5, 10 and 20 μM for 12 h to determine the protein expression level of COX-2 in LPS induced microglia cells. As shown in Figure 4.17, 5, 10 and 20 μM doses significantly reduced COX-2 protein expression levels by 46%, 58%, and 67%, respectively, in contrast to LPS control group.

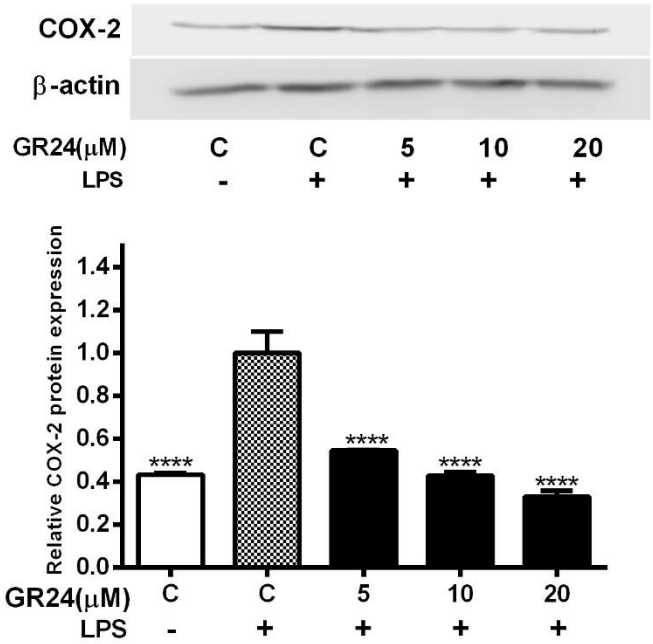

Figure 4.17. GR24 reduced COX-2 protein expression levels in SIM-A9 cells following LPS treatment. C: Control.

GR24 at 5, 10 and 20 μM doses were applied to LPS induced microglia cells for 12 h to detect TNF-α protein expression levels as represented in Figure 4.18. Accordingly, 10 and 20 μM doses significantly decreased TNF-α protein expression levels by 52% and 87%, respectively in contrast to LPS control group.

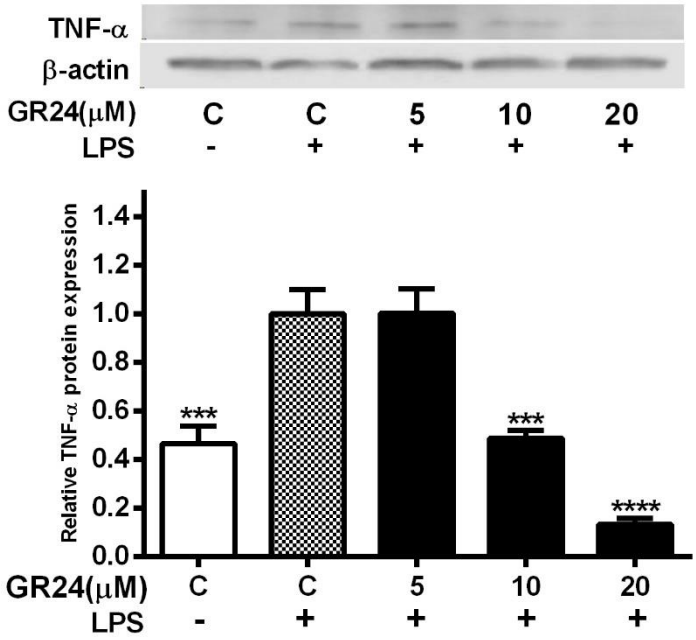

Figure 4.18. GR24 reduced TNF-α protein expression levels in SIM-A9 cells following LPS treatment. C: Control.

PPARγ plays crucial role in immune response due to its capability of inhibiting the expression of inflammatory cytokines. 5, 10 and 20 µM doses of GR24 and LPS (1 µg/mL) were used for the treatment of cells for 12 h. LPS treatment of SIM-A9 cells significantly suppressed PPARγ protein expression by 21%. This inhibitory effect was repressed dose-dependently by GR24 treatment and almost reversed to the control level at 20 µM dose via enhancing the expression by 32% (Figure 4.19).

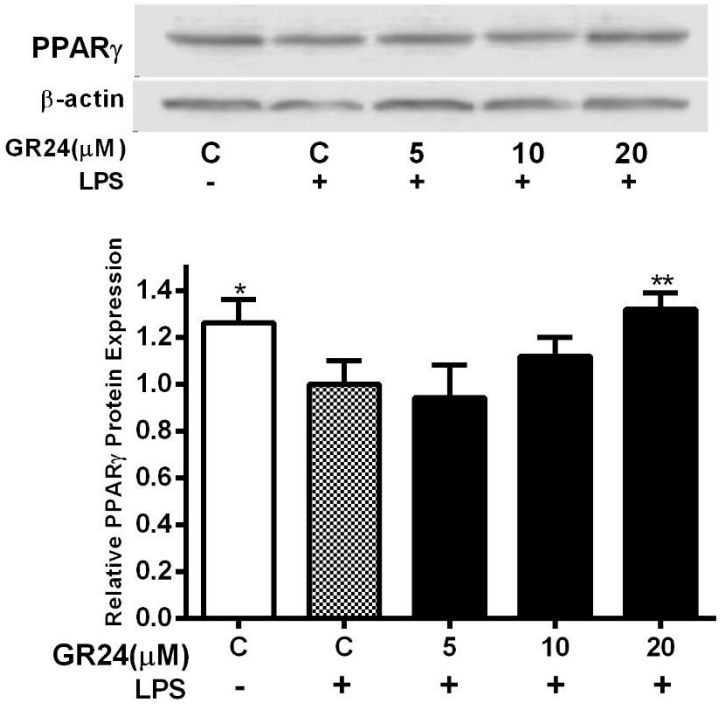

Figure 4.19. GR24 induced PPARγ protein expression levels in SIM-A9 cells following LPS treatment. C: Control.

AMPK activation alleviates the inflammatory response in microglia cells and it is recently identified as one of the potential target for terminating inflammation-related neurodegeneration. Under LPS-stimulated inflammatory conditions, 10 and 20 μM of GR24 enhanced the expression level of AMPK by 21 and 65%, respectively as represented in Figure 4.20.

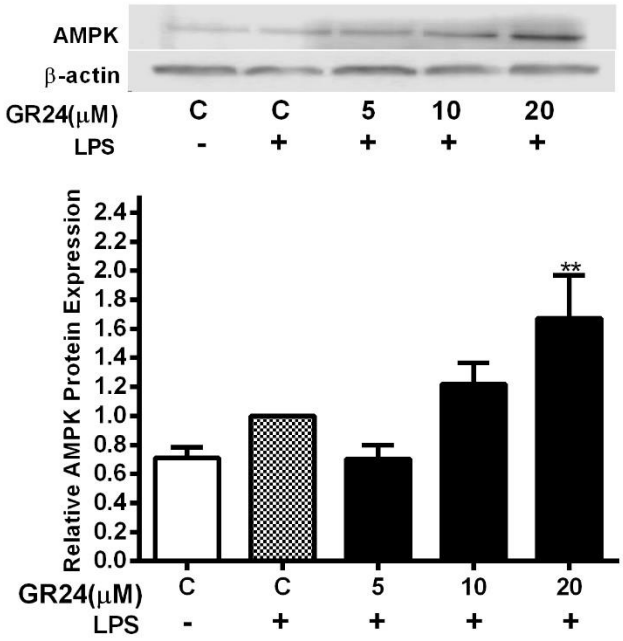

Figure 4.20. GR24 induced AMPK protein expression levels in SIM-A9 cells following LPS treatment. C: Control.

NF-kB, one of the key transcription factor of proinflammatory mediators and cytokines was investigated for its nuclear accretion in LPS induced microglia cells. 1 µg/mL of LPS and 20 µM of GR24 were used for 2, 4 and 6 h. The accumulation levels of NF-kB were detected at the maximum level for 4 h as compared to other incubation times in response to LPS. This effect is reduced 2.5 fold when treated by GR24 at 20 µM dose of GR24 (Figure 4.21). The protein bands obtained after Western blotting were analyzed densitometrically by Image Studio Digits software program. Lamin B is used for endogeneous control for NF-kB expression levels.

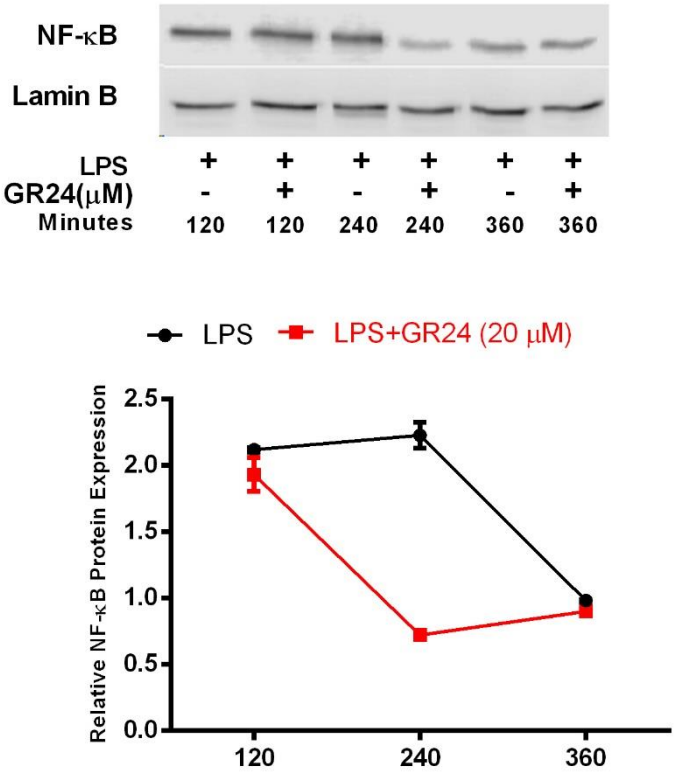

Figure 4.21. GR24 reduced nuclear acuumulation of NF-κB in SIM-A9 cells following LPS treatment. C: Control.

4.1.10.2. GR24 Induced the Cytoprotective Protein Expression Levels in SIM-A9 Cells Following LPS Treatment

The nuclear accumulation levels of Nrf2, the main transcription factor regulation antioxidant genes, were determined with in a time-related manner in LPS induced microglia cells. These cells were incubated with LPS and GR24 during 2, 4 and 6 h. The accumulation levels of Nrf2 were detected at the maximum level for 4 h in comparison with other groups (Figure 4.22).

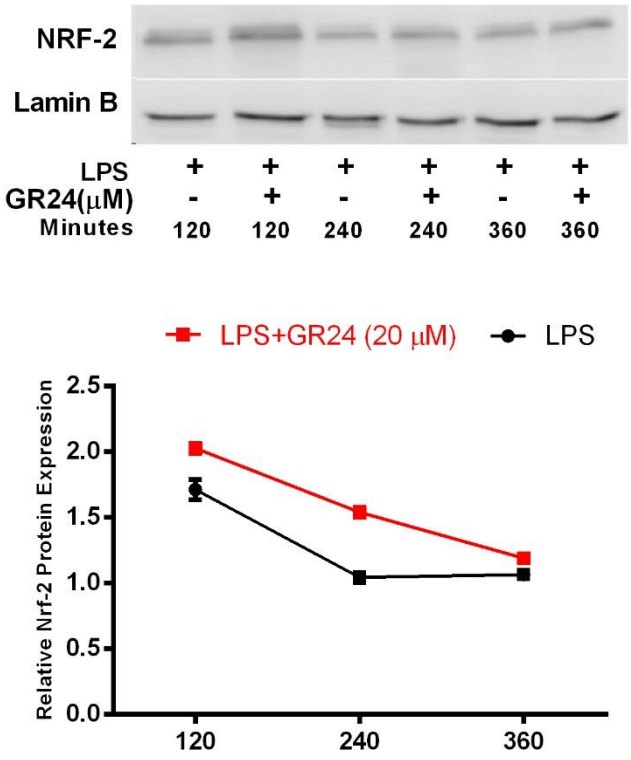

Figure 4.22. GR24 induced nuclear accumulation of Nrf2 in SIM-A9 microglia following LPS treatment. C: Control.

Microglia cells were incubated with GR24 (5, 10 and 20 µM) and LPS (1 µg/mL) to evaluate the nuclear levels of Nrf2 following the treatment of GR24 (Figure 4.23). GR24 at 5 µM dose was not increased the expression level of Nrf2 according to statistical analysis. However, compared to LPS induced control, at 10 and 20 µM doses were resulted in activation of the expression Nrf2 signaling 93% and 52%, relatively.

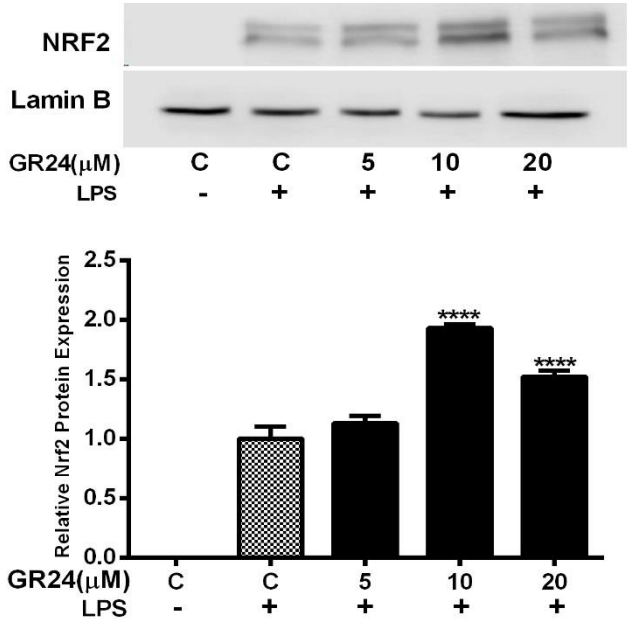

Figure 4.23. GR24 induced Nrf2 protein expression levels in SIM-A9 cells following LPS treatment. C: Control.

4.2.1. Cell Viability in Brain Endothelial Cells Following GR24 Treatment

Cells were incubated with LPS (1 µg/mL) and variable doses of GR24 (5, 10 and 20 µM). GR24 were given no toxic effects in LPS induced bEnd.3 cells, even GR24 promoted the amount of viable cell in a dose-related manner (Figure 4.24.).

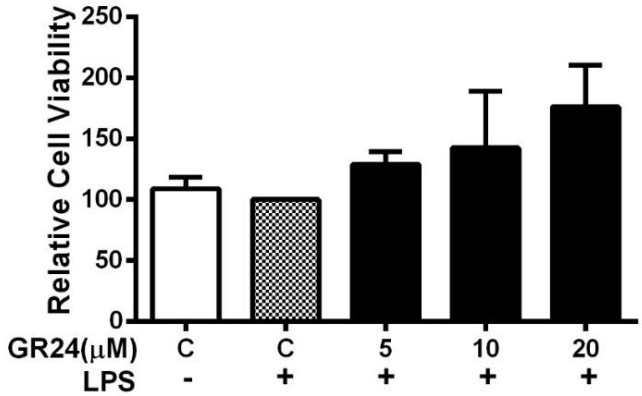

Figure 4.24. Effect of GR24 on cell viability in brain endothelial cell line. C: Control.

4.2.2. The Proinflammatory and Cytoprotective Gene Expression Levels in Brain Endothelial Cells Following LPS and GR24 Treatment

To find out the potent cytoprotective effects of GR24 on endothelial cells, GR24 was

applied in LPS induced bEnd.3 cells (1 µg/mL) for 5 and 10 µM doses at 24 h. After RNA extraction, cDNA synthesis and qPCR analysis, mRNA expression levels of proinflammatory (iNOS, COX-2, IL-1β, TNF-α) and cytoprotective genes (Nrf2, HO-1, and NQO1) were determined. Normalization of interested gene expression levels was organized to β-actin. Every experiments were analyzed by using GraphPad Prism 6 software program ($p<0.05$).

4.2.2.1. GR24 Reduced the Proinflammatory Gene Expression Levels in Brain Endothelial Cells Following LPS Treatment

The effects of GR24 on iNOS gene expression levels were determined with qPCR analysis. After treated with LPS and GR24 at 5 and 10 µM doses, cells were incubated for 24 h. GR24 did not represent a statistically significant difference at 5 µM. However at 10 µM dose, GR24 significantly suppressed the mRNA transcription levels of iNOS in LPS-induced bEnd.3 cells by 40% in comparison to LPS-induced control group (Figure 4.25.).

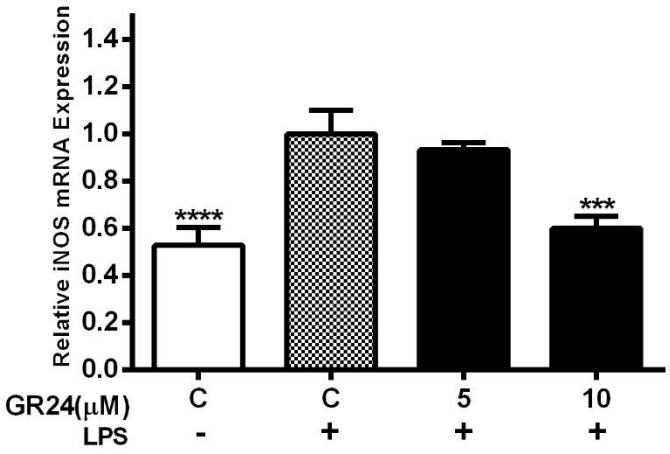

Figure 4.25. GR24 reduced iNOS mRNA expression levels in brain endothelial cells following LPS treatment. C: Control.

One of the proinflammatory genes, IL-1β was investigated in LPS induced brain endothelial cells. After treated with LPS and GR24 at 5 and 10 µM, cells were incubated for 24 h. Although 5 µM did not cause a meaningful change, GR24 at 10 µM dose resulted in highly potent downregulation in the gene expression of IL-1β by 96% (Figure 4.26).

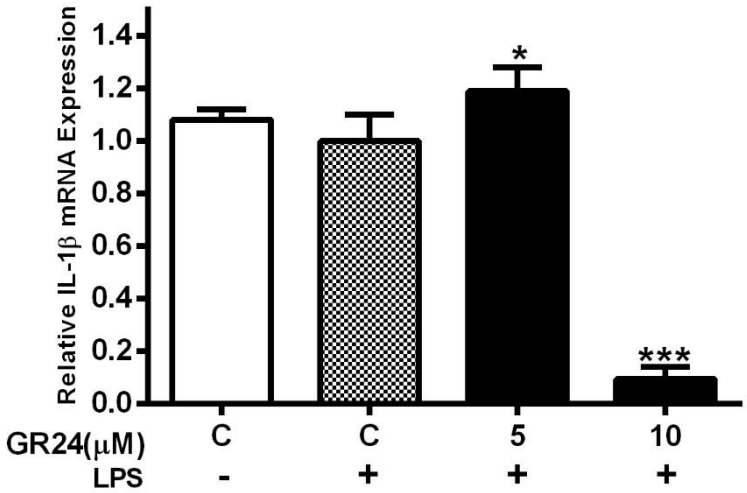

Figure 4.26. GR24 reduced IL-1β mRNA expression levels in brain endothelial cells following LPS treatment. C: Control.

As represented in Figure 4.27, the effects of GR24 were investigated for TNF-α mRNA expression levels. After treated with LPS and GR24 at 5 and 10 μM, these cells were incubated during 24 h. GR24 significantly reduced the TNF-α expression level by 49%.

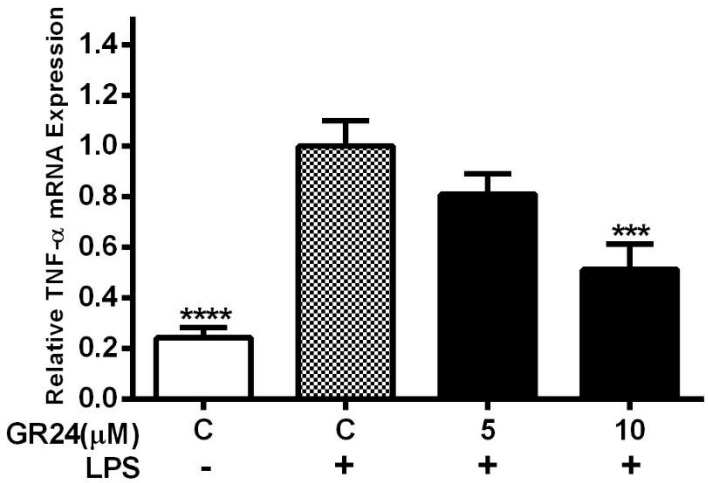

Figure 4.27. GR24 reduced TNF-α mRNA expression levels in brain endothelial cells following LPS treatment.. C: Control.

COX-2 produces prostaglandins which are related to the inflammatory process. After the LPS and GR24 treatments for 24 h, the results were showed that LPS treatment was slightly upregulated the mRNA transcription levels of COX-2 but not significantly. Similar

to this , GR24 at both 5 and 10 μM slightly upregulated the COX-2 transcription in LPS stimulated bEnd.3 cells. However, the levels did not significant (Figure 4.28.).

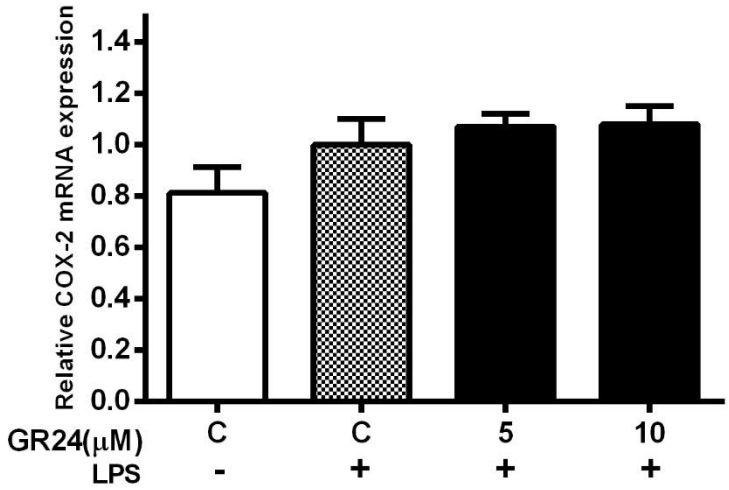

Figure 4.28. COX-2 mRNA expression levels in brain endothelial cells following LPS and GR24 treatment. C: Control.

4.2.2.2 GR24 Induced the Cytoprotective Gene Expression Levels in Brain Endothelial Cells Following LPS Treatment

The effects of GR24 on Nrf2 gene expression levels were investigated with qPCR analysis. After treated with LPS and GR24 at 5 and 10 μM, cells were incubated for 24 h. GR24 treatment did not show any effect on the mRNA expression level of Nrf2 gene in LPS induced bEnd.3 cells (Figure 4.29).

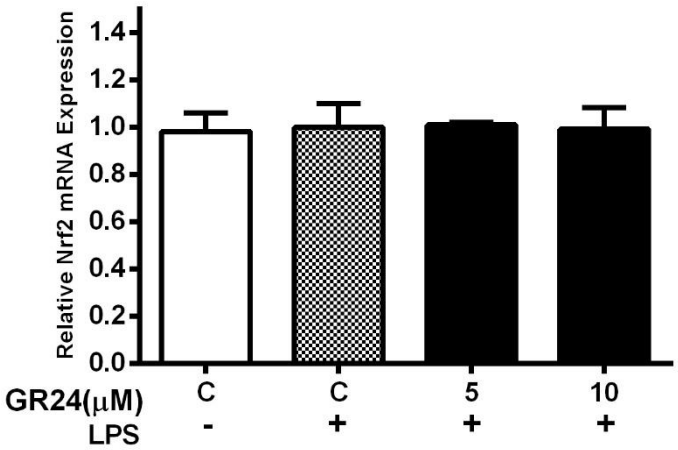

Figure 4.29. Nrf2 mRNA expression levels in brain endothelial cells following the LPS and

GR24 treatment. C: Control.

After 24 h incubation of LPS and GR24 (5 and 10 μM) treatments , the HO-1 mRNA expression were slightly increased. However, GR24 was not significantly effective in bEnd.3 cells for inducing HO-1 expression levels (Figure 4.30).

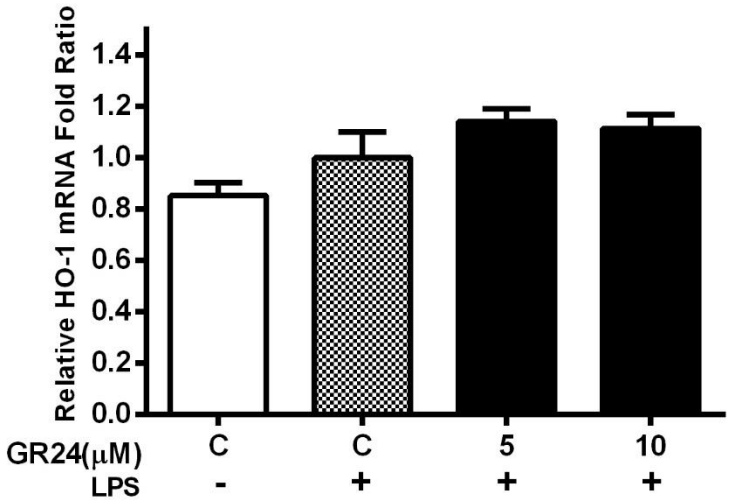

Figure 4.30. HO-1 mRNA expression in brain endothelial cells following LPS and GR24 treatment. C: Control.

As represented in Figure 4.31, another cytoprotective enzyme NQO1 was remarkably upregulated by GR24 treatment in LPS induced bEnd.3 cells for 24 h. The NQO1 mRNA expression level of GR24 groups at 5 and 10 μM doses were significantly upregulated in 1.8 and 3.2 fold, respectively.

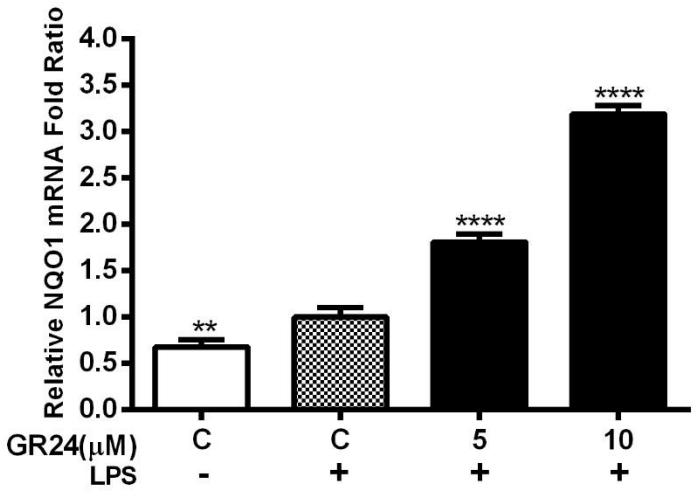

Figure 4.31. GR24 induced NQO1 mRNA expression levels in brain endothelial cells following LPS treatment. C: Control.

4.2.3. Permeability assay on Brain Endothelial Cells

To investigate the effect of GR24 on LPS stimulated endothelial cell permeabilization, Evans Blue dye was used for measuring the permeability of BBB to macromolecules (Evans, 1914). GR24 (5, 10 and 20 μM) and LPS (1 μg/mL) treatment on cells was carried out for 24 h. Following the incubation, EBA mixture was applied on the luminal side for 2 h. After 2 h, the assay buffer in the abluminal side was collected and each group was measured with i-control ™ microplate reader software (Tecan Infinite® M200 PRO) at 650 nm. Every experiments and analysis were repeated at least three times and analyzed by use of GraphPad Prism 6 software program ($p<0.05$).

Results showed that in LPS stimulated bEnd.3 cells permeability increased significantly (33%) compared to nontreated control group (Figure 4.32). After 5, 10 and 20 μM of GR24 treatments, the permeability of brain endothelium decreased in dose dependent manner. 10 and 20 μM of GR24 remarkably reduced EBA transfer through the abluminal side by 6% and 64%, respectively.

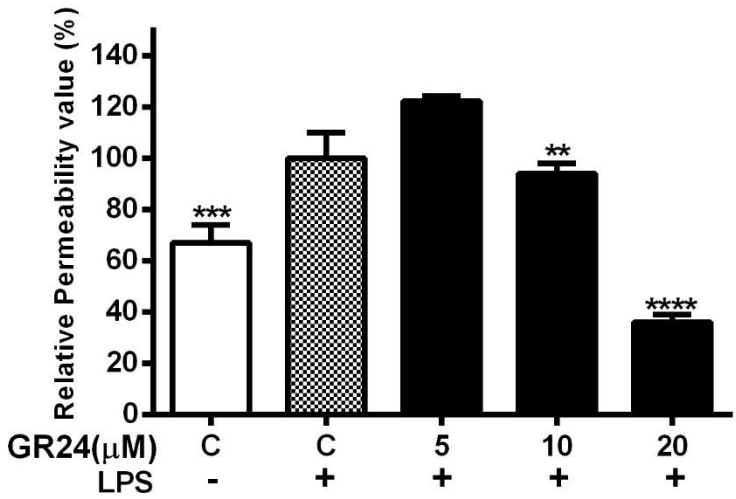

Figure 4.32. GR24 reduced the permeability in brain endothelial cells following LPS treatment. C: Control.

4.3. Discussion

Cognitive disorders are classified as progressive NVU losses in particular areas of CNS which result in serious motor, cognitive or behavioral deficits (Iuvone, 2009). Even neurodegenerative diseases have been categorized and identified diagnostically, the causes of neurodegeneration and its full mechanism are generally unknown. As an example, Huntington Disease has been well established more than two decades before, however how mutant huntingtin genes triggers the disorders are remain unclear. The recent reports emphasize the importance of neuroinflammation and related processes in the molecular etiology of neurodegenerative disorders.

Neuroinflammation can be triggered by several impulses, such as injury, chemical exposures, stress and pathogen invasions (LPS) (Carson, Cameron Thrash, & Walter, 2006). Systemic LPS is known as a potent trigger of cytokine release both periphery and central nervous system. Recently, it has become evident that peripheral inflammation following an injury or infection is initiated a site of a systemic response. Macrophages can directly interact with the site of inflammation and lead to the infiltration of proinflammatory cytokines into the blood. Through the cellular or humoral pathways, cytokines reach to the brain and interacted with the brain endothelium (Perry, Cunningham, & Holmes, 2007). Systemic inflammation has a wide range and many conditions in the periphery can cause inflammation. Recent studies have shown that gut microbiota can promote chronic low-grade inflammation through mechanisms involving increased exposure of the host to

harmful bacterial products and particularly to lipopolysaccharides (LPS) leaking from the intestinal barrier to blood circulation (Manco, Putignani, & Bottazzo, 2010). The increased level of cytokines in the bloodstream can promote an inflammatory environment in the CNS (Figure 4.33.) (Holmes, 2013).

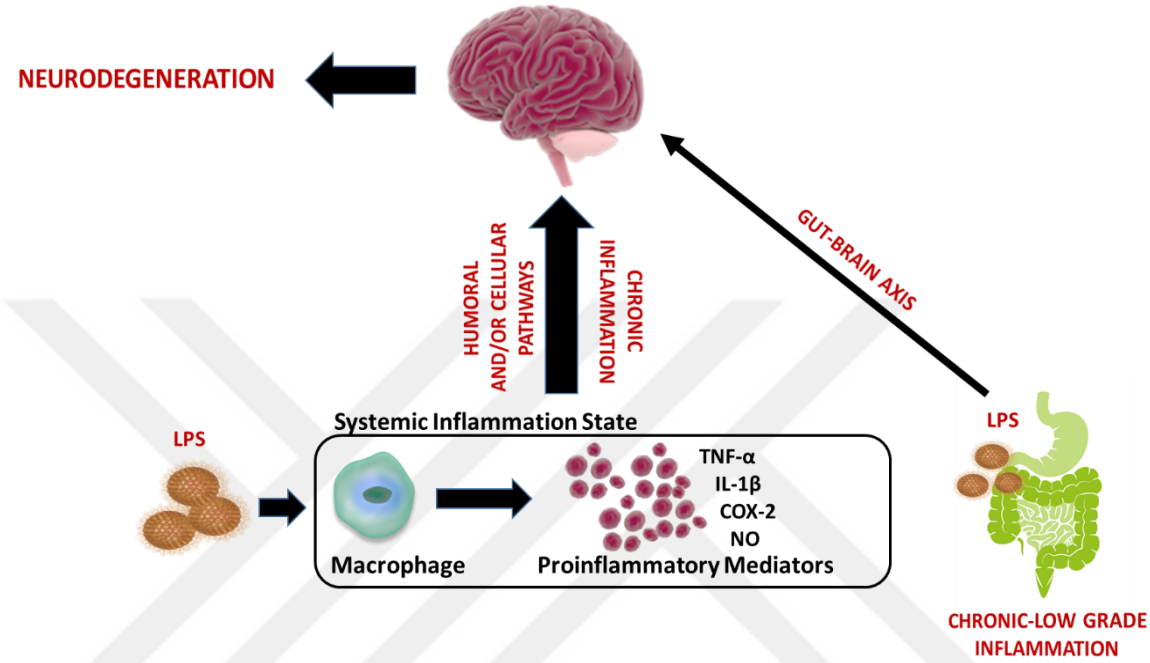

Figure 4.33. Development of neurodegeneration from chronic low grade inflammation in peripheral

Emerging evidences reveal that peripheral LPS stimulations could have an enormous outcome to the CNS. Qin et al. (2007) demonstrated that LPS administration from periphery induced some of the NVU units, such as astrocytes and microglia activation, resulting in the induction of some of the proinflammatory mediators, COX-2 and iNOS in the brain. Recent transcriptome analysis showed that the hippocampus and hypothalamus regions have the most abundant microglial populations in the brain (Grabert., 2016). Another study supported this transcriptome data, the peripheral administration of LPS (10 µg/kg) induced IL-1β, IL-6 and TNF-α secretion in the spleen as well as in hippocampus and hypothalamus of mice (Layé, Parnet, Goujon, & Dantzer, 1994). It has been known that the proinflammatory cytokine levels in the blood of the AD patients are considerably higher than healthy individuals. Recent studies revealed that a major acute inflammation during adulthood such as pneumonia increased the risk of dementia or reduced brain volumes in gray matter which are the most prominent symptoms of AD, in the late adulthood (Holmes, 2013).

As seen in the literature, one of the main target of the peripheral inflammation in the CNS is microglial cells. However, NVU is a compact unit and every cell types within this interacting population has great potential to affect each other. Before reaching to microglia, the components of systemic inflamation are first detected by BBB. BBB and the peripheral immune system show a bidirectional relation. Cytokines from the periphery can penetrate the BBB and reach the brain via humoral, neural and cellular pathways. When the stimulation reaches the brain, these inputs strongly activate microglia to alter their morphology and functioning and they further secrete excessive levels of proinflammatory cytokines and mediators (Buttini et al., 1996). Through the interaction between the cells of the brain-immune interface and cytokine signaling, astrocytes are also activated to secrete inflammatory cytokines particularly, TNF-α and interleukins such as IL-1 and IL-6 (Lee et al., 1993) which interfere with the integral role of these cells in neural and synaptic functioning. Finally, overproduction of high levels of inflammatory mediators disrupt the delicate balance and produce detrimental effects on neural precursor cells, neuronal functioning, and neurogenesis (Figure 4.34.). Alteration of the systemic immune response can affect brain immunity and homeostasis. For instance, vascular inflammation may be caused by Aβ accumulation in brain blood vessels. As a result, BBB became leaky due to the damage of blood vessels (Kinnecom et al., 2007).

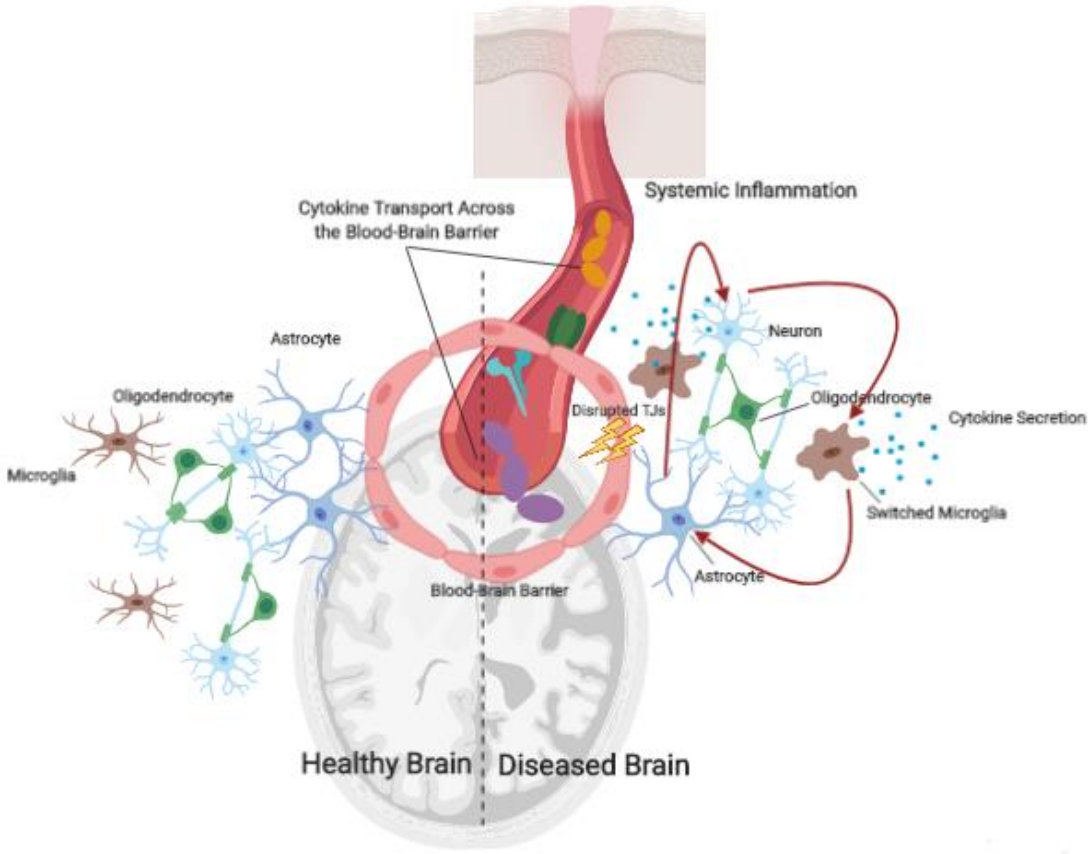

Figure 4.34. Systemic Inflammation and its Effects on NVU in the CNS

Although the mechanisms of neuroinflammation have been well studied, there is still no permanent treatment for neurodegenerative disorders. During the last few decades, researches have reformed in the field of neurodegeneration. Enlightening the mechanism of genetics and neuronal pathogenesis has contributed greatly and created a wealth of information and became the basis of new technologies and multiple therapeutic targets for these neurodegenerative diseases. From the 19th century to present, synthetic drugs are used for disease management. Yet, many serious side effects of synthetic drugs have been reported. Today, natural compounds have gained attention as a potential therapeutic agents due to the increasing need for safe with few side effects and efficient treatment strategies in the treatment of CNS diseases (Rasool et al., 2014, Cooper & Ma, 2017). A large number of studies admitted that phytochemicals are found to be involved in many crucial mechanisms for the homeostasis of healthy CNS (Starkov & Beal, 2008). For this reason, natural compounds are thought to have the potential to treat and/or prevent neurodegenerative diseases.

In this study, apigenin, luteolin, caffeic acid, rosmarinic acid, caffeoyl-rosmarinic acid,

and a representative strigolactone analog, GR24 has been selected as candidate phytochemicals to investigate the potenital anti-neuroinflammatory and cytoprotective effects in LPS induced microglial and brain endothelial cells. In the first stage of the study, the effects of the test compounds on NO increase in LPS-induced SIM-A9 cells were observed and as a result of the screening studies, GR24 was determined as the most effective compound to suppress increased NO production under inflammatory conditions. Our previous studies showed that the GR24 held a great potential against systemic inflammation in LPS stimulated RAW 264.7 cells (Tumer et al., 2018). GR24 remarkably suppressed NO production by iNOS down-regulation, as well as by decreasing nuclear accumulation of NF-κB. GR24 also increased cytoprotective enzymes by destroying Nrf2-Keap1 interaction and promoted the nuclear accumulation of Nrf2 in systemic macrophage cells. Through this pathway, HO-1 and NQO1, the phase II detoxifying enzymes were also upregulated. These results clearly showed that GR24 is a potential proinflammatory and cytoprotective compound againts peripheral inflammation. Based on the protective effects of GR24 against systemic inflammation, we hypothesized that GR24 may be protective against neuroinflammation in the CNS.

SIM-A9 cells described in 2014 as the first spontaneously immortalized microglia, isolated from murine cerebral cortices (Nagamoto-Combs et al., 2014). Their responses to inflammatory stimulus are similar to primary microglia when they are treated with LPS and Aβ. Therefore, these cells are used as an alternative tool for primary microglia culture. Alhadidi and Shah. (2018) showed that the LPS-induced SIM-A9 cells released a large amount of NO. In a siRNA study in SIM-A9 cells, coifilin is a protein closely related to pathways responsible for the synthesis of reactive oxygen species was silenced. The study revealed that after the coifilin silencing, iNOS, COX-2 and the NO level in the environment and activation of cytokines such as TNF-α, IL-1β was significantly suppressed (Alhadidi & Shah, 2018). Another study (Farrell, Borazjani, Damaser, & Kothapalli, 2016), showed that the SIM-A9 cells co-cultured with neuronal nerve cells released 1.7 times more TNF-α after LPS administration than the control group. All these recent studies show that LPS-induced SIM-A9 cells can be effectively used as a model in neuroinflammation studies.

iNOS-derived NO is one of the major sources of toxic free radicals in the brain. Abnormal NO production leads to the inflammatory process and due to its free radical properties, its overproduction has been found to be associated with a variable of neurodegenerative disorders (Tse, 2017). In an in vitro study, it was reported that LPS increased the production of NO in microglia cells via inducible iNOS and caused death in

co-cultured neuronal cells within 48 h (Bal-Price and Brown, 2001). Due to the important role of NO in neurotoxicity, inhibition of NO could be a possible route of therapeutic intervention in neuropathogenesis. The effect of GR24 on NO synthesis was determined in LPS stimulated SIM-A9 microglia cells. GR24 was applied to SIM-A9 microglia cells at different doses ranging from 5 to 50 μM. As a result of these studies, the IC50 value for GR24 on NO inhibition was calculated as 6.157 μM. These results showed that the low doses of GR24 remarkably suppressed NO levels in LPS induced microglia cells. Also, results showed that GR24 could be more effective for supressing LPS-induced NO levels in inflammed microglial cells than some of the natural compounds which have been noted for their remarkable roles in the amelioration of inflammation according to the literature. For instance, phytosterol-derived saponins significantly inhibited NO production at 40 μM of dose in LPS stimulated RAW 264.7 macrophage cells (Huang et al., 2014). Another study revealed that syringaresinol and pinoresinol, polyphenolic compounds, suppressed NO production in RAW 264.7 macrophage cells with IC50 value of 8.30 and 7.89 μM and in BV-2 microglia cells with IC50 value of 13.48 and 26.19 μM, respectively (Li et al., 2013).

Neuroinflammation-related neuronal cell death is often caused by increasing long-term ROS and nitric oxide synthase (NOS). This process plays a major role in the emergence of apoptotic cell death due to irreversible oxidative or nitrosative damage in the CNS (Yuste, 2015). iNOS-derived NO is one of the major sources of toxic free radicals in the brain. The expression of the iNOS enzyme can be regulated at transcriptional, post-transcriptional and translational levels (Galea & Feinstein, 1999). In this study, the iNOS enzyme is regulated at both transcriptional and translational levels. In LPS induced microglia cells, GR24 significantly decreased the expression levels of iNOS transcript 87%, 77% and 79% at 5, 10 and 20 μM doses, respectively as compared to LPS-induced control group. On the other hand, 1400W, a selective iNOS inhibitor, at 10 μM downregulated iNOS mRNA expression only 37%. Therefore, the results showed that GR24 was an effective iNOS suppressor. For iNOS protein expression levels, results showed that 5 μM of GR24 did not show a significant difference . On the other hand, compared to LPS induced control group, the protein expression levels of iNOS were suppressed by 31% and 32% at 10 and 20 μM, relatively.

Some of the flavonoids, which are known to effective against LPS induced inflammation in BV2 microglia cells, showed their anti-inflammatory effects at higher doses compared to GR24. Treatment of BV2 microglia cells with orientin showed a significant downregulation in iNOS mRNA expression levels at 50 μM by 31% only (Zhou et al., 2014).

Nobiletin, known as a protective agent againts neuronal apoptosis (Qu et al., 2018) and also neuroprotective flovanoid (Marsh, Das, Ridell, & Smid, 2017) inhibited iNOS gene expression levels by 71.2% at 50 µM. Therefore, as compared to some other phytochemicals which are noted for their considerable neuroprotective and antineuroinflammatory effects, GR24 exerts its desirable biological activities at relativeley lower concentrations.

iNOS enzyme shares a common pathway with another proinflammatory mediator enzyme COX-2. COX-2 is a key enzyme of the activation of inflammatory process including LPS, IL-1, TNF-α, and arachidonic acid (Yucel-Lindberg et al., 1999). The overexpression of COX-2 results in accumulation of PGE2. The target molecules found on the downstream of PGE2 regulate several signaling pathways. PGE2 contributes to various physiological processes (Satoh et al., 2013). iNOS and COX-2 are involved in the tumorogenesis via several molecular mechanisms including loss of apoptosis, uncontrolled cell proliferation and cancer-leading angiogenesis (Gandhi et al., 2017). NO production from activated iNOS enzyme promotes PGE2 production through COX-2 in both cancer and inflammation. (Figure 4.35.) Gallo et al. (2003) revealed that iNOS inhibitors could be used for the suppression of COX-2 enzyme activation in carcinogenic cells. In this study, GR24 significantly and dose dependently decreased the COX-2 mRNA expression levels by 58, 72 and 78% at 5, 10 and 20 µM doses, respectively. GR24 showed similar reduction pattern in both COX-2 and iNOS mRNA expression levels. The same doses of GR24 showed a similar effect of COX-2 protein expression levels; at 5, 10 and 20 µM doses, the protein expression of COX-2 was decreased by 46%, 58%, and 67%.

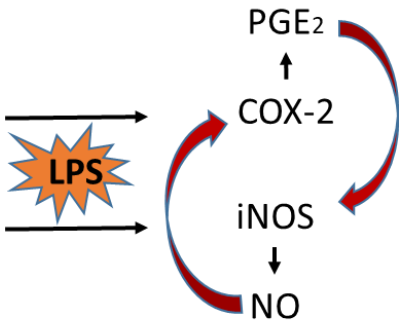

Figure 4.35. The crosstalk between iNOS and COX-2 under the stimulation of LPS

COX-2 derived PGE2 is not only linked with iNOS enzyme but also related with other cytokines. Zasłona et al. (2017) revealed that the LPS-induced PGE2 increased the mRNA transcription levels of IL-1β in macrophages. In vitro studies demonstrated the ability of

murine astrocytes to release prostaglandins and express mRNA COX-2 following treatment with IL-1β (Molina-Holgado et al., 2000). Our previous study showed that GR24 decreased IL-1β cytokine secretion level significantly only at 50 μM dose by 50%, and mRNA expression levels decreased at 10, 20 and 50 μM doses by 28, 30, 70%, relatively in RAW 264.7 cells (Tumer et al., 2018). In SIM-A9 cells, our results showed that GR24 at low doses strongly reduced IL-1β secretion level compared to LPS induced control group by 30% and 40%, respectively. IL-1β mRNA expression levels showed a similar pattern, GR24 at 5,10 and 20 μM doses remarkably decreased the IL-1β mRNA expression levels at dose dependent manner by 40%, 86% and 99%.

In normal conditions, TNF-α levels are sustained in a very low amount both in peripheral and the CNS for maintaining brain development, neurogenesis, and synaptogenesis by activated astrocytes (Di Benedetto, Müller, Wenger, Düzel, & Pawelec, 2017). However, in AD patients, TNF-α levels are significantly elevated in blood and the CNS (Chang, Yee, & Sumbria, 2017). Recent studies have showed that the TNF-α inhibitors (TNFIs) could be a therapeutic approach against AD. Sulforaphane (SFN) is known for its antiinflammatory, antioxidant and cytoprotective activity and it is widely used as a dietary supplement either in powder or capsule form. Eren et al. (2018) showed that SFN decreased secretion levels of TNF-α in LPS (100 ng/mL) induced N9 murine microglia cells approximately by 60% at 10 μM dose. In this study, GR24 decreased TNF-α cytokine secretion levels at low doses. For protein and gene expression level of TNF-α, GR24 treatment also resulted in dramatic reductions. 5, 10 and 20 μM doses of GR24 significantly decreased mRNA expression level by 32%, 67%, and 57%, respectively. Also, 10 and 20 μM doses of GR24 strongly suppressed protein expression level 52% and 87%, respectively as compared to the LPS induced control group.

Briefly, GR24 remarkably decreased both the release and the expressions of proinflammatory mediators that have vital roles in systemic- and neuro-inflammation. Among this mediators, TNF-α has a crucial role in the degenerative processes of both systemic and neuro-inflammation. It expresses its effect by binding to TNF-α receptor superfamily (TNFR) (Idriss & Naismith, 2000). When TNF-α binds to its receptors under the inflammatory conditions, one of the important molecular complex (NF- κB) involved in the control of a large number of molecular pathway responds to this call. NF-κB is a DNA binding complex and a regulator of many different signaling pathways linking to inflammation and it is abundantly expressed in glial cells and cerebral blood vessels (Wan & Lenardo, 2010). NF-κB responds to different stimuli including ligands of various

receptors (cytokine receptors, PPRs, TNFR members), inflammatory transcription factors, chemokines, and adhesion molecules. As a central regulator of inflammation, NF-κB has been known and studied widely and well established for its role of inflammation. For instance, smooth muscle cells treated with IL-1β can increase COX-2 mRNA expression Yang et al., 2002). Another study showed that LPS induction also can increase COX-2 mRNA expression bEnd.3 cells (Shih, 2010). These proteins are mediated and synthesized via NF-κB-dependent signaling mechanisms. In the inactive form, NF-κB appears in two dimers, p50and p60 which is bound with its inhibitors (IκBs), IκBα and IκBβ. These inhibitors hide nuclear location signals (NLS) of NF-κB. Upon activation, IκB kinase complex phosphorylate IκB and this result in the proteasomal degredation of IκB by ubiquitination. Finally, the NLSs are uncovered and these two dimers are translocated from cytoplasm into the nucleus and binds their promoter regions. Finally, this process triggers the transcription of responsive genes (Mikenberg et al., 2007). Today, the relation between inflammatory process and NF-κB activation has been well known and this pathway considered as an attractive approach for therapeutic drug development studies. Today, a large number of NF-κB blockers are widely used. Aspirin and salicylate are used as an inhibitor of IKK to prevent the phosphorylation of IkBα (Yin, Yamamoto, & Gaynor, 1998). Some drugs such as PPAR agonists and glucocorticoids inhibit the DNA binding activity of NF-κB. Generally, these drugs are used as a blocker of nuclear accumulation and transcription of NF-κB.

In this study, the nuclear accumulation levels of NF-κB were investigated in a time-dependent manner. Results were showed that the nuclear accumulation of NF-κB increased to its maximum level after 4 h incubation at which 20 μM GR24 treatment significantly and dramatically (about three folds) decreased the nuclear accumulation of NF-κB.

As mentioned before, NF-κB has a broad range of signaling crosstalks with many different molecules including cytoprotective signaling pathways. Nrf2 is the main transcription factor regulating the expression of cytoprotective enzymes (Wakabayashi, Slocum, Skoko, Shin, & Kensler, 2010). Antioxidant target molecules including HO-1 and NQO1 block the activation of the classical pro-inflammatory transcription factor NF-κB. HO-1 leads to the production of biliverdin, CO, and free iron by breaking down heme. Especially bilirubin and free iron modulate TNF-α dependent NF-κB activation through inhibiting RelA phosphorylation (Soares et al., 2014). The action of CO and free iron are able to inhibit the iNOS and prevent NO production by scavenger molecule biliverdin (Figure 4.36). In THP-1 cells, the upregulation of NQO1 decreases LPS-stimulated

expression of the TNF-a and IL-1 (Rushworth, MacEwan, & O'Connell, 2014).

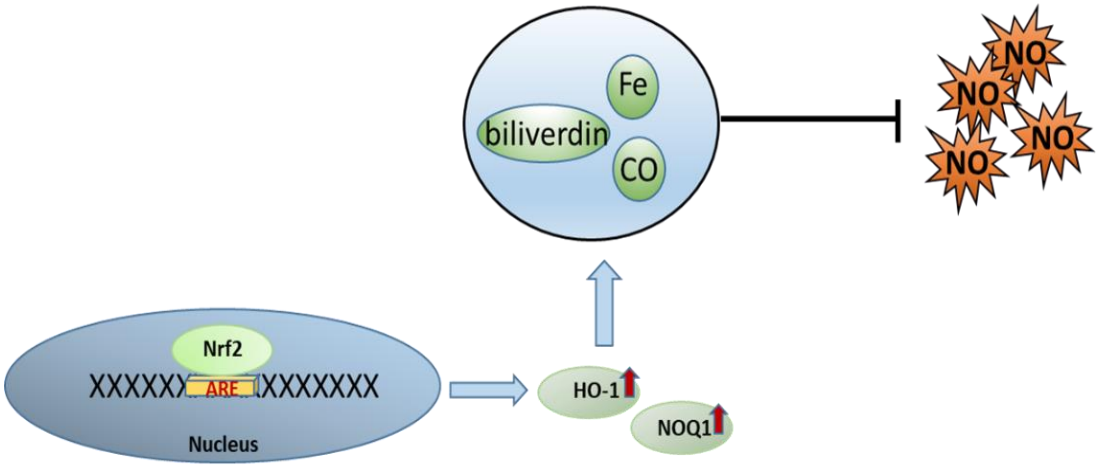

Figure 4.36. Interactions between Nrf2-dependent enzymes and nitric oxide

In the present study, Nrf2 and its downstream enzymes HO-1 and NQO1 were investigated in LPS stimulated microglia following GR24 treatment. Results showed that expressed mRNA levels of Nrf2 in control group with LPS treatment were decreased by 1.67 fold. GR24 treatments at 5 and 10 µM did not result in significant change, however, suppressive effect of LPS on Nrf2 gene expression was repressed at 20 µM dose and reversed 2.02 fold. For cytoprotective enzymes, GR24 treatment gave rise to a remarkable and dose-dependent increase in the NQO1 expression which was 4.5, 9.5 and 13 fold for 5, 10 and 20 µM of GR24, respectively, in LPS treated SIM-A9 cells. Although the increase was not as high as NQO1 gene, the transcriptional expression of HO-1 was also upregulated by GR24 treatment 1.5 and 2.4 fold at 10 and 20 µM.

In normal conditions, Nrf2 is a short-lived molecule and remained inactive form in the cytoplasm by binding with Keap1 (Dinkova-Kostova, Kostov, & Kazantsev, 2018). During inflammatory stimulus, Nrf2 is dissociated from Keap1, then translocated to the nucleus where it binds to the ARE (antioxidant response elements) and activates the transcription of some antioxidant and detoxifying enyzmes. When Nrf2 is activated in the nucleus, it stimulates molecules that responsible for the destruction of free radicals and thus exhibit antioxidant action. Increased expression of Nrf-2 also inhibits the release of NF-κB-dependent free radicals to the cytoplasm. (Figure 4.37).

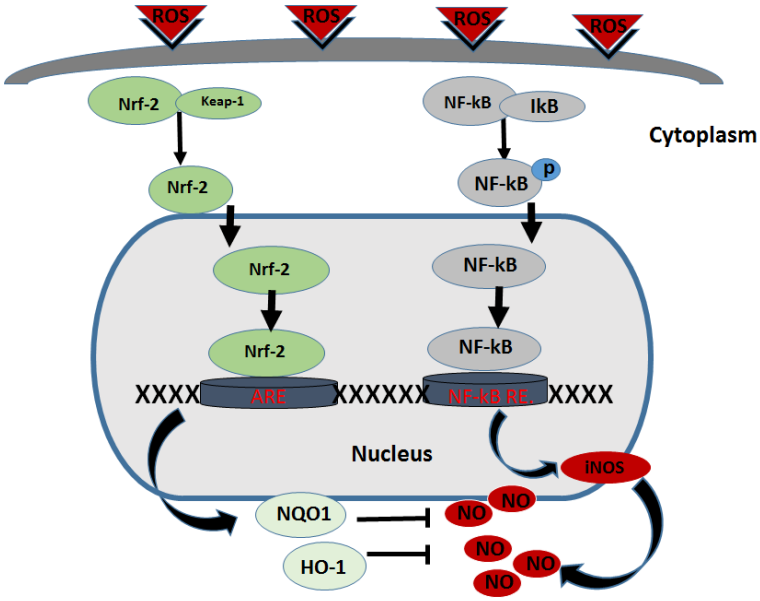

Figure 4.37. The crosstalk between NF-kB and Nrf2 transcription factors during inflammation

Nrf2 activation in pharmalogical aspect especially by natural compounds such as carnasoic acids, sulforaphane holds promise for combating oxidative stress-related cognitive disorders in Alzheimer's, Parkinson's and Huntington's diseases (Dinkova-Kostova, Kostov, & Kazantsev, 2018). Recent evidence reveals that the transcription of proinflammatory mediators including TNF-α and IL-1β are suppressed following Nrf2 activation both in peripheral and CNS cells. Towsend et al. (2016) showed that sulforaphane upregulated the nuclear translocation of Nrf2 and upregulated Nrf2 target genes in primary mouse microglia (Townsend & Johnson, 2016). Several human studies revealed that compounds which are used for the prevention of early stages of mild cognitive impairment are tightly related with the Nrf2 and its antioxidant effects on microglia.

In present study, we investigated the possible effect of GR24 in the nuclear accumulation levels of Nrf2 under the inflammatory conditions. Our study indicated that GR24 for 2, 4 and 6 h of incubation period significantly increased Nrf2 accumulation from the cytoplasm to the nucleus especially for 4 h by 1.53 fold. We know that Nrf2 is short-lived protein and we concluded that 4 h of LPS stimulation gave the maximum response of Nrf2, as well as GR24. According to our findings, GR24 at 5, 10 and 20 µM doses were tested for protein expression levels of Nrf2 for 4 h in SIM-A9 cells. Results showed that 10 and 20 µM of GR24 significantly increased Nrf2 protein expression levels by 93% and 52%,

respectively, as compared to the LPS induced control group.

Although the mechanisms still remain unclear, the cellular energy metabolism is greatly involved in the prevention of inflammatory process (Z. Yang, Kahn, Shi, & Xue, 2010). As a sensor of the cellular energy status of an organism, AMPK is an evolutionarily conserved protein and its role in chronic inflammatory disorders has been well established.. AMPK dysregulation may participate in neurodegenerative diseases, such as MS, AD, PD, ALS, Huntington's disease and neuropathies (Peixoto et al., 2017). AMPK and its role on NF-κB-related molecular oxidative stress revealed that this molecule may have a common mechanism with Nrf2. Mo et al. (2014) identified AMPK-Nrf2-NF-κB crosstalk after berberine treatment in LPS stimulated macrophage cells and LPS-shocked mice models. Berberine suppressed the NF-κB activation, in the same time upregulated Nrf2 translocation to the nucleus and elevated the expression of Nrf2-related enzymes (Mo et al., 2014). However, when AMPK is silenced in these cells, berberine had no effect on cytoprotective enzyme activation and Nrf2 nuclear translocation. In the current study, we investigated the possible involvement of AMPK to the anti-neuroinflammatory action mechanism of GR24 in microglial cells under the LPS induced inflammatory conditions. Results indicated that GR24 at 10 and 20 μM doses, AMPK was upregulated by 21% and 65%, respectively as compared to LPS induced control group. Further studies are needed to elucidate the AMPK mechanism after GR24 application and its interaction with other molecules involved in inflammation, for instance, Nrf2 in the CNS. Understanding the mechanisms of target molecules and the role of NVU should be helpful to develop effective therapeutic approaches against neuroinflammation.

Another signaling molecule which has been recently identified as an important molecule in inflammatory processes is PPARγ. Researches have recently focused on potential anti-inflammatory effects of PPARγ in both peripheral and in CNS inflammation due to its regulatory role in glucose and lipid metabolism (Kapadia, Yi, & Vemuganti, 2008). In normal conditions, PPARγ expression levels remain at a very low level in peripheral. Moreno et al. (2004) showed that the expression level of PPARγ was found in a certain level in some brain regions (Moreno, Farioli-vecchioli, & Cerù, 2004). PPARγ has been observed also in the NVU and the expressed in microglia and astrocytes

In this study, results supported to the literature; the control group without LPS treatment expressed PPARγ at much higher levels than the LPS induced control group in microglia cells. LPS treatment of SIM-A9 cells significantly suppressed PPARγ protein expression 21% as compared to the non-treated control. According to the results, this

inhibitory effect of LPS was repressed dose dependently by GR24 treatment and upregulated by 32% at 20 µM dose as compared to LPS induced control group.

PPARγ agonists such as ciglitazone and troglitazone abolished LPS-induced NO/PGE release by suppressing T-cell activation and prevented LPS-induced neuronal death. In MS, T-cell is activated in the presence of cytokine secretion. In vivo studies supported that the PPARγ agonists suppressed T-cell activation via decreasing proinflammatory mediators, microglial and astrocyte activation and improved homeostasis in the CNS (Steinman, Martin, Bernard, Conlon, & Oksenberg, 2002). Synthetic agonists of PPARγ has been approved by the FDA against type-II diabetes and diabetes-related stroke. PPARγ agonists were found to protect against stroke, and this beneficial result was associated with endothelial relaxation and decreased oxidative stress (Bordet et al., 2006). In this aspect, the promoting effect of GR24 on PPARγ seem to be beneficial.

bEnd.3 cells are used effectively in drug development studies by the screening of active compounds against BBB injury and neuroinflammation in preclinical in vitro studies. A limited number of research have been conducted with LPS-activated brain endothelial cells. In a recent study, Astragaloside IV (ASIV) isolated from Astragalus membranaceus in LPS-stimulated bEnd.3 brain endothelial cells induced the expression levels of TJs proteins and upregulated the integrity of BBB (Li et al., 2018). In addition, ASIV showed an antioxidant effect due to suppressing ROS production and increased the transcription activity of Nrf2 and its downstream enzymes (Li et al., 2018). A considerable number of researches point to the Nrf2 signaling has a neurovascular protective role to BBB. According to the literature, activation of the Nrf2-ARE is a potential mechanism to prevent the loss of BBB integrity and BBB-related disorders. Alfieri et al. (2013) suggested that sulforaphane induced Nrf2 and HO-1 against ROS-induced ischemic stroke in the blood-brain barrier in mice. Although no specific effect of GR24 on the mRNA expression of cytoprotective genes Nrf2 and HO-1 was observed, NQO1showed a remarkable increase 1.8 and 3.2 fold at 5 and 10 µM doses of GR24 respectively as compared to the LPS induced control group. According to the results, we assumed that GR24 may upregulate NQO1 cytoprotective enzyme by a different signaling pathway than Nrf2 in brain endothelial cells. For instance, Wang et al. (2013) revealed that aryl-hydrocarbon receptor (AhR) pathway is directly related with NQO1 activation in a nuclear level in mouse hepa1c1c7 liver hepatoma. An AhR agonist, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) linked with AhR and activated AhR translocation into the nucleus. Dimerized AhR bound to NQO1 enhancer region and as a result, NQO1 synthesis occurred. However, this assumption might be addressed in future

studies.

LPS has been shown to aggregate inflammatory mediators such as ROS overproduction in HUVEC cells. A number of studies claimed that ROS alter TJs and participate in BBB disruption which lead BBB permeability. Excess generation of reactive oxygen/nitrogen species and inflammatory cytokines are two main mechanisms of BBB disruption and increased permeability. Van Horssen et al. (2008) concluded that a large amount of ROS in CNS and leakage from BBB was observed in encephalomyelitis (EAE) brain (van Horssen et al., 2008). In this study, we investigated the expression levels of proinflammatory mediators in LPS induced brain endothelial cells. GR24 did not show significant statistical change at 5 µM for iNOS gene expression level, However, at 10 µM, 40% decrease of iNOS expression has been observed. For IL-1β and TNF-α, 10 µM dose of GR24 showed a significant decrease in expression levels by 96% and 49%.

Vascular leakage is a main trait in several diseases including hypoxia, septic shock, viral hemorrhagic fever and metastasis of cancer cells. Performing endothelial cell permeability assay will allow an insight into the development of these disorders. Li et al. (2018) revealed that ASIV decreased the permeability of BBB according to the TEER analysis. Also, ASIV upregulated the TJ proteins and it could protect BBB from CNS inflammation in vitro and in vivo. In the current work, LPS induced control group showed a significant increase of permeability (33%) compared to tcontrol group without LPS indicating that LPS significantly induced the BBB permeability in bEnd.3 cells,. At 10, 20 and 50 µM doses, GR24 showed notable reduction of LPS-induced permeability by 6%, 64% and 60% respectively, as compared to the LPS-induced control group. GR24 may be a potential lead compound targeting brain endothelial cells. However, in future work, investigating the possible therapeutic effects of GR24 on LPS disrupted TJ proteins might be important in the scope of BBB targeted drug design studies.

.

CHAPTER 5
CONCLUSION

Neuroinflammation is a complex process in which proinflammatory mediators play a crutial role during the progress of various neurodegenerative disorders. Currently, suppressing the formation of proinflammatory factors/mediators and activating the molecular pathways of cytoprotective mechanism has been becoming popular approach to fight against the formation of neuroinflammation. In order to achieve this, phytochemicals with high antiinflammatory and antioxidant potential have been extensively investigated and this approach sheds light on in vivo and clinical studies.

Our previous in vitro and in silico studies revealed that GR24 was an effective compound against systemic inflammation especially as a Nrf2 activator in LPS induced RAW 264.7 macrophage cells (Tumer et al., 2018). In this study, GR24 showed a remarkable results in both proinflammatory and cytoprotective perspective in SIM-A9 brain microglial and bEnd.3 brain endothelial cells, which are the main members of the NVU. GR24 suppressed the proinflammatory mediators, and increased the expressions of cytoprotective molecules in the NVU cells at low doses compared to the compounds known as antiinflammatory subtances which are commonly used in in vitro and in vivo studies worldwide according to the literature. Additionally, we showed that GR24 significantly decreased the permeability levels of LPS stimulated brain endothelial cells. According to the results, GR24 may be the possible suppressor of proinflammatory molecules both in cytoplasmic and nuclear levels. In additionaly may have regulatory role in the energy metabolism of a the cell damaged by inflammation and be able to induce phase II enzymes that are fundamental for protecting against ROS damage and several of endogenous metabolites. Also, GR24 may improve the integrity of the BBB during neuroinflammatory disease conditions (Figure 5.1.). However, the mechanisms which GR24 affects the blood brain barrier remain unclear.

In summary, GR24 may be developed as a potential complementary and / or alternative approach for the prevention and treatment of neuroinflammatory diseases. To elucidate GR24 and its modes of action as a possible drug candidate and to identify its therapeutic potential further in vivo studies are needed.

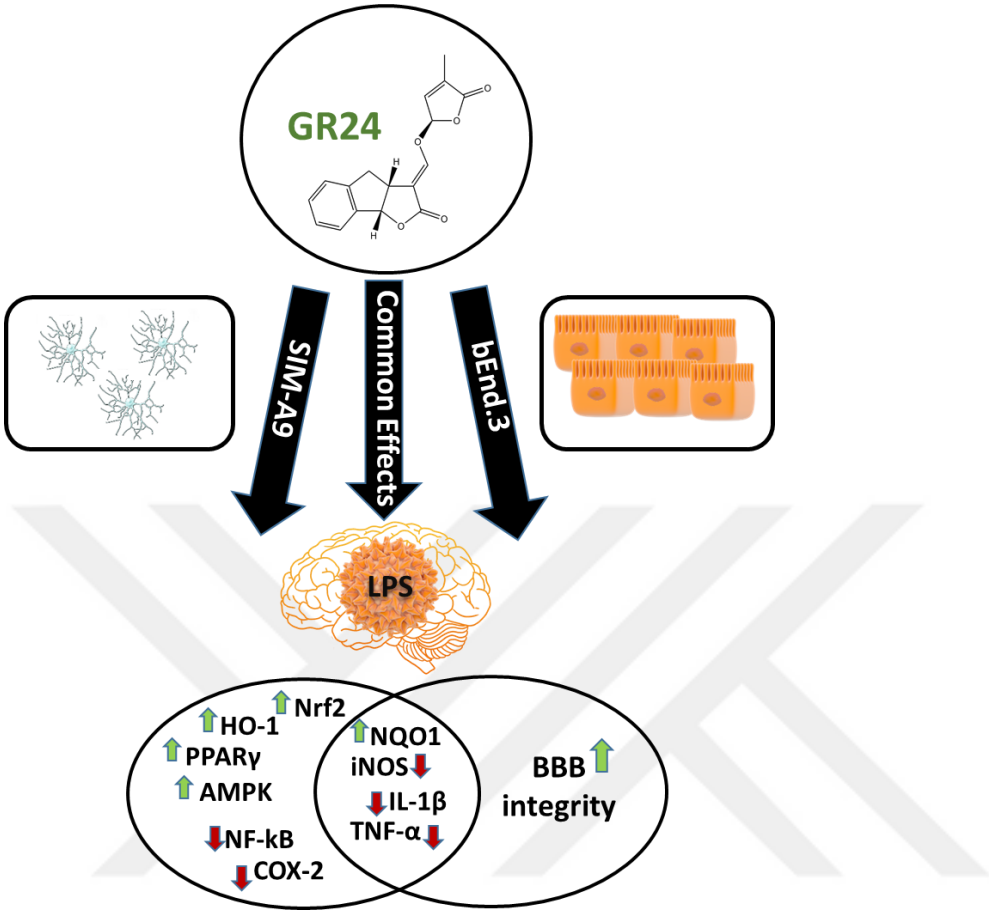

Figure 5.1. Multitarget potential of GR24 in LPS activated components of NVU

REFERENCES

Abbott N. J., Friedman A., 2012. Overview and Introduction: The Blood-Brain Barrier in Health and Disease. Epilepsia, 53: 1–6.

Abbott N. J., Patabendige A. A. K., Dolman D. E. M., Yusof S. R., Begley D. J. 2010. Structure and Function of the Blood-Brain Barrier. Neurobiology of Disease, 37(1): 13–25.

Abbott N. J., Rönnbäck L., Hansson, E. 2006. Astrocyte-Endothelial Interactions at the Blood-Brain Barrier. Nature Reviews Neuroscience, Vol. 7, pp. 41–53.

Alfieri A., Mann G. E. 2014. Bioactive Nutraceuticals and Stroke: Activation of Endogenous Antioxidant Pathways and Molecular Mechanisms Underlying Neurovascular Protection. In Bioactive Nutraceuticals and Dietary Supplements in Neurological and Brain Disease: Prevention and Therapy 25: 365–379.

Alhadidi Q., Shah Z. A. 2018. Cofilin Mediates LPS-Induced Microglial Cell Activation and Associated Neurotoxicity Through Activation of NF-κB and JAK–STAT Pathway. Molecular Neurobiology, 55(2): 1676–1691.

Bain J. M., Moore L., Ren Z., Simonishvili S., Levison S. W. 2013. Vascular Endothelial Growth Factors A and C are Induced in the SVZ Following Neonatal Hypoxia-Ischemia and Exert Different Effects on Neonatal Glial Progenitors. Translational Stroke Research, 4(2): 158–170.

Bal-Price A., Brown G. C. 2001. Inflammatory Neurodegeneration Mediated by Nitric Oxide from Activated Glia-Inhibiting Neuronal Respiration, Causing Glutamate Release and Excitotoxicity. The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 21(17): 6480–6491.

Balez R., Steiner N., Engel M., Muñoz S. S., Lum J. S., Wu Y., Ooi L. 2016. Neuroprotective Effects of Apigenin Against Inflammation, Neuronal Excitability and Apoptosis in an Induced Pluripotent Stem Cell Model of Alzheimer's Disease. Scientific Reports, 6.

Bauer H. C., Krizbai I. A., Bauer H., Traweger A. 2014. "You Shall Not Pass"-Tight Junctions of the Blood Brain Barrier. Frontiers in Neuroscience, Vol. 8.

Bechmann I., Galea I., Perry V. H. (2007). What Is the Blood-Brain Barrier (not)? Trends in Immunology, 28(1): 5–11.

Bordet R., Ouk T., Petrault O., Gelé P., Gautier S., Laprais M., Bastide M. 2006. PPAR: a New Pharmacological Target for Neuroprotection in Stroke and Neurodegenerative Diseases. Biochemical Society Transactions, 34(6): 1341–1346.

Brahmachari G. 2017. Discovery and Development of Neuroprotective Agents From Natural Products: An Overview. In Discovery and Development of Neuroprotective Agents from Natural Products: Natural Product Drug Discovery (pp. 1–7).

Brochard V., Combadière B., Prigent A., Laouar Y., Perrin A., Beray-Berthat V., Hunot S. 2009. Infiltration of CD4+ Lymphocytes into the Brain Contributes to Neurodegeneration In a Mouse Model of Parkinson Disease. Journal of Clinical Investigation, 119(1): 182–192.

Butovsky O., Jedrychowski M. P., Moore C. S., Cialic R., Lanser A. J., Gabriely G., Weiner H. L. 2014. Identification of a Unique TGF-β-Dependent Molecular and Functional Signature in Microglia. Nature Neuroscience, 17(1): 131–143.

Buttini M., Appel K., Sauter A., Gebicke-Haerter P. J., Boddeke H. W. G. M. 1996. Expression of Tumor Necrosis Factor Alpha After Focal Cerebral Ischaemia in the Rat. Neuroscience, 71(1): 1–16.

Carson M. J., Cameron Thrash J., Walter B. 2006. The Cellular Response in Neuroinflammation: The Role of Leukocytes, Microglia and Astrocytes in Neuronal Death and Survival. Clinical Neuroscience Research, 6(5): 237–245.

Chang R., Yee K.-L., Sumbria R. K. 2017. Tumor Necrosis Factor α Inhibition for Alzheimer's Disease. Journal of Central Nervous System Disease, 9(1): 3-7.

Chen W. W., Zhang X., Huang W. J. 2016. Role of Neuroinflammation in Neurodegenerative Diseases (Review). Molecular Medicine Reports, 13(4): 3391–3396.

Cherry J. D., Olschowka J. A., O'Banion M. K. 2014. Are "Resting" Microglia More "M2"? Frontiers in Immunology, Vol. 5.

Coelho V. R., Viau C. M., Staub R. B., De Souza M. S., Pflüger P., Regner G. G., Saffi J.

2017. Rosmarinic Acid Attenuates the Activation of Murine Microglial N9 Cells through the Downregulation of Inflammatory Cytokines and Cleaved Caspase-3. NeuroImmunoModulation, 24(3): 171–181.

Cook C. E., Whichard L. P., Turner B., Wall M. E., Egley G. H. 1966. Germination of Witchweed (striga lutea lour.): Isolation and Properties of a Potent Stimulant. Science, 154(3753): 1189–1190.

Cooper E. L., Ma M. J. 2017. Alzheimer Disease: Clues from Traditional and Complementary Medicine. Journal of Traditional and Complementary Medicine, 7: 380–385.

Croglio M. P., Haake J. M., Ryan C. P., Wang V. S., Lapier J., Schlarbaum J. P., Yarden R. I. 2016. Analogs of the Novel Phytohormone, Strigolactone, Trigger Apoptosis and Synergize with PARP Inhibitors by Inducing DNA Damage and Inhibiting DNA Repair. Oncotarget, 7(12): 256-262.

Cunningham C. 2013. Microglia and Neurodegeneration: The Role of Systemic Inflammation. Glia, 61(1): 71–90.

D'Ambrosi N., Finocchi P., Apolloni S., Cozzolino M., Ferri A., Padovano V., Volonte C. 2009. The Proinflammatory Action of Microglial P2 Receptors Is Enhanced in SOD1 Models for Amyotrophic Lateral Sclerosis. The Journal of Immunology, 183(7): 4648–4656.

da Fonseca A. C. C., Matias D., Garcia C., Amaral R., Geraldo L. H., Freitas C., Lima F. R. S. 2014. The Impact of Microglial Activation on Blood-Brain Barrier in Brain Diseases. Frontiers in Cellular Neuroscience, 8.

de Haas A. H., Boddeke H. W. G. M., Biber K. 2008. Region-Specific Expression of Immunoregulatory Proteins on Microglia in the Healthy CNS. Glia, 56(8): 888–894.

Dendrou C. A., McVean G., Fugger L. 2016. Neuroinflammation-Using Big Data to Inform Clinical Practice. Nature Reviews Neurology, 12: 685–698.

Di Benedetto S., Müller L., Wenger E., Düzel S., Pawelec G. 2017. Contribution of Neuroinflammation and Immunity to Brain Aging and the Mitigating Effects of Physical and Cognitive Interventions. Neuroscience and Biobehavioral Reviews, 75: 114–128.

Dinarello C. A. 2000. Proinflammatory Cytokines. Chest, 118(2): 503–508.

Dinkova-Kostova A. T., Kostov R. V., Kazantsev A. G. 2018. The Role of Nrf2 Signaling in Counteracting Neurodegenerative Diseases. FEBS Journal, 285: 3576–3590.

DiSabato D. J., Quan N., Godbout J. P. 2016. Neuroinflammation: the Devil Is in the Details. Journal of Neurochemistry, 139: 136–153.

Engelhardt B. 2008. Immune Cell Entry into the Central Nervous System: Involvement of Adhesion Molecules and Chemokines. Journal of the Neurological Sciences, 274(1–2): 23–26.

Eren E., Tufekci K. U., Isci K. B., Tastan B., Genc K., Genc S. 2018. Sulforaphane Inhibits Lipopolysaccharide-Induced Inflammation, Cytotoxicity, Oxidative stress, and miR-155 Expression and Switches to Mox Phenotype Through Activating Extracellular Signal-Regulated Kinase 1/2-Nuclear Factor Erythroid 2-Related Factor 2. Frontiers in Immunology, 9.

Esposito E., Di Matteo V., Benigno A., Pierucci M., Crescimanno G., Di Giovanni G. 2007. Non-Steroidal Anti-Inflammatory Drugs in Parkinson's Disease. Experimental Neurology, 205: 295–312.

Evans H. M., Schulemann W. 1914. The Action of Vital Stains Belonging to the Benzidine Group. Science, 39(1004): 443–454.

Fantin A., Plein A., Ruhrberg C. 2012. Neuropilin Signalling in Vascular Development and Pathology. Current Angiogenesise, 1(2): 125–132.

Farrell K., Borazjani A., Damaser M., Kothapalli C. R. 2016. Differential Regulation of NSC Phenotype and Genotype by Chronically Activated Microglia Within Cocultures. Integrative Biology (United Kingdom), 8(11): 1145–1157.

Furuse M., Hirase T., Itoh M., Nagafuchi A., Yonemura S., Tsukita S., Tsukita S. 1993. Occludin: A novel Integral Membrane Protein Localizing at Tight Junctions. Journal of Cell Biology, 123(6 II): 1777–1788.

Galea E., Feinstein D. L. 1999. Regulation of the Expression of the Inflammatory Nitric Oxide Synthase (NOS2) by Cyclic AMP. FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 13(15): 2125–2137.

Galea I., Bechmann I., Perry V. H. 2007. What is Immune Privilege (not)? Trends in Immunology, 28(1): 12–18.

Gallo O., Schiavone N., Papucci L., Sardi I., Magnelli L., Franchi A., Capaccioli S. 2003. Down-Regulation of Nitric Oxide Synthase-2 and Cyclooxygenase-2 Pathways by p53 in Squamous Cell Carcinoma. American Journal of Pathology, 163(2): 723–732.

Gandhi J., Khera L., Gaur N., Paul C., Kaul R. 2017. Role of Modulator of Inflammation Cyclooxygenase-2 in Gammaherpesvirus Mediated Tumorigenesis. Frontiers in Microbiology, 8: 86-95.

Garvey E. P., Oplinger J. A., Furfine E. S., Kiff R. J., Laszlo F., Whittle B. J. R., Knowles R. G. 1997. 1400W Is a Slow, Tight Binding, and Highly Selective Inhibitor of Inducible Nitric-Oxide Synthase in vitro and in vivo. Journal of Biological Chemistry, 272(8): 4959–4963.

Grabert K., Michoel T., Karavolos M. H., Clohisey S., Kenneth Baillie J., Stevens M. P., McColl B. W. 2016. Microglial Brain Regionâ 'Dependent Diversity and Selective Regional Sensitivities to Aging. Nature Neuroscience, 19(3): 504–516.

Griess P. 1879. Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt „Ueber einige Azoverbindungen". Berichte Der Deutschen Chemischen Gesellschaft, 12(1): 426–428.

Hamaguchi T., Ono K., Murase A., Yamada, M. 2009. Phenolic Compounds Prevent Alzheimer's Pathology Through Different Effects on the Amyloid-β Aggregation Pathway. American Journal of Pathology, 175(6): 2557–2565.

Hanisch U.-K. 2013. Functional Diversity of Microglia – How Heterogeneous Are They to Begin With? Frontiers in Cellular Neuroscience, 7(89): 1379-1390.

Holmes C. 2013. Review: Systemic Inflammation and Alzheimer's Disease. Neuropathology and Applied Neurobiology, 39: 51–68.

Hu Y., Wang Z., Pan S., Zhang H., Fang M., Jiang H., Lin Z. 2017. Melatonin Protects Against Blood-Brain Barrier Damage by Inhibiting the TLR4/ NF-kB Signaling Pathway After LPS Treatment in Neonatal Rats. Oncotarget, 8(19): 31638-31654.

Huang X. J., Tang J. Q., Li M. M., Liu Q., Li Y. L., Fan C. L., Ye W. C. 2014.

Triterpenoid Saponins from the Rhizomes of Anemone Flaccida and Their Inhibitory Activities on LPS-Induced NO Production in Macrophage RAW264.7 Cells. Journal of Asian Natural Products Research, 16(9): 910–921.

Hüntelmann A. C., Hüntelmann A. C. 2019. Paul Ehrlich als Assistenzarzt an der Charité (1878-1885). In Paul Ehrlich (pp. 45–73).

Idriss H. T., Naismith J. H. 2000. TNFα and the TNF Receptor Superfamily: Structure-Function Relationship(s). Microscopy Research and Technique, 50(3): 184–195.

Iuvone T., Esposito G., De Filippis D., Scuderi C., Steardo L. 2009. Cannabidiol: A Promising Drug for Neurodegenerative Disorders? CNS Neuroscience and Therapeutics, 15(1): 65–75.

Jimbo D., Kimura Y., Taniguchi M., Inoue M., Urakami K. 2009. Effect of Aromatherapy on Patients with Alzheimer's Disease. Psychogeriatrics, 9(4): 173–179.

Johnson S. L., Kirk R. D., DaSilva N. A., Ma H., Seeram N. P., Bertin M. J. 2019. Polyphenol Microbial Metabolites Exhibit Gut and Blood–Brain Barrier Permeability and Protect Murine Microglia against LPS-Induced Inflammation. Metabolites, 9(4): 78.

Kapadia R., Yi J.-H., Vemuganti R. 2008. Mechanisms of Anti-Inflammatory and Neuroprotective Actions of PPAR-Gamma Agonists. Frontiers in Bioscience : A Journal and Virtual Library, 13: 1813–1826.

Kapche D. W. F. G., Lekane N. M., Kulabas S. S., Ipek H., Tok T. T., Ngadjui B. T., Tumer T. B. 2017. Aryl Benzofuran Derivatives from the Stem Bark of Calpocalyx Dinklagei Attenuate Inflammation. Phytochemistry, 141: 70–79.

Kennedy D., Scholey A. 2006. The Psychopharmacology of European Herbs with Cognition-Enhancing Properties. Current Pharmaceutical Design, 12(35): 4613–4623.

Khan N., Mukhtar H. 2010. Cancer Chemoprevention. In Comprehensive Toxicology, Second Edition, 14: 417–431.

Kinnecom C., Lev M. H., Wendell L., Smith E. E., Rosand J., Frosch M. P., Greenberg S. M. 2007. Course of Cerebral Amyloid Angiopathy-Related Inflammation.

Neurology, 68(17): 1411–1416.

Kofler J., Wiley, C. A. 2011. Microglia:Key Innate Immune Cells of the Brain. Toxicologic Pathology, 39(1): 103–114.

Kreutzberg G. W. 1996. Microglia: A Sensor for Pathological Events in the CNS. Trends in Neurosciences, 19(1): 312–318.

Laemmli U. K. 1970. Cleavage of Structural Proteins During the Assembly of the Head of Bacteriophage T4. Nature, 227(5259): 680–685.

Layé S., Parnet P., Goujon E., Dantzer R. 1994. Peripheral Administration of Lipopolysaccharide Induces the Expression of Cytokine Transcripts in the Brain and Pituitary of Mice. Molecular Brain Research, 27(1): 157–162.

Lee S. C., Dickson D. W., Liu W., Brosnan, C. F. 1993. Induction of Nitric Oxide Synthase Activity in Human Astrocytes by Interleukin-1β and Interferon-γ. Journal of Neuroimmunology, 46(1–2): 19–24.

Lenz K. M., Nelson L. H. 2018. Microglia and Beyond: Innate Immune Cells as Regulators of Brain Development and Behavioral Function. Frontiers in Immunology, 9: 698.

Li C. S., Black W. C., Chan C. C., Ford-Hutchinson A. W., Gauthier J. Y., Gordon R., Prasit P. 1995. Cyclooxygenase-2 Inhibitors. Synthesis and Pharmacological Activities of 5-Methanesulfonamido-1-indanone Derivatives. Journal of Medicinal Chemistry, 38(25): 4897–4905.

Li H., Wang P., Huang F., Jin J., Wu H., Zhang B., Wu X. 2018. Astragaloside IV Protects Blood-Brain Barrier Integrity from LPS-Induced Disruption via Activating Nrf2 Antioxidant Signaling Pathway in Mice. Toxicology and Applied Pharmacology, 340: 58–66.

Li J., Zhang S., Lu M., Chen Z., Chen C., Han L., Xu Y. 2013. Hydroxysafflor Yellow A Suppresses Inflammatory Responses of BV2 Microglia After Oxygen-Glucose Deprivation. Neuroscience Letters, 535(1): 51–56.

Manco M., Putignani L., Bottazzo G. F. 2010. Gut Microbiota, Lipopolysaccharides, and Innate Immunity in the Pathogenesis of Obesity and Cardiovascular Risk. Endocrine

Reviews, 31(6): 817-844.

Marsh D. T., Das S., Ridell J., Smid S. D. 2017. Structure-Activity Relationships for Flavone Interactions with Amyloid β Reveal a Novel Anti-Aggregatory and Neuroprotective Effect of 2′,3′,4′-trihydroxyflavone (2-D08). Bioorganic and Medicinal Chemistry, 25(14): 3827–3834.

Matter K., Balda M. S. 2003. Holey Barrier: Claudins and the Regulation of Brain Endothelial Permeability. Journal of Cell Biology, 161(3): 459–460.

Mayzlish-Gati E., Laufer D., Grivas C. F., Shaknof J., Sananes A., Bier A., Koltai H. 2015. Strigolactone Analogs Act as New Anti-Cancer Agents in Inhibition of Breast Cancer in Xenograft Model. Cancer Biology and Therapy, 16(11): 1682–1688.

Mikenberg I., Widera D., Kaus A., Kaltschmidt B., Kaltschmidt C. 2007. Transcription Factor NF-κB is Transported to the Nucleus via Cytoplasmic Dynein/Dynactin Motor Complex in Hippocampal Neurons. PLoS ONE, 2(7): 589.

Mo C., Wang L., Zhang J., Numazawa S., Tang H., Tang X., Xiao H. 2014. The Crosstalk Between Nrf2 and AMPK Signal Pathways Is Important for the Anti-Inflammatory Effect of Berberine in LPS-Stimulated Macrophages and Endotoxin-Shocked Mice. Antioxidants & Redox Signaling, 20(4): 574–588.

Modi S., Yaluri N., Kokkola T., Laakso M. 2017. Plant-Derived Compounds Strigolactone GR24 and Pinosylvin Activate SIRT1 and Enhance Glucose Uptake in Rat Skeletal Muscle Cells. Scientific Reports, 7(1): 17606.

Molina-Holgado E., Ortiz S., Molina-Holgado F., Guaza C. 2000. Induction of COX-2 and PGE2 Biosynthesis by IL-1β is Mediated by PKC and Mitogen-Activated Protein Kinases in Murine Astrocytes. British Journal of Pharmacology, 131(1): 152–159.

Moreno S., Farioli-vecchioli S., Cerù M. P. 2004. Immunolocalization of Peroxisome Proliferator-Activated Receptors and Retinoid X Receptors in the Adult Rat CNS. Neuroscience, 123(1): 131–145.

Mosmann T. 1983. Rapid Colorimetric Assay for Cellular Growth and Survival: Application to Proliferation and Cytotoxicity Assays. Journal of Immunological Methods, 65(1–2): 55–63.

Muoio V., Persson P. B., Sendeski M. M. 2014. The Neurovascular Unit - Concept Review. Acta Physiologica, 210(4): 790–798.

Nagamoto-Combs K., Kulas J., & Combs C. K. 2014. A Novel Cell Line from Spontaneously Immortalized Murine Microglia. Journal of Neuroscience Methods, 233: 187–198.

Nakajima K., Kohsaka S. 2001. Microglia: Activation and Their Significance in the Central Nervous System. Journal of Biochemistry, 130(2): 169–175.

Nimmerjahn A., Kirchhoff F., Helmchen F. 2005. Neuroscience: Resting Microglial Cells are Highly Dynamic Surveillants of Brain Parenchyma in vivo. Science, 308(5726): 1314–1318.

Noh H., Jeon J., Seo H. 2014. Systemic Injection of LPS Induces Region-Specific Neuroinflammation and Mitochondrial Dysfunction in Normal Mouse Brain. Neurochemistry International, 69(1): 35–40.

Obermeier B., Daneman R., Ransohoff R. M. 2013. Development, Maintenance and Disruption of the Blood-Brain Barrier. Nature Medicine, 19(12): 1584–1596.

Ortiz G. G., Pacheco-Moisés F. P., Macías-Islas M. Á., Flores-Alvarado L. J., Mireles-Ramírez M. A., González-Renovato E. D., Alatorre-Jiménez M. A. 2014. Role of the Blood-Brain Barrier in Multiple Sclerosis. Archives of Medical Research, 45(8): 687–697.

Pardridge W. M. 2015. Blood–brain Barrier Endogenous Transporters as Therapeutic Targets: A New Model for Small Molecule CNS Drug Discovery. Expert Opinion on Therapeutic Targets, 19(8): 1059–1072.

Peixoto C. A., Oliveira W. H., de Araújo S. M. da R., Nunes, A. K. S. 2017. AMPK Activation: Role in the Signaling Pathways of Neuroinflammation and neurodegeneration. Experimental Neurology, 298: 31–41.

Perry V. H., Cunningham C., Holmes C. 2007. Systemic Infections and Inflammation Affect Chronic Neurodegeneration. Nature Reviews Immunology, 7(2): 161–167.

Perry V. H., Nicoll J. A. R., Holmes C. 2010. Microglia in Neurodegenerative Disease. Nature Reviews Neurology, 6(4): 193–201.

Pollock C. B., McDonough S., Wang V. S., Lee H., Ringer L., Li X., Yarden R. I. 2014. Strigolactone Analogues Induce Apoptosis Through Activation of p38 and the Stress Response Pathway in Cancer Cell Lines and in Conditionally Reprogrammed Primary Prostate Cancer Cells. Oncotarget, 5(6): 1683-1698.

Qin L., Wu X., Block M. L., Liu Y., Breese G. R., Hong J. S., Crews F. T. 2007. Systemic LPS Causes Chronic Neuroinflammation and Progressive Neurodegeneration. Glia, 55(5): 453–462.

Qu Y., Liu Y., Chen L., Zhu Y., Xiao X., Wang D., Zhu Y. 2018. Nobiletin Prevents Cadmium-Induced Neuronal Apoptosis by Inhibiting Reactive Oxygen Species and Modulating JNK/ERK1/2 and Akt/mTOR Networks in Rats. Neurological Research, 40(3): 211–220.

Ransohoff R. M., Perry V. H. 2009. Microglial Physiology: Unique Stimuli, Specialized Responses. Annual Review of Immunology, 27(1): 119–145.

Rasool M., Malik A., Qureshi M. S., Manan A., Pushparaj P. N., Asif M., Sheikh I. A. 2014. Recent Updates in the Treatment of Neurodegenerative Disorders Using Natural Compounds. Evidence-Based Complementary and Alternative Medicine, 2014, 1–7.

Réu P., Khosravi A., Bernard S., Mold J. E., Salehpour M., Alkass K., Frisén J. 2017. The Lifespan and Turnover of Microglia in the Human Brain. Cell Reports, 20(4): 779–784.

Rezai-Zadeh K., Gate D., Town T. 2009. CNS Infiltration of Peripheral Immune Cells: D-Day for Neurodegenerative Disease? Journal of Neuroimmune Pharmacology, 4(4): 462–475.

Rushworth S. A., MacEwan D. J., O'Connell M. A. 2014. Lipopolysaccharide-Induced Expression of NAD(P)H:Quinone Oxidoreductase 1 and Heme Oxygenase-1 Protects against Excessive Inflammatory Responses in Human Monocytes. The Journal of Immunology, 181(10): 6730–6737.

Ryu J. K., Cho T., Choi H. B., Wang Y. T., McLarnon J. G. 2009. Microglial VEGF Receptor Response Is an Integral Chemotactic Component in Alzheimer's Disease Pathology. Journal of Neuroscience, 29(1): 3–13.

Saitou M., Furuse M., Sasaki H., Schulzke J.-D., Fromm M., Takano H., Tsukita S. 2000. Complex Phenotype of Mice Lacking Occludin, a Component of Tight Junction Strands. Molecular Biology of the Cell, 11(12): 4131–4142.

Salter M. W., Stevens B. 2017. Microglia Emerge as Central Players in Brain Disease. Nature Medicine, 23(9): 1018–1027.

Satoh H., Amagase K., Ebara S., Akiba Y., Takeuchi K. 2013. Cyclooxygenase (COX)-1 and COX-2 Both Play an Important Role in the Protection of the Duodenal Mucosa in Cats. Journal of Pharmacology and Experimental Therapeutics, 344(1): 189–195.

Schain M., Kreisl W. C. 2017. Neuroinflammation in Neurodegenerative Disorders—a Review. Current Neurology and Neuroscience Reports, 17(3):25.

Scuderi C., De Filippis D., Iuvone T., Blasio A., Steardo A., Esposito G. 2009. Cannabidiol in Medicine: A Review of Its Therapeutic Potential in CNS Disorders. Phytotherapy Research, 23(5):597–602.

Seegobin M., Kisiala A., Noble A., Kaplan D., Brunetti C., Emery R. J. N. 2018. Canis Familiaris Tissues Are Characterized by Different Profiles of Cytokinins Typical of the tRNA Degradation Pathway. FASEB Journal, 32(12): 6575–6581.

Shamsizadeh A., Roohbakhsh A., Ayoobi F., Moghaddamahmadi A. 2017. The Role of Natural Products in the Prevention and Treatment of Multiple Sclerosis. In Nutrition and Lifestyle in Neurological Autoimmune Diseases: Multiple Sclerosis. 249–260 p.

Shields M. 2016. Chemotherapeutics. In Pharmacognosy: Fundamentals, Applications and Strategy. 295–313 p.

Smith R. H. F. 1951. Permeability of the Blood-Brain Barrier to Penicillin in Cases of Parenchymatous Neurosyphilis. Journal of Mental Science, 97(407): 340–361.

Smith S. E., Barker S. J., Zhu Y. G. 2006. Fast Moves in Arbuscular Mycorrhizal Symbiotic Signalling. Trends in Plant Science, 11(8): 369–371.

Soares M. P., Seldon M. P., Gregoire I. P., Vassilevskaia T., Berberat P. O., Yu J., Bach F. H. 2014. Heme Oxygenase-1 Modulates the Expression of Adhesion Molecules Associated with Endothelial Cell Activation. The Journal of Immunology, 172(6): 3553–3563.

Starkov A. A., Beal F. M. 2008. Portal to Alzheimer's Disease. Nature Medicine, 14(10): 1020–1021.

Steinman L., Martin R., Bernard C., Conlon P., Oksenberg J. R. 2002. Multiple Sclerosis: Deeper Understanding of Its Pathogenesis Reveals New Targets for Therapy. Annual Review of Neuroscience, 25(1): 491–505.

Sul D., Kim H. S., Lee D., Joo S. S., Hwang K. W., Park S. Y. 2009. Protective Effect of Caffeic Acid Against Beta-Amyloid-Induced Neurotoxicity by the Inhibition of Calcium Influx and Tau Phosphorylation. Life Sciences, 84(9–10): 257–262.

Tovey E. R., Ford S. A., Baldo B. A. 1987. Protein Blotting on Nitrocellulose: Some Important Aspects of the Resolution and Detection of Antigens in Complex Extracts. Journal of Biochemical and Biophysical Methods, 14(1): 1–17.

Townsend B. E., Johnson R. W. 2016. Sulforaphane Induces Nrf2 Target Genes and Attenuates Inflammatory Gene Expression in Microglia from Brain of Young Adult and Aged Mice. Experimental Gerontology, 73(1): 42–48.

Tsai C. F., Kuo Y. H., Yeh W. L., Wu C. Y. J., Lin H. Y., Lai S. W., Lu D. Y. 2015. Regulatory Effects of Caffeic Acid Phenethyl Ester on Neuroinflammation in Microglial Cells. International Journal of Molecular Sciences, 16(3): 5572–5589.

Tse J. K. Y. 2017. Gut Microbiota, Nitric Oxide, and Microglia as Prerequisites for Neurodegenerative Disorders. ACS Chemical Neuroscience, 8(7): 1438–1447.

Tumer T. B., Rojas-Silva P., Poulev A., Raskin I., Waterman C. 2015. Direct and Indirect Antioxidant Activity of Polyphenol- and Isothiocyanate-Enriched Fractions from Moringa Oleifera. Journal of Agricultural and Food Chemistry, 63(5): 1505–1513.

Tumer T. B., Yılmaz B., Ozleyen A., Kurt B., Tok T. T., Taskin K. M., Kulabas S. S. 2018. GR24, A Synthetic Analog of Strigolactones, Alleviates Inflammation and Promotes Nrf2 Cytoprotective Response: In Vitro and In Silico Evidences. Computational Biology and Chemistry, 76(10): 179–190.

Turner M. R., Cagnin A., Turkheimer F. E., Miller C. C. J., Shaw C. E., Brooks D. J., Banati R. B. 2004. Evidence of Widespread Cerebral Microglial Activation in Amyotrophic Lateral Sclerosis: An [11 C](R)-PK11195 Positron Emission Tomography Study. Neurobiology of Disease, 15(3): 601–609.

van Horssen J., Schreibelt G., Drexhage J., Hazes T., Dijkstra C. D., van der Valk P., de Vries H. E. 2008. Severe Oxidative Damage in Multiple Sclerosis Lesions Coincides With Enhanced Antioxidant Enzyme Expression. Free Radical Biology and Medicine, 45(12): 1729–1737.

Varnum M. M., Ikezu T. 2012. The Classification of Microglial Activation Phenotypes on Neurodegeneration and Regeneration in Alzheimer's Disease Brain. Archivum Immunologiae et Therapiae Experimentalis, 60(4): 251–266.

Vlad S. C., Miller D. R., Kowall N. W., Felson D. T. 2008. Protective Effects of NSAIDs on the Development of Alzheimer Disease. Neurology, 70(19): 1672–1677.

Voller A., Bartlett A., Bidwell D. E., Clark M. F., Adams A. N. 1976. The Detection of Viruses by Enzyme Linked Immunosorbent Assay (ELISA). Journal of General Virology, 33(1): 165–167.

Wan F., Lenardo M. J. 2010. The Nuclear Signaling of NF-κB: Current Knowledge, New Insights, and Future Perspectives. Cell Research, 20(1): 24–33.

Wang L., He X., Szklarz G. D., Bi Y., Rojanasakul Y., Ma Q. 2013. The Ayl Hydrocarbon Receptor Interacts with Nuclear Factor Erythroid 2-Related Factor 2 to Mediate Induction of NAD(P)H: Quinoneoxidoreductase 1 by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Archives of Biochemistry and Biophysics, 537(1): 31–38.

Waters M. T., Gutjahr C., Bennett T., Nelson D. C. 2017. Strigolactone Signaling and Evolution. Annual Review of Plant Biology, 68(1): 291–322.

Wruck C. J., Claussen M., Fuhrmann G., Römer L., Schulz A., Pufe T., Götz M. E. 2007. Luteolin Protects Rat PC 12 and C6 Cells Against MPP+ Induced Toxicity via an ERK Dependent Keapl-Nrf2-ARE Pathway. Journal of Neural Transmission, Supplementa, (72):57–67.

Yang C. M., Chien C. S., Hsiao L. Der Luo S. F., Wang C. C. 2002. Interleukin-1β-Induced Cyclooxygenase-2 Expression is Mediated Through Activation of p42/44 and p38 MAPKS, and NF-κB Pathways in Canine Tracheal Smooth Muscle Cells. Cellular Signalling, 14(11): 899–911.

Yang Z., Kahn B. B., Shi H., Xue B. Z. 2010. Macrophage α1 AMP-Activated Protein Kinase (α1AMPK) Antagonizes Fatty Acid-Induced Inflammation Through SIRT1.

Journal of Biological Chemistry, 285(25): 19051–19059.

Yin M. J., Yamamoto Y., Gaynor R. B. 1998. The Anti-Inflammatory Agents Aspirin and Salicylate Inhibit the Activity of IκB Kinase-β. Nature, 396(6706): 77–80.

Yu L., Wang L., Chen S. 2010. Endogenous Toll-Like Receptor Ligands and Their Biological Significance. Journal of Cellular and Molecular Medicine, 14(11): 2592–2603.

Yucel-Lindberg T., Ahola H., Carlstedt-Duke J., Modéer T. 1999. Involvement of Tyrosine Kinases on Cyclooxygenase Expression and Prostaglandin E2 Production in Human Gingival Fibroblasts Stimulated with Interleukin-1β and Epidermal Growth Factor. Biochemical and Biophysical Research Communications, 257(2): 528–532.

Yuste J. E., Tarragon E., Campuzano C. M., Ros-Bernal F. 2015. Implications of Glial Nitric Oxide in Neurodegenerative Diseases. Frontiers in Cellular Neuroscience, 9:322.

Zaitone S. A., Ahmed E., Elsherbiny N. M., Mehanna E. T., El-Kherbetawy M. K., ElSayed M. H., Moustafa Y. M. 2019. Caffeic Acid Improves Locomotor Activity and Lessens Inflammatory Burden in A Mouse Model of Rotenone-Induced Nigral Neurodegeneration: Relevance to Parkinson's Disease Therapy. Pharmacological Reports, 71(1): 32–41.

Zasłona Z., Pålsson-McDermott E. M., Menon D., Haneklaus M., Flis E., Prendeville H., O'Neill L. A. J. 2017. The Induction of Pro–IL-1β by Lipopolysaccharide Requires Endogenous Prostaglandin E2 Production. The Journal of Immunology, 198(9):3558-3564.

Zhao X., Chen R., Liu M., Feng J., Chen J., Hu K. 2017. Remodeling the Blood–Brain Barrier Microenvironment by Natural Products for Brain Tumor Therapy. Acta Pharmaceutica Sinica B, 7(5): 541–553.

Zhao Z., Nelson A. R., Betsholtz C., Zlokovic B. V. 2015. Establishment and Dysfunction of the Blood-Brain Barrier. Cell, 163(5): 1064–1078.

Zhou X., Gan P., Hao L., Tao L., Jia J., Gao B., Zhen X. 2014. Antiinflammatory Effects of Orientin-2″-O-Galactopyranoside on Lipopolysaccharide-Stimulated Microglia. Biological and Pharmaceutical Bulletin, 37(8): 1282–1294.

Zwanenburg B., Regeling H., Van Tilburg-Joukema C. W., Van Oss B., Molenveld P., De Gelder R., Tinnemans P. 2016. Securing Important Strigolactone Key Structures: Orobanchol and 5-Deoxystrigol. European Journal of Organic Chemistry, 2016(12): 2163–2169.

BIOGRAPHY

PERSONAL DETAILS

Name Surname: Begüm KURT

Place of Birth: İstanbul/Kadıköy

Date of Birth: 28.09.1992

EDUCATION STATUS

Bachelor's Degree: Çanakkale Onsekiz Mart University Faculty of Arts and Sciences, Department of Biology

Erasmus Programme: University of Naples, Federico II, Department of Biology

Master's Degree: Çanakkale Onsekiz Mart University Graduate School of Natural and Applied Sciences, Department of Biomolecular Sciences

Foreign Languages: English

SCIENTIFIC ACTIVITY

a. Publishing -SCI –Other

Tumer, T.B., Yılmaz, B., Ozleyen, A., **Kurt, B**., Tok, T.T., Taskin, K.M., Kulabas, S.S., 2018. GR24, a synthetic analog of Strigolactones, alleviates inflammation and promotes Nrf2 cytoprotective response: In vitro and in silico evidences. Computational biology and chemistry 76, 179-190.

b. Notifications -International -National

1) **Begum Kurt**, Adem Ozleyen, Elif Turkdonmez, Gizem Antika, Berkay Yilmaz, Tugba Boyunegmez Tumer. Neuroprotective Potential of Strigolactones on LPS-activated brain endothelial and microglial cells. FENS Regional Meeting, 10-13 July 2019, Belgrade, Serbia.

2) Tugba Boyunegmez Tumer, Berkay Yılmaz, **Begum Kurt**, Saliha Busra Kurt, Kemal

Melih Taskin, Seda Savranoglu Kulabas. A synthetic Analog of Novel Phytohormones Strigolactones Attenuates Inflammation and Improves Insulin Response In Vitro. 3rd International Conference on Natural Products Utilization from Plants to Pharmacy Shelf, 18-21 October 2017, Bansko, Bulgaria.

c. Projects

Project Title: Strigolakton Analoglarının in vitro Nöroinflamasyon Modellerinde Antiinflamatuvar Etkilerinin Araştırılması ve Potansiyel Aktif Bileşiklerin Belirlenmesi, TUBİTAK 1002 Project, (Researcher), 2018-Ongoing.

Project Title: Effects of Cholesterol on metabolism of glial cells, University of Naples, Federico II, Department of Biology (Erasmus Student- Guest Project Researcher), 2018.

Project Title: Chemopreventive Approaches in In Vitro Neuroinflammation Models: Therapeutic Effects and Molecular Mechanisms of Novel and Known Bioactive Molecules, ÇOMÜ-BAP Master's Thesis Project (FYL-2017-1339), 2017-2019 (Researcher).

d. Scholarships

Travel Grant - Federation of European Neuroscience Societies (FENS) Regional Meeting 10-13 July 2019 in Belgrade, Serbia.

Abstract Title: Neuroprotective Potential of Strigolactones on LPS-activated brain endothelial and microglial cells, poster presentation.

e. Certificates

13th Turkish Neuroscience Congress, 2015 - certificate of participation

14th Turkish Neuroscience Congress, 2016 - certificate of participation

13th International Congress of Cognitive Neuroscience, 2016 - certificate of participation

Practices of stimulated potentials and clinical applications in human brain

Practices of Functional magnetic resonance imaging (fMRI)

f. Memberships

Neuroscience Society of Turkey

Federation of European Neuroscience Societies (Fens Member ID: TR99523)

CONTACT

E-mail address: begum@stu.comu.edu.tr