EVALUATION OF THE MECHANISMS OF ANTINEUROINFLAMMATORY EFFECTS OF ETHANOL EXTRACT OF *Moringa oleifera* LAM. (MORINGACEAE) LEAVES

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BY

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ABSTRACT

Neuroinflammation is the hallmark of neurodegenerative diseases which, causes dementia and ataxia reducing the quality of life in the aged population. Conventional treatments are not very effective in targeting the underlying pathology of the diseases. *Moringa oleifera* (MO) has been used for centuries to treat a variety of diseases whose pathogenesis have lately been established to be inflammatory. The study was designed to evaluate the antineuroinflammatory mechanisms of ethanol extract of MO leaves (EMOL).

Moringa oleifera obtained from a domestic garden at Ojoo Ibadan was authenticated at Forest Herbarium Ibadan with voucher number FHI 109601. Pulverized leaves (500 g) of MO were extracted by maceration in 50% aqueous ethanol at room temperature. Twenty-five Swiss male mice (18-22 g) were allotted into 5 treatment groups (n=5): 5% Tween80 (10 mL/kg), EMOL (250, 500, 1000 and 2000 mg/kg) were used for central nervous system studies. In Lipopolysaccharide (LPS) cognitive deficit (LCD), thirty male mice were distributed into groups 1-5 (n=6) and treated orally for 7 days: 5% Tween80 (10 mL/kg), LPS and EMOL (100, 200, 400 mg/kg) before intraperitoneal administration of 250 µg/kg LPS to groups 2-5. The LCD was assessed by Y-maze test. The EMOL was partitioned into 20%, 50%, 80% and 100% methanol fractions (F20, F50, F80, and F100), respectively. Bioactivities of the fractions were evaluated using MTT and nitrite assays. The F50 was further purified to isolate compounds using HPLC, ^{1}H NMR and ^{13}C NMR. Isolated compounds were screened by MTT assay in the presence of compounds on murine microglia (BV-2) and macrophages (RAW 264.7). Lipopolysaccharides was used to induce inflammation either in the presence and absence of various EMOL (100, 150 and 200 µg/mL), fractions (12.5, 25 and 50 µg/mL) and three compounds (12.5, 12.5 and 25µM) in BV-2 cells. Nitric oxide (NO), cytokine (TNF-α) and PGE_2 production were evaluated in the supernatants using spectrophotometry and ELISA. Expression of cyclooxygenase-2 (COX-2), inducible nitric oxide (iNOS) and p38 proteins were determined using western blots. The effect of the isolated compounds on NF-κB transactivation was evaluated using luciferase reporter gene assay. Data were analysed using descriptive statistics and ANOVA at $\alpha_{0.05}$

The EMOL (100-400 mg/kg) significantly increased % alternation in LCD (61.55±1.162, 59.68±1.948, 64.25±1.938) compared with LPS (49.13±1.225). The MTT assay revealed that EMOL, fractions (F20 and F50) and the compounds (kaempferol, quercetin and rutin), had no

effect on viability of BV-2 and RAW 264.7 cells. The EMOL (150 and 200 µg/mL) and kaempferol (12.5 µM) significantly reduced NO (43.82±4.23, 38.68±12.71), (16.39±1.48); PGE_2 (45.05±1.30, 59.30±3.20), (51.73±1.48); and TNF-α (57.67±2.38, 60.43±8.07), (42.31±5.1) compared with LPS. Kaempferol, quercetin and rutin inhibited COX-2 and iNOs protein expressions in LPS stimulated BV-2 cells. Kaempferol, quercetin and rutin significantly reduced NF-κB transcriptional activation (40.49±10.01, 20.74±7.54, 41.68±8.32) in HEK 293 cells compared with TNF-α, and also significantly inhibited p-38 expression (58.06±18.17, 52.78±11.81, 26.86±3.96) in RAW 264.7 cells, respectively.

The antineuroinflammatory effect of *Moringa oleifera* leaves was mediated vin inhibition of p-38 protein expression, nuclear factor kappa-B transactivation and tumor necrosis factor-α release.

Keywords: *Moringa oleifera*, Antineuroinflammatory effect, Cyclooxygenase, Cognitive deficit

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Adewale C. Bakre

CERTIFICATION

I certify that this work was carried out by Mr. A. G. Bakre in the Department of Pharmacology and Therapeutics, University of Ibadan, Ibadan, Nigeria.

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ALS	amyotrophic lateral sclerosis
AP	Activator Protein-1
ATCC	American Type Culture Collection
Aβ	amyloid-β
BACE	β-site APP–cleaving enzyme
BAFF	B-cell activating factor
BDNF	brain-derived neurotrophic factor
CD	cluster differentiation
CDK	cyclin-dependent kinase
CR	complement receptor
DAMP	damage associated molecular pattern
DCF-DA	2', 7' dichloroflouresccin diacctate
DMEM	Dulbecco's Modified Essential Medium (DMEM)
EPM	Elevated plus maze
ERK	extracellular signal-regulated kinase
FADD	Fas-associated protein with a death domain
FHI	Forest Herbarium Ibadan
FRIN	Forestry Research Institute of Nigeria
GC-MS	Gas chromatography-mass spectrophotometry
HPLC-DAD	High performance liquid chromatography-Diode Array Detector (DAD).
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon-gamma
IgE	immunoglobin E
IRAK	IL-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase (JNK1/2/3
LFA-1	leucocyte function-associated antigen-1
LTβR	lymphotoxin β receptor
MAP	mitogen activated protein kinase (MAP kinase)

MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
MD	myeloid differentiation factor
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
NADPH	nicotinamide adenine dinucleotide phosphate
NFT	neurofibrillary tangles
NFκB	nuclear factor-kappa B
NIB	Novelty Induced Behavior
NIH	National Institute of Health
NLR	Nod-like receptor
OTC	Over The Counter
PAMPs	pathogen-associated molecular patterns
PHOX	phagocyte oxidase
PI3	phosphoinositide 3-kinase
PKC	protein kinase C
PTZ	pentylenetetrazole
PVDF	polyvinylidene fluoride
RANTES	regulated upon activation, normal T-cell expressed and secrete
STAT	signal transducer and activator of transcription
TAM	Traditional African Medicine
TCR	T cell receptors
TIR	Toll/interleukin-1 receptor
TLR4	toll like receptor
TRKB	tropomyosin-related kinase
VCAM-1	vascular cell adhesion molecule-1

DEDICATION

To the lovely babes in my life

Oluwaseun, my wife

Eyitemi, Eyitoluwa, and Eyilolufunmi my daughters

You babes are my joy

CHAPTER ONE

INTRODUCTION

1.1 Global burden of Neurodegenerative diseases

Neurodegenerative diseases include a wide range of incurable and debilitating conditions that result in progressive degeneration or death of nerve cells in human brain. The global burden of neurodegenerative diseases has continued to increase yearly accounting for at least 15% of the burden of diseases (Shrestha *et al.*, 2014). Most neurodegenerative diseases cause problems with movement (ataxias), or mental functioning (dementias). Alzheimer's disease (AD), a neurodegenerative disease is the world's most common dementing illness, affecting over 150 million people worldwide (Heneka *et al.*, 2015). This debilitating disease has remained incurable after several decades of research. It is the fifth leading cause of death for people of age ≥65 and a leading cause of morbidity (Alzheimer's-Association, 2015). Although research has revealed a great deal about AD, much is yet to be discovered about the precise biologic changes that cause the disease. Epidemiologic and laboratory evidence attribute the progression of the disease to inflammation (Sastre *et al.*, 2003). The pathogenic importance of neuroinflammation in AD is becoming increasingly evident.

Neuroinflammation is a defense mechanism aimed at protecting the central nervous system (CNS) against infectious insults and injury (Spencer *et al.*, 2012). It constitutes a beneficial process in most cases and ceases once the threat has been eliminated and homeostasis has been restored (Glass *et al.*, 2010). However, sustained neuroinflammatory processes may contribute to the cascade of events culminating in the progressive neuronal damage observed in many neurodegenerative disorders, most notably Parkinson's disease (PD) and Alzheimer's disease (AD) (McGeer and McGeer, 2003; Hirsch *et al.*, 2005).

Acute inflammatory diseases in the brain are caused by injury or trauma, while the chronic ones also referred to as neurodegenerative diseases in most cases do not have specific cause but some have established autoimmunity involvement. Inflammatory process has a significant participation in host defense against infectious agents and injury, but it is implicated in pathophysiology of many chronic diseases. The innate immune cells mediate acute inflammation and interact with adaptive immune cells via the inflammatory mediators to orchestrate aspects of the acute and chronic inflammation that underlie many diseases including AD.

Putative anti-inflammatory and neuroprotective agents that can affect the neuropathology of various neurodegenerative diseases are confounding. This could be as a result of the ambiguities and gaps in knowledge of neuroinflammation and neurodegeneration. Although neuroinflammation is evident in many chronic neurodegenerative diseases such as epilepsy, Alzhemiers disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and stroke, it is the inflammatory response that predisposes or exacerbates the neuropathology. As a result, the use of non-steroidal anti-inflammatory drugs, such as ibuprofen, has been proposed to delay or even prevent the onset of such neurodegenerative disorders (Casper *et al.*, 2000; Chen *et al.*, 2003). Epidemiologic studies have indicated that the risk for developing AD was reduced in regular users of anti-inflammatory drugs (Vlad *et al.*, 2008). However, majority of drug treatments only ameliorate the symptoms of these neurodegenerative disorders rather than preventing the underlying degeneration of neurons. Consequently there is a desire to develop novel therapies capable of preventing the progressive loss of specific neuronal populations that underlie pathology in these diseases (Legos *et al.*, 2002; Narayan *et al.*, 2002).

1.2 Study rationale

The therapeutic approaches for neurodegenerative diseases are symptomatic; in AD cholinergic transmission is enhanced using cholinesterase inhibitors (donepezil, rivastigmine and galanthamine). All of the approaches elude the holy grail of neurodegenerative diseases which is the retardation or inhibition of neurodegeneration. This is partly due to the inability of the intervention to affect the underlying course of the disease. Most of the current therapies for neurodegenerative diseases are symptomatic and the therapy for AD is particularly much less effective (Standaert and Young, 2012). Current drug treatments for neurodegenerative diseases including NSAIDs (non-steroidal anti-inflammatory drugs) only treat the symptoms or can delay the onset of disease rather than preventing the underlying degeneration of neurons (Casper *et al.*, 2000; Chen *et al.*, 2003).

With the increasing burden of AD's mortality and mordibity, there is a great need for the development of novel therapeutics. The ideal therapeutic target for AD should target the tightly controlled kinetics of amyloid-β peptides in the brain parenchyma. Aβ oligomers are considered to be the most neurotoxic form when added directly to neuronal cultures (Walsh *et al.*, 2002). The toxicity observed due to aggregation of Aβ *in vivo* could be mediated partly

via proinflammatory cytokines derived from activated microglia. Microglia cells are principally involved in clearance of Aβ. Microglial cells are the primary immune cells in the CNS and have similar actions to that of peripheral macrophages (Kreutzberg, 1996). Being immune cells, their primary functions are to promote host defense by destroying invading pathogens, removing deleterious debris, promoting tissue repair and facilitating tissue homeostasis, partly through their influence on surrounding astrocytes and neurons (Glass *et al.*, 2010). However, sustained, uncontrolled activation of microglia can lead to excess production of various factors that contribute to neuronal injury; most notably, nitric oxide, pro-inflammatory cytokines (IL-1β, TNF-α) (Gibbons and Dragunow, 2006), reactive oxygen species (ROS) (Wang *et al.*, 2006) and glutamate (Takeuchi *et al.*, 2006). As impaired microglial clearance has been identified as a disease-promoting factor, several attempts have been made to positively influence microglia by pharmacological, vaccine-based or gene-therapy strategies (Heneka *et al.*, 2015). The same receptors that sense pathogen-associated molecular patterns (PAMPs) such as bacterial lipopolysaccharide (LPS) and viral surface proteins are instrumental for responses triggered by Aβ. Combating AD by pharmacological modulation of microglia would have a great impact on the progression of neuroinflammation and consequently neurodegeneration. However, the development of such drug acting on microglia cells will propose a mixed opportunity of studying the interference of adaptive immune cells in AD.

During neurodegenerative diseases, peripheral immune cells, such as T cells, and CNS resident immune competent cells such as microglia as well as neurons, astrocytes and oligodendrocytes, release inflammatory mediators to recruit more peripheral immune cells including lymphocytes leading to CNS inflammation (Block and Hong, 2005). The key features of neuroinflammation are microglia activation, local production of inflammatory mediators, expression of Major Histocompatibility Complex (MHC) and adhesion molecules, release of free-radicals and recruitment of immune cells (Lucas *et al.*, 2006). Microglia activated via toll like receptor (TLR4) produce several mediators (TNF-α, IL-6, iNOS, COX and PGE_2) via NFκB. Macrophages and other cells of the innate immune system activate NFκB via triggering of toll like receptors (TLR) expressed on them by various molecules including components of the bacterial cell wall (lipopolysaccharide LPS), microbial nucleic acid (pathogen associated molecular pattern, PAMP or damage associated molecular pattern (DAMP) (Medzhitov, 2001; Takeda *et al.*, 2003). NFκB is essential for the induction of a wide variety of genes important for immune response including genes for TNF-α, IL-1 and IL-6, chemokines (macrophage inflammatory protein-1α, MIP-1α), RANTES (regulated upon

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activation, normal T-cell expressed and secreted) and adhesion molecules [E-selectin and VCAM-1(vascular cell adhesion molecule-1)] which collectively regulate recruitment of immune cells to sites once NFκB is activated (Zhang and Ghosh, 2001). Apart from TLR activation, stimulation of the receptors for TNF-α and IL-1 amplifies and extends the duration of innate response by strongly activating NFκB (O'Neil and Dinarello, 2000). NFκB transcriptionally induce enzymes which generates reactive intermediates (iNOS, inducible nitric oxide synthase) Beinke and Ley, 2004). NFκB activation also upregulates MHC proteins and CD80/86 on APC which is involved in the activation of T and B lymphocytes in adaptive immune response (Li and Verma, 2002). Additionally, NFκB is required for LTβR (lymphotoxin β receptor) regulation of peripheral lymphoid organogenesis and stimulation of B-cell differentiation and survival by B-cell activating factor (BAFF) (Claudio *et al.*, 2002). NFκB plays an important role in regulating the expression of antiapoptotic proteins (c-IAP-1/2, Al, Bcl-2 and Bel-X_1) and cell cycle regulator cyclin (D1) which increase cellular survival and proliferation respectively (Karin *et al.*, 2002). Dysregulation of NFκB can lead to the constitutive overproduction of proinflammatory cytokines, which are associated with chronic inflammatory disorders and has been implicated in cell transformation (Girardin *et al.*, 2003). It is the link between chronic inflammation and some cancers (Nonnark *et al.*, 2003).

Also, activation of microglia causes expression of MHC and adhesion molecules leading to recruitment of lymphocytes. Lymphocytes, particularly T cells have been detected in the brain of AD patients (Togo *et al.*, 2002). Studies have also shown that upregulation of T cells, with increased activity of Th-17 and Th-9 subsets and the cytokines (IL-9, IL-21 and IL-23) released from these T cells in AD (Saresella, 2011).

The idea of a protective autoimmunity of the brain has been developed in the last few years and has brought the devise of immunomodulatory therapies for neurodegenerative diseases. It involves the augmenting of the protective and regenerative aspects of the immune system for neuroprotection in brain diseases (Polazzi and Monti, 2010).

There is a growing interest in the neuroprotective effects of flavonoids which have been shown to be effective in protecting against both age-related cognitive and motor decline neurodegenerative disease *in-vivo* (Joseph *et al.*, 1999; Vauzour *et al.*, 2007; Williams *et al.*, 2008). Neuroprotective potential may reside in a number of physiological functions, including their antioxidant properties and ability to modulate intracellular signaling pathways including regulation of cell survival/apoptotic genes and mitochondrial function (Bastianetto *et al.*, 2000; Williams *et al.*, 2004; Spencer, 2009a; Spencer *et al.*, 2009b). Flavonoids and

their *in-vivo* metabolites have been shown to modulate signaling through phosphoinositide 3-kinase (PI3 kinase) and mitogen activated protein kinase (MAP kinase) pathways that are also critical signaling cascades for the control of inflammatory processes in the brain including the activation of microglia in response to cytokines and the induction of iNOS and nitric oxide production (Bhat *et al.*, 1998; Kaminska *et al.*, 2009; Spencer, 2009b); Wen *et al.*, 2011). As a consequence, flavonoids have been suggested as novel therapeutic agents for the reduction of the deleterious effects of neuroinflammation in the brain and thus also as potential preventive drugs for neurodegenerative disease development.

Flavonoids are secondary metabolites derived from plants. The anecdotal use of *Moringa oleifera* has taken a new dimension in Nigeria in the last few years. Various parts of the plant are used in ethnopharmacology and Traditional African Medicine (TAM) for centuries in treatment of diseases ranging from infectious diseases to chronic neurodegenerative diseases (Fahey 2005; Patel *et al.*, 2010; Mishra *et al.*, 2011). In recent years the World Health Organization (WHO), National Institute of Health (NIH) and many peer reviewed journals have published many articles indicating that *Moringa oleifera* might contain many promising immunity boosting principles. Of the 418 articles on *Moringa* on Pubmed, about 21 are on diabetes, 18 on cancer and 12 on immunity. Most of the sighted works tends to measure the protective ability of the plant in disease conditions. These are in tandem with the exorbitant use of the plant lately.

The Moringa tree has great use medicinally both as preventative and treatment. It is folk remedy for stomach complaints, catarrh, cancer, gastric ulcers, skin diseases and lowering blood sugar (Mishra *et al.*, 2011). It is also used in diabetes, fatigue, increase lactation, hay fever, impotence, edema, cramps, hemorrhoids, headaches, epilepsy, respiratory diseases, immune system booster, blood cleanser and blood builder (Fahey, 2005). Quite a number of patients also use it in the management of specific diseases like hypertension, diabetes, epilepsy among others. Convulsion is one of the many disorders which *Moringa oleifera* is used for suggesting that it may have centrally mediated effect.

Moringa oleifera, otherwise known as the 'miracle plant' is just one of the many herbal remedies claimed to have several benefits with very little information on scientific proofs. These herbal drugs seem to be making their way to orthodox medical practice as they are now made into various pharmaceutical dosage forms and sold almost at every corner as Over The Counter (OTC) drugs.

Moringa oleifera is used as food and drug by many people for many reasons. it is very important to know the effect the plant has on behavior and other CNS parameters. With the significant advances over the past two decades in the fields of immunology and neurobiology, new avenues to explore the mechanism of these diseases have been provided.

1.3 Aim and objectives of the study

The aim of this study is to identify and isolate active principles with antineuroinflammatory property from the leaves of *Moringa oleifera*

The specific objectives are to:

I. Evaluate the neuropharmacological activities of the ethanol extract of *Moringa oleifera* leaf.

II. Investigate bioactivity guided isolation of compounds from the extract of *Moringa oleifera*

III. Evaluate the mechanisms of antineuroinflammatory and immunomodulatory actions of isolated compounds on microglia and macrophage cell lines in LPS-induced neuroinflammation.

IV. Evaluate the effect of the extract of *Moringa oleifera* on proliferation, apoptosis, and homing pattern in pre-stimulated T-cells.

CHAPTER TWO

LITERATURE REVIEW

2.1 Inflammation

Inflammation has been known and documented as far back as the 1st century AD. Then, it was understood that tissue response to injury resulted in rubor (redness, due to hyperemia), tumor (swelling, caused by increased permeability of the microvasculature and leakage of protein into the interstitial space), calor (heat, associated with the increased blood flow and the metabolic activity of the cellular mediators of inflammation), and dolor (pain, in part due to changes in the perivasculature and associated nerve endings). Loss of function or dysfunction of the organs (*Functio laesa*), the fifth characteristic of inflammation was included by Rudolf Virchow in the 1850s. By the late 19th century, Elie Metchnikoff introduced the concept of phagocytosis, a fundamental aspect of innate immunity in inflammation after watching protozoa engulf particulate matter and examining blood leukocytes ingest foreign bodies. Metchnikoff later received Nobel Prize for Physiology or Medicine in 1908 for this discovery, jointly with Paul Ehrlich for his work on humoral immunity, a key component of adaptive immunity.

Inflammation provides a unifying pathophysiological mechanism underlying many chronic disease including diabetes, cardiovascular disease, certain cancers and bowel diseases, arthritis, neurodegeneratives, epilepsy to mention just a few. A common pathophysiologic scenario applies in the progression of many of these diseases (Libby *et al*., 2002). Some of the predispositions to chronic inflammation diseases are aging in population, conquest by communicable diseases and changing lifestyles.

2.2 Innate and Adaptive Immunity

The perspective of inflammation in the 21st century provides a detailed knowledge of the cells and mediators that produce the characteristic signs of inflammation as observed by the ancients. The response mechanisms of the host can be divided into two distinct, but inextricably linked, pathways; innate and adaptive pathways (Hansson *et al*., 2002). The innate response detect a broad range of molecular patterns (pathogen-associated molecular

CHAPTER TWO

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patterns, PAMPs) and (damage associated molecular pattern, DAMP). Macrophages usually over express these receptors which detects this molecular patterns; including Toll-like receptors. Engagement of Toll-like receptors results in activation of nuclear factor-kappa B (NFκB) and mitogen-activated protein kinase (MAPK) pathways (Karin, 2009). Ligation of Toll-like receptors can also heighten phagocytosis, production of reactive oxygen species, and release of cytokines, autacoids, and lipid mediators that coordinate and amplify the local inflammatory response. Innate immune response is rapid but lacks structural specificity and memory while the adaptive immune response, mounts a slower and specific response with memory (Tufecki *et al.*, 2011). The adaptive immune response requires the recognition of specific molecular structures which depends on the generation of large numbers of antigen receptors expressed on T and B-cells. Immunoglobulins are free B cell receptors. T cell receptors (TCR) becomes activated when they recognize the foreign antigen presented to them, and initiate responses that target precisely that antigen, including a direct attack against the antigen presenting cell by cytotoxic T-cells, stimulation of more antibody production by B-cells, and induction of a local inflammatory response ie necrosis. T helper cells can differentiate into at least two subtypes of T helper (Th) cells (Th1 and Th2). Th1 cells release several cytokines including interferon-gamma (IFNγ), a prominent cytokine which coordinates crosstalk between immunity (both innate and adaptive) and inflammatory responses by stimulating the macrophage to increase its production of a broad range of mediators including autacoids, reactive oxygen species, lipid species, and pro-inflammatory cytokines. Also, Th2 cells release several cytokines which are involved in stimulating B-cell maturation into antibody-producing plasma cells and promotion of B-cell class-switching to increase production of immunoglobin E (IgE) antibodies. Although, Th2 cells aids the recruitment and activation of mast cells involved in pathophysiology of chronic inflammation it also produces cytokines with antiinflammatory properties such as interleukin-10 (IL-10) (Hansson *et al.*, 2002).

2.3 Ideal Model of Chronic Inflammatory Disease

According to this model, signals from the innate and adaptive immune systems interact and converge on two prototypic cell types: an epithelial cell and a mesenchymal cell of the affected organs. These signals orchestrate a repertoire of tissue responses such as recruitment of leukocytes involved in chronic inflammation, extracellular matrix remodeling, cellular proliferation or death, and angiogenesis. While the diseases may manifest in very different ways based on organ involved, the same fundamental mechanisms and mediators drive the

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disease process. Helper T-cells abound in the lesions of chronic inflammation in many organs. The mononuclear phagocyte, cloaked variously as a foam cell, osteoclast, histiocyte, microglia, or alveolar macrophage, also characteristically populates such lesions. The basic aspects of inflammation involve selective and sequential migration of blood cells into tissues and then local activation and interaction of these blood-based cells with resident tissue cells (Tufecki *et al.*, 2011). Some conditions display only limited elements of the classic inflammatory processes while in other conditions, key inflammatory mediators dominate but without the context of the classic inflammatory mechanisms. For example, in Alzheimer's disease, blood cells do not migrate into the brain tissue, but a resident monocytic cell (microglia cell) is activated locally expressing pro-inflammatory mediators. The microglia participates prominently in innate immune responses of Alzheimers. The resultant responses in either case can, in time, impair the function of the organ or tissue involved.

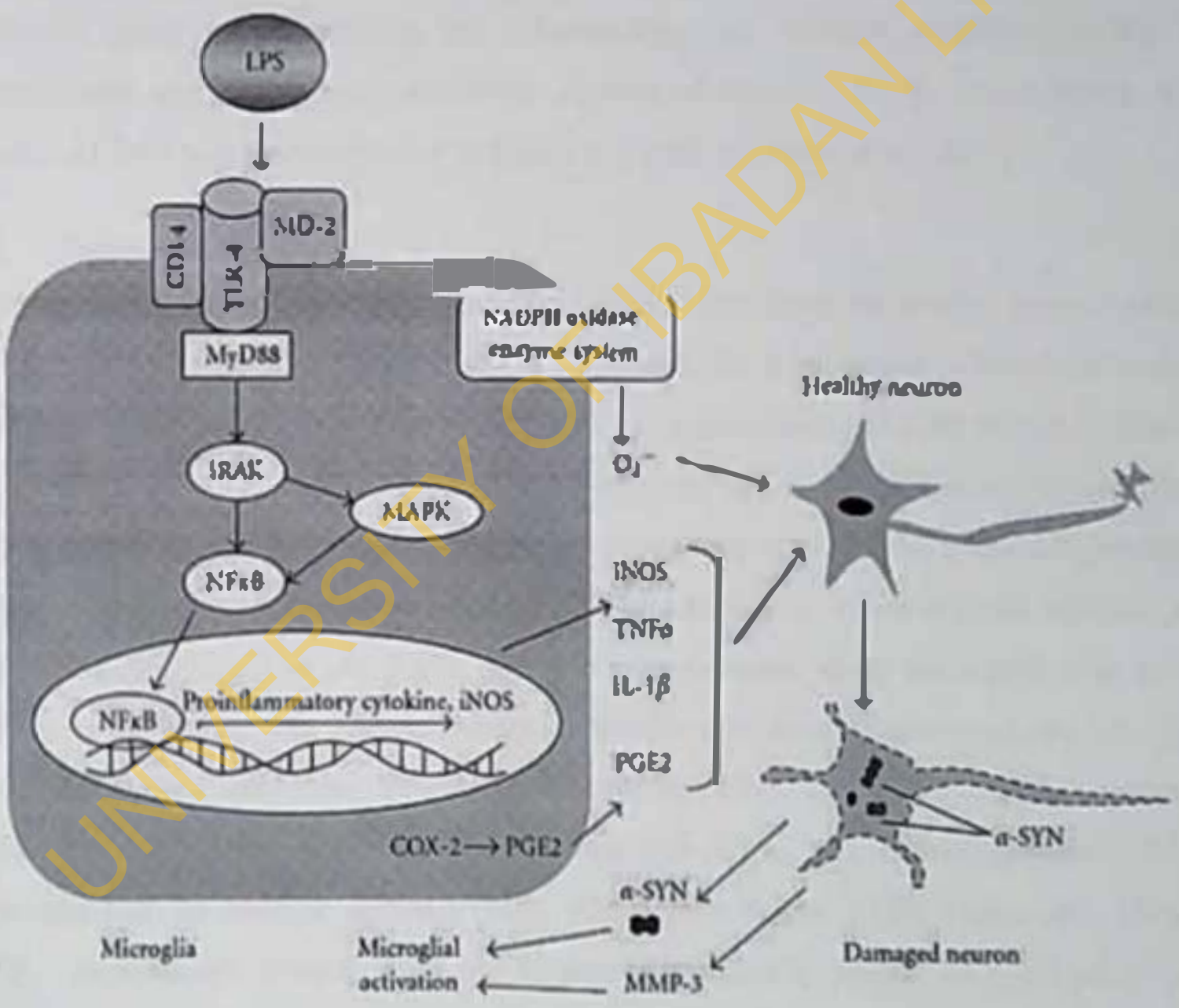

Figure 2.1: Simplified schematic representation of the link between LPS-induced microglia activation, inflammatory mediators, and dopaminergic neurodegeneration. Microglia responds to pathogens, proinflammatory cytokines, neuronal dysfunction, and cellular debris after injury or necrosis (Tufekci *et al.*, 2011).

2.4 Immune and Inflammatory Mechanisms in the Initiation and Progression of Chronic Diseases

The cells of the immune system are involved in the initiation of most chronic diseases. However, most neurodegenerative diseases attribute their progression to these cells of immunity. Once present and active in tissues, these cells of the innate immune system elaborate reactive oxygen species, cytokines, procoagulants, and other small molecules that amplify and sustain the inflammatory response. The resident local epithelial and mesenchymal cells both respond to pro-inflammatory signals elaborated by the mononuclear phagocytes and, when thus activated, can actively participate in propagating the inflammatory response by generating a similar spectrum of mediators as the "professional" phagocytes (McGeer and McGeer, 2003). The palette of the cytokines and other pathogenic proteins expressed in response include IL-1, IL-6, IL-18, TNF, M-CSF, MCP-1, intercellular adhesion molecule-1 (ICAM-1) etc. The inflammatory mediator CD40 ligand (CD40L or CD154) has a particular place in perpetuating the inflammatory and immune responses during the development and progression of chronic diseases (Saresella, 2010). More recent work localized CD40 to macrophages and its ligand to T cells (Shrestha *et al.*, 2014)

2.5 Neuroinflammation

Neuroinflammation is a defense mechanism aimed at protecting the central nervous system (CNS) against infectious insults and injury. In most cases, it constitutes a beneficial process that ceases once the threat has been eliminated and homeostasis has been restored (Glass *et al.*, 2010). However, sustained neuroinflammatory processes may contribute to the cascade of events culminating in the progressive neuronal damage observed in many neurodegenerative disorders, most notably Parkinson's disease (PD) and Alzheimer's disease (AD) (McGeer and McGeer, 2003; Hirsch *et al.*, 2005), and also with neuronal injury associated with stroke (Zhang *et al.*, 2006). The process principally involve activation of astrocytes and microglia by inflammatory mediators as proven in various CNS pathologies, including brain inflammation, trauma, ischemia, stroke, brain infections, and neurodegenerative CNS disorders such as multiple sclerosis (MS), Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS) (Amor *et al.*, 2012).

Microglia are the resident immune cells in the central nervous system and are now considered to be the primary component of the brain immune system. In neuroinflammation, microglia become activated, undergo a change in morphology, and release various cytotoxic mediators,

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such as nitric oxide (NO), tumour necrosis factor-alpha (TNFα), interleukin-1β (IL-1β), prostaglandin E2, (PGE_2), and reactive oxygen species (ROS). Overproduction of these mediators has been shown to be toxic to neurons and results in a vicious and self-propagating cycle of neuronal death (Olajide *et al.*, 2013). As such, the use of non-steroidal anti-inflammatory drugs, such as ibuprofen, has been proposed to delay or even prevent the onset of such neurodegenerative disorders (Casper *et al.*, 2000; Chen *et al.*, 2003) and epidemiologic studies have indicated that the risk for developing AD was reduced in regular users of anti-inflammatory drugs (Vlad *et al.*, 2008).

However, till date, most indicated drugs treat the symptoms of these neurodegenerative disorders rather than preventing the underlying degeneration of neurons. Consequently there is a desire to develop novel therapies capable of preventing the progressive loss of specific neuronal populations that underlie pathology in these diseases (Legos *et al.*, 2002; Narayan *et al.*, 2002). Microglial inflammation therefore serves as an important model for investigating potential therapeutic entities for slowing the progression of neuronal cell death in neurodegenerative disorders.

The transcription factor, nuclear factor kappa B (NFκB), has been shown to control inflammatory responses in microglia cells. Activation of NF-κB is triggered by phosphorylation and subsequent degradation of inhibitor of κB (IκB). This process subsequently leads to translocation of the free NFκB to the nucleus where it promotes the expression of proinflammatory genes such as the proinflammatory cytokines (TNFα, IL-6, IL-1β, etc.), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS). Mitogen-activated protein kinases (MAPK) are critical regulators of pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) during inflammation (Soliman *et al.*, 2012). Of the MAPKs, the p38 has been central to anti-inflammatory drug discovery for years due to its importance in the production of the proinflammatory cytokines and other mediators (Schlapbach and Huppertz, 2009). p38 produces inflammation by acting on MAPK-activated protein kinase-2 (MAPKAPK2 or MK2). MAPKAPK2 is stimulated in a wide range of inflammatory conditions and is a potential target for anti-inflammatory drug development (Duraisamy *et al.*, 2008). MK2 activation and expression have been shown to be increased in microglia cells stimulated with LPS and gamma interferon (Culbert *et al.*, 2006).

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2.6 Neurodegeneration and Inflammation

The adult brain contains 10^{11}–10^{12} neurons supported by at least twice as many neuroglial cells (Emerit *et al.*, 2004). There are different types of glial cells: oligodendrocytes, microglial, and astrocytes. These cells, especially microglia, are the equivalent monocytes/macrophages of the central nervous system (CNS). Recent studies have demonstrated a strong link between chronic inflammation and neurodegeneration. Alzheimer's disease (AD) is characterized by the death of cells in the hippocampus and the frontal cortex secondary to chronic inflammation. In Parkinson's disease (PD), chronic inflammation leads to loss of dopaminergic receptors in the substantia nigra. Amyotrophic lateral sclerosis (ALS) is another inflammatory condition in which motor neurons are ultimately destroyed. Multiple sclerosis (MS) is an autoimmune disorder in which inflammatory cells attack the myelin sheath. Although activation of an acute inflammatory event is a necessary self-defense mechanism of the CNS against foreign antigens, prolonged activation of the inflammatory response can lead to chronic inflammation and cell death (Campbell, 2004). The CNS has very limited, if any, regenerative capacity; therefore, it is very important to limit cell death in that region (Rossi and Cattaneo, 2002). Neural cell death occurs by either necrosis or apoptosis (Kanduc *et al.*, 2002). In necrosis, there is often a definitive temporal cause of the death of the cell. In apoptosis, the stimulus for death initiates a cascade of events that ultimately leads to cell destruction. Necrosis in the CNS generally follows an acute ischemic or traumatic injury to the brain (Emery *et al.*, 1998). Abrupt biochemical collapse in an area of the CNS leads to the generation of reactive oxygen species (ROS) and excitotoxins such as glutamate, calcium, and cytokines. The hallmark histologic features of necrotic cell death are mitochondrial and nuclear swelling, and chromatin dissolution. This ultimately leads to nuclear and cytoplasmic membrane degeneration (Kerr *et al.*, 1972). Apoptosis is also known as programmed cell death and often demonstrates histologic features of acute and chronic neurologic diseases (Yuan and Yanker, 2000). After an acute insult in the CNS, apoptosis often occurs in areas that are not as severely affected by the acute injury. Apoptosis is the secondary cause of the neuronal cell death after an acute CNS injury, such as ischemia (MacManus *et al.*, 1993). In contrast, in chronic neurodegenerative diseases; apoptosis is the predominant form of cell death (Smale *et al.*, 1995). In an apoptotic event, a cascade of biochemical reactions occurs, activating proteases that destroy molecules necessary for cell survival. Histologically, the cytoplasm condenses, mitochondria and ribosomes aggregate, the nucleus condenses, and chromatin aggregates. Within the apoptotic process, intracellular acidification occurs and ROS are generated. The

major executioners in apoptosis are proteases known as caspases (Alnemri *et al.*, 1996). Upstream caspases are activated by cell-death signals (eg. tumor necrosis factor). The upstream caspases activate downstream caspases that directly lead to the death of the cell (Shi, 2002). In the cascade of apoptosis, cytochrome T (from the mitochondrial electron transport chain) is released. Members of a group of proteins, known as the BCL-2 family, are either apoptotic or antiapoptotic. The balance of these proteins is crucial in stimulating or blocking the release of cytochrome T and initiating or blocking the apoptosis cycle (Gross *et al.*, 1999). In chronic neurodegenerative diseases, caspase-mediated apoptotic pathways have the dominant role in causing cell dysfunction and cell death (Friedlander, 1997)

2.7 Rodent Models of Neuroinflammatioo

In conventional transgenic animal models of AD, neuroinflammation is mainly known as a secondary response to sustained amyloid-β (Aβ) overproduction and deposition. It includes microglial activation and variable involvement of the complement system and production of cytokines (Wyss-Coray, 2006; Schwab *et al.*, 2010; Krstic and Knuesel, 2013). Altogether, in these models, the inflammatory response is incomplete and less severe compared to AD in humans (Wyss-Coray, 2006). Janelsins and colleagues detected early activation of inflammatory processes in the entorhinal cortex (but not hippocampus) of the triple transgenic model (3xTg) of AD at 3 months of age (Janelsins *et al.*, 2005). Interestingly, the neuroinflammation process was concurrent with the production and accumulation of intracellular Aβ but occurred prior to any significant extracellular Aβ plaque deposition, which manifests at about 12 months of age in the 3xTg mice (Janelsins *et al.*, 2005). Of note, this neuroinflammatory process was characterized by a selective trend of increasing expression of TNF-α and monocyte chemoattractant protein-1 (MCP-1), which was not detected for 21 other cytokines tested (Janelsins *et al.*, 2005). Moreover, a substantial microgliosis was detectable at 6 months of age. Although, this study provided valuable evidence for a contributory role of inflammatory factors like TNF-α and MCP-1 in AD pathology, the model system replicates the familial but not sporadic type of AD (Janelsins *et al.*, 2005).

An ideal disease model should recapitulate causes, lesions, and symptoms in a chronological order similar to the actual disease (Duyckaerts *et al.*, 2008). A faithful model to the inflammation hypothesis of AD should be an aged animal that recapitulates early chronic neuroinflammation prior to hyperphosphorylation of tau and Aβ plaque deposition. In rats, a neuroinflammatory process lasting more than 7 days is considered chronic

neuroinflammation (Moore *et al.*, 2009); and rodents older than 22 months are considered senescent (Burton and Johnson. 2012). The following table presents potential rodent models of AD that present early neuroinflammation in the disease process and are not genetically manipulated by mutations related to Aβ or tau production.

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Models	Predisposing factors/causes	Time of appearance of lesions		Signs (time detectable)
		hp-Tau	Aβ	
LPS	Peripheral immune challenge, chronic neuroinflammation	?	?	Fear memory (?) Spatial memory (?)
PolyI:C	Peripheral immune challenge, chronic neuroinflammation	3m (PHF, but not NFTs)	12m (APP depositions)	Spatial memory (20 m)
ICV-STZ	Disrupted insulin signaling, chronic neuroinflammation	6-7w	12w	Spatial memory Visual recognition memory (3w)
ICV-OKA	Inhibition of serine/threonine phosphatases 1 and 2A	2w (PHF, but not NFTs)	6w (Non-fibrillar Aβ deposits)	Spatial memory (?)
ICV-colchicine	Inhibition of tubulin formation/ microtubule breakdown	? (Tau dephosphorylation)	? (Amyloid plaque)	Spatial memory (14d to 21d)
p25 Tg	Upregulation of cPLA2, neuroinflammation	4w	8w	Contextual fear memory (6w)
IL-1β Tg	Chronic neuroinflammation	?	? (Increased clearance of amyloid plaques)	Contextual fear memory (12w)
Anti-NGF antibody Tg	Blockade of NGF signaling pathway	? (Neurofibrillary pathology)	? (Amyloid plaques)	Visual recognition memory (4 m); Spatial memory (9 m)

This table summarizes the suggested models of late-onset AD (LOAD) displaying neuroinflammation as one of the prominent pathological events (Abbreviations: ? unavailable data; LPS: lipopolysaccharide; PolyI:C: polyriboinosinic-polyribocytidilic acid; p25 Tg :p25 transgenic model; NGF:nerve growth factor; IL-1β Tg: interleukin-1β transgenic model; ICV: intracerebroventricular; STZ: streptozotocin; OKA: okadaic acid; hp-Tau: hyperphosphorylated tau; Aβ: amyloid-β; PHF:paired helical filaments; NFT:neurofibrillary tangles; cPLA2: cytosolic phospholipase 2; w: week; m:month). (Nazem *et al.*, 2015).

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Polyl:C	Peripheral immune challenge, chronic neuroinflammation	3m (PHF, but not NFTs)	12m (APP depositions)	Spatial memory (20 m)
ICV-STZ	Disrupted insulin signaling, chronic neuroinflammation	6-7w	12w	Spatial memory Visual recognition memory (3w)
ICV-OKA	Inhibition of serine/threonine phosphatases 1 and 2A	2w (PHF, but not NFTs)	6w (Non-fibrillar Aβ deposits)	Spatial memory (?)
ICV-colchicine	Inhibition of tubulin formation/ microtubule breakdown	? (Tau dephosphorylation)	? (Amyloid plaque)	Spatial memory (14d to 21d)
p25 Tg	Upregulation of cPLA2, neuroinflammation	4w	8w	Contextual fear memory (6w)
IL-1β Tg	Chronic neuroinflammation	?	? (Increased clearance of amyloid plaques)	Contextual fear memory (12w)
Anti-NGF antibody Tg	Blockade of NGF signaling pathway	? (Neurofibrillary pathology)	? (Amyloid plaques)	Visual recognition memory (4 m); Spatial memory (9 m)

This table summarizes the suggested models of late-onset AD (LOAD) displaying neuroinflammation as one of the prominent pathological events (Abbreviations: ? unavailable data; LPS: lipopolysaccharide; PolyI:C: polyriboinosinic-polyribocytidilic acid; p25 Tg :p25 transgenic model; NGF:nerve growth factor; IL-1β Tg: interleukin-1β transgenic model; ICV: intracerebroventricular; STZ: streptozotocin; OKA: okadaic acid; hp-Tau: hyperphosphorylated tau; Aβ: amyloid-β; PHF:paired helical filaments; NFT:neurofibrillary tangles; cPLA2: cytosolic phospholipase 2; w: week; m:month). (Nazem *et al.*, 2015).

2.7.1 LPS INDUCED NEURODEGENERATION

Toll like receptors (TLRs) recognize invariant microbial molecules, including components of the bacterial cell wall such as lipopolysaccharide (LPS) and microbial nucleic acids (Takeda *et al.*, 2003). The proinflammatory cytokine, TNF-α kills neurons and is elevated in the brains of patients with neuroinflammatory diseases. LPS is a potent inducer of TNF-α and its administration results in significant microglia activation and sustained elevation of TNF-α in both the substantia nigra and the corpus striatum, even several weeks after the sole initial exposure. (Ling *et al.*, 2004). Microglia, the sentinel cell of the brain responds to pathogens, proinflammatory cytokines, neuronal dysfunction, and cellular debris after injury or necrosis. These cells are at the forefront of the defense mechanisms that could set the conditions for repair or contribute to neuronal damage. Such equilibrium might depend on the expression and function of specific TLRs and how they are activated by endogenous and exogenous ligands and signals. Recognition of such signals leads to transcriptional activation of innate immune genes. Bacterial endotoxin LPS is a TLR4 potent stimulator of macrophages, monocytes, microglia, and astrocytes causing release of various immunoregulatory and proinflammatory cytokines and free radicals. Neurons do not express functional TLR4. Thus, LPS does not appear to have a direct effect on neurons, making it an ideal activator to study indirect neuronal injury mediated by microglia activation (Dutta *et al.*, 2008). LPS binds to its intermediate receptor CD14 and in concert with TLR4 and accessory adaptor protein MD2 causing a triggers the activation of kinases of various intracellular signaling pathways. The MyD88-dependent cascade initiates NFκB activation through the IKKs and/or the MAPK pathway, leading to the upregulated expression of proinflammatory cytokines (TNFα, IL-1β) and increased production of other inflammatory mediators (NO and PGE_2, synthesized by iNOS and COX-2, respectively.). These soluble mediators collectively damage neuron. MMP-3and αSYN released by stressed neurons aggravate microglial activation.

The inflammatory process in the brain, accompanied by changes in the levels of proinflammatory cytokines and neurotrophins, along with the presence of activated microglia, has gained much attention in the area of neurodegenerative diseases. Activated microglia produce either neuroprotective or neurotoxic factors. Unlike the direct death of neurons caused by neurotoxins, endotoxin mediated neurodegeneration seems to result from indirect neuronal death due to inflammatory reactions. Bacterial endotoxin LPS is capable of activating glial cells, predominantly microglia, to release a wide variety of proinflammatory and neurotoxic factors that include reactive oxygen and nitrogen species, proinflammatory

cytokines, and lipid mediators (Long-Smith *et al.*, 2009). Cell culture studies have a number of mechanisms by which inflammatory-activated microglia and astrocytes kill neurons (Brown and Neher, 2010). Studies employing enzyme inhibitors, neutralizing antibodies, specific inhibitors of inflammatory signaling pathways, and knockout animals have identified the soluble factors and signaling molecules involved in microglia activation as major contributors to the endotoxin mediated neurodegeneration (Dutta *et al.*, 2008). The toll encoding gene was first identified in Drosophila embryos, where it has a role in dorsoventral axis determination (Anderson *et al.*, 1985; Hashimoto et al., 1988). Many organisms have multiple homologues of the Drosophila toll gene, which is highly conserved among species (Medzhitov *et al.*, 1997). In vertebrates, TLR (Toll-like receptors) recognize pathogen associated molecular patterns of bacteria, fungi, and viruses and play roles in host defense mechanism. TLR4 takes part in recognition of strongly conserved patterns of gram-negative cell wall components, LPS and discriminates indigenous from foreign molecules (Ganglo *et al.*, 2003). In TLR4 signaling, TLR4 must first associate with its extracellular binding partner, myeloid differentiation factor 2 (MD-2), before ligands can bind to the TLR4-MD-2 complex (Shimazu *et al.*, 1999; Nagai *et al.*, 2002). The TLR4-MD-2-Ligand complex forms a heterodimer with another TLR4-MD-2 ligand complex and the signal is transferred to the TLR4's Toll/interleukin-1 receptor (TIR) domain. The signal is then further transduced via an unknown mechanism (Ganglo *et al.*, 2003; Kobayashi *et al.*, 2006). The signal is then transmitted to two separate pathways; MyD88 path activating NF-κB and IFN-β (TRIF) path induced by adaptor for Toll/IL-1 receptor. In the MyD88 path, MyD88 adaptor-like protein (Mal or TIRAP) mediates the TIR-TIR association between TLR4 and MyD88 (Horng, *et al.*, 2002). This results in an interaction between IL-1 receptor-associated kinase (IRAK) and MyD88 causing activation of the cascade that leads to the phosphorylation of NF-κB transcription factors (RelA and p50 heterodimers) and Activator Protein-1 (AP-1) which regulates expression of proinflammatory cytokines (Akira *et al.*, 2006; Kawai and Akira, 2007). In the other pathway, TRIF and TLR4 require an adaptor molecule called TRAM (TRAF3- or TRAF6) for transduction of its signal (endocytosis of the TLR4 receptor complex) (Rowe *et al.*, 2006; Tanimura *et al.*, 2008). After incorporation of TRAF3- or TRAF6, TRIF forwards the signal to the TRAM adaptor molecules (TRIF-binding kinase-(TBK-) IKK or RIP) (Hacker *et al.*, 2006). TBK-IKK terminates interferon regulatory factor-3 (IRF-3) dimerization and translocation into nucleus to induce IFNβ synthesis; in this way, TBK-IKK regulates cellular response to inflammation (Poikonen *et al.*, 2009). On the other

hand, RIP which interacts with TRAF6 activates NF-κB through TAK1, which operates the same as in the MyD88 pathway, causing late phase NF-κB activation (Hacker *et al.*, 2006).

2.7.1.1 Nitric Oxide

Nitric oxide (NO) is an important messenger molecule involved in many normal physiological functions such vasodilation of blood vessels and mediating communication between cells of the nervous system. It is a gas produced from L-arginine by different isoforms of nitric oxide synthase (NOS) involved in variety of physiological systems. In addition to its physiological actions, free radical activity of NO can cause cellular damage through a phenomenon known as nitrosative stress (Knott and Bossy-Wetzel, 2009). There are several evidence from studies supporting the notion that excessive production and accumulation of NO in the LPS-induced neuroinflammation leads to neurodegeneration (Dutta *et al.*, 2008). Thus, increased NO availability subsequent to iNOS induction seems to play an important role in the initial phase of neurodegeneration. Hunter *et al.* (2009) have suggested that permanent expression of the iNOS plays a role in the progressive loss of neurons but not the initial loss induced by LPS. Although the mechanism of NO mediated neurodegeneration still remains uncertain, it has been suggested that NO contributes to LPS-induced neurodegeneration through several mechanisms. NO has been shown to modify protein function by nitrosylation and nitrotyrosination, contribute to glutamate excitotoxicity, inhibit mitochondrial respiratory complexes, participate in organelle fragmentation, and mobilize zinc from internal stores (Knott and Bossy-Wetzel, 2009; Tsang and Chung, 2009). NO can react with superoxide radicals to form peroxynitrite radicals that are short-lived oxidants and highly damaging to neurons (Szabo *et al.*, 2007; Dutta *et al.*, 2008). Mitochondrial injury is prevented by treatment with L-N(6)-(liminoethyl) lysine, an iNOS inhibitor, suggesting that iNOS-derived NO is also associated with the mitochondrial impairment (Choi *et al.*, 2009). NO inhibits cytochrome oxidase in competition with oxygen, resulting in glutamate release and excitotoxicity (Brown and Neher, 2010). The main cellular source of NO in the CNS is microglia whereas astroglia constitute the main defense system against oxidative stress. However, under patbological or chronic inflammatory conditions, astroglial cells may also release neurotoxic mediators.

2.7.1.2 Reactive Oxygen Species

A large body of evidence supports the involvement of oxidative stress in the pathogenesis of neurodegenerative diseases (Tsang and Chung, 2009). Besides NO, ROS generated by activated glia, especially microglia are major mediators of the neurodegeneration caused by inflammation (Dutta *et al.*, 2008). ROS can cause lipid peroxidation, protein oxidation, DNA damage, and mitochondrial dysfunction. LPS-induced ROS production in microglia is mediated by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a multisubunit enzyme (Brown and Neher, 2010). This complex is responsible for the production of both extracellular and intracellular ROS by microglia. Activation of microglia NADPH oxidase causes neurotoxicity through two mechanisms. Firstly, extracellular ROS released from activated microglia are directly toxic to neurons. Secondly, intracellular ROS amplifies the production of several proinflammatory and neurotoxic cytokines compounds such as TNFα, prostaglandin E2 (PGE_2), COX-2, and IL-1β (Wang *et al.*, 2004). The activation of the phagocyte NADPH oxidase (PHOX) by cytokines, LPS, or arachidonic acid metabolites causes microglia proliferation and inflammatory activation. PHOX is a key regulator of inflammation. Pharmacologic inhibition of NADPH oxidase provides protection against LPS-induced neurotoxicity and PHOX knockout mice have been shown to be resistant to LPS-induced loss of neurons (Qin *et al.*, 2004; 2005a). Gene expression and release of TNFα was much lower in PHOX−/− mice than in control PHOX+/+ mice (Qin *et al.*, 2004). By injecting LPS into the striatum of wild type and Noxl knockout mice, it has been shown that Noxl, a subunit of NADPH oxidase, also enhances microglia production of cytotoxic nitrite species and promotes loss of presynaptic proteins in striatal neurons (Ch'eret *et al.*, 2008). Activation of PHOX alone causes no cell death, but when combined with expressed iNOS, it results in extensive neuronal cell death via the production of peroxynitrite (Brown and Neher, 2010). The relationship between the signaling pathway downstream of TLR4, after LPS stimulation, and the activation of the oxidase remains elusive. Using mice lacking a functional TLR4, it has been demonstrated that TLR4 and ROS work in concert to mediate microglia activation (Qin *et al.*, 2005b). Both TLR4(−/−) and TLR4(+/+) microglia display a similar increase in extracellular superoxide production when exposed to LPS. These data indicate that LPS-induced superoxide production in microglia is independent of TLR4 and that ROS derived from the production of extracellular superoxide in microglia mediates the LPS-induced TNF-α response of both the TLR4-dependent and independent pathway (Qin *et al.*, 2005b).

The integrin CD11b/CD18 (MAC1, macrophage antigen complex-1) pattern recognition receptor mediates LPS induced production of superoxide by microglia (Pei *et al.*, 2007).

MAC1 is a TLR4-independent receptor for the endotoxin LPS. MAC1 is essential for LPS-induction of superoxide in microglia, implicating that MAC1 acts as a critical trigger in microglia-derived oxidative stress during inflammation mediated neurodegeneration.

2.7.1.3 Proinflammatory Cytokines

Cytokines are small, nonstructural proteins with molecular weights ranging from 8 to 40,000 d. Originally called lymphokines and monokines to indicate their cellular sources, it became clear that the term "cytokine" is the best description, since nearly all nucleated cells are capable of synthesizing these proteins and, in turn, of responding to them. There is no amino acid sequence motif or three-dimensional structure that links cytokines. Rather, their biological activities allow grouping them into different classes. For the most part, cytokines are primarily involved in host responses to disease or infection, and any involvement with homeostatic mechanisms has been less than dramatic. Although cytokines are similar to hormones, they differ in that they are synthesized by nearly all cells accounting for less amount of the synthetic output, whereas hormones are produced by highly specialized cells accounting for primarily all the cells synthetic output. Also, hormones are expressed in response to homeostatic control signals, many of which are part of a daily cycle, but in contrast, most cytokine genes are not expressed (at least at the translational level) unless specifically stimulated by noxious events. In fact, it has become clear that the triggering of cytokine gene expression is nearly identical to "cell stressors." For example, ultraviolet light, heat-shock, hyperosmolarity, or adherence to a foreign surface activate the mitogen-activated protein kinases (MAPKs), which phosphorylate transcription factors for cytokine gene expression. Of course, infection and inflammatory products also use the MAPK pathway for initiating cytokine gene expression. One concludes then that cytokines themselves are produced in response to "stress," whereas most hormones are produced by a daily intrinsic clock (Janeway *et al.*, 2001).

There are presently 18 cytokines with the name interleukin (IL). Other cytokines have retained their original biological description, such as tumor necrosis factor (TNF). Another way to look at some cytokines is their role in infection and/or inflammation. Some cytokines clearly promote inflammation and are called proinflammatory cytokines, whereas other cytokines suppress the activity of proinflammatory cytokines and are called anti-inflammatory cytokines. For example, IL-4, IL-10, and IL-13 are potent activators of B lymphocytes. However, IL-4, IL-10, and IL-13 are also potent anti-inflammatory agents

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They are anti-inflammatory cytokines by virtue of their ability to suppress genes for proinflammatory cytokines such as IL-1, TNF, and the chemokines (Owen *et al.*, 2013). Interferon (IFN)-γ is another example of the pleiotropic nature of cytokines. Like IFN-α and IFN-β, IFN-γ possesses antiviral activity. IFN-γ is also an activator of the pathway that leads to cytotoxic T cells. However, IFN-γ is considered a proinflammatory cytokine because it augments TNF activity and induces nitric oxide (NO). Therefore, listing cytokines in various categories should be done with an open mind, in that, depending on the biological process, any cytokine may function differentially (Janeway *et al.*, 2001).

The concept that some cytokines function primarily to induce inflammation while others suppresses inflammation is fundamental to cytokine biology and also to clinical medicine. The concept is based on the genes coding for the synthesis of small mediator molecules that are up-regulated during inflammation. For example, genes that are proinflammatory are type II phospholipase (PL) A2, cyclooxygenase (COX)-2, and inducible NO synthase. These genes code for enzymes that increase the synthesis of platelet-activating factor and leukotrienes, prostanoids, and NO. Another class of genes that are proinflammatory are chemokines, which are small peptides (8,000 d) that facilitate the passage of leukocytes from the circulation into the tissues. The prototypical chemokine is the neutrophil chemoattractant IL-8. IL-8 also activates neutrophils to degranulate and cause tissue damage. IL-1 and TNF are inducers of endothelial adhesion molecules, which are essential for the adhesion of leukocytes to the endothelial surface prior to emigration into the tissues. Taken together, proinflammatory cytokine-mediated inflammation is a cascade of gene products usually not produced in healthy persons. What triggers the expression of these genes? Although inflammatory products such as endotoxins trigger it, the cytokines IL-1 and TNF are particularly effective in stimulating the expression of these genes. Moreover, IL-1 and TNF act synergistically in this process. Whether induced by an infection, trauma, ischemia, immune-activated T cells, or toxins, IL-1 and TNF initiate the cascade of inflammatory mediators by targeting the endothelium (Owen *et al.*, 2013).

Anti-inflammatory cytokines block this process or at least suppress the intensity of the cascade. Cytokines such as IL-4, IL-10, IL-13, and transforming growth factor (TGF)-β suppress the production of IL-1, TNF, chemokines such as IL-8, and vascular adhesion molecules. Therefore, a "balance" between the effects of proinflammatory and anti-inflammatory cytokines is thought to determine the outcome of disease, whether in the short term or long term. In fact, some studies have suggest that susceptibility to disease is

genetically determined by the balance or expression of either proinflammatory or anti-inflammatory cytokines. However, gene linkage studies are often difficult to interpret. Nevertheless, the deletion of the IL-10 gene in mice results in the spontaneous development of a fatal inflammatory bowel disease. Deletion of the TGF-β1 gene also results in a spontaneous inflammatory disease. In mice deficient in IL-1 receptor antagonist (IL-Ra), spontaneous disease that is nearly identical to rheumatoid arthritis is observed (Owen *et al.*, 2013).

The synergism of IL-1 and TNF is a commonly reported phenomenon. Clearly, both cytokines are being produced at sites of local inflammation, and, hence, the net effect should be considered when making correlations between cytokine levels and severity of disease. There is also synergism between IL-1 and bradykinin as well as between IL-1 or TNF and mesenchymal growth factors. Most relevant to pain is the increase in prostaglandin (PG)-E_2 stimulated by IL-1 or the combination of IL-1 and TNF. IL-1 also lowers the threshold of pain primarily by increasing PGE_2 synthesis (Schweizer *et al.*, 1988).

Humans injected with IL-1 experience fever, headache, myalgias, and arthralgias, each of which is reduced by the coadministration of COX inhibitors (Smith *et al.*, 1991). One of the more universal activities of IL-1 is the induction of gene expression for type II PLA_2 and COX-2. IL-1 induces the transcription of COX-2 and seems to have little effect on the increased production of COX-1. Moreover, once triggered, COX-2 production is elevated for several hours and large amounts of PGE_2 are produced in cells stimulated with IL-1. Therefore, it comes as no surprise that many biological activities of IL-1 are actually due to increased PGE_2 production. There appears to be selectivity in COX inhibitors, in that some nonsteroidal anti-inflammatory agents are better inhibitors of COX2 than of COX-1. Similar to COX-2 induction, IL-1 preferentially stimulates new transcripts for the inducible type II form of PLA_2, which cleaves the fatty acid in the number 2 position of cell membrane phospholipids. In most cases, this is arachidonic acid. The release of arachidonic acid is the rate-limiting step in the synthesis of PGs and leukotrienes. IL-1 also stimulates increased leukotriene synthesis in many cells.

2.7.1.4 Cyclo-Oxygenase-2 and Prostaglandin E_2

Prostaglandins are potent autocrine and paracrine oxygenated lipid molecules that contribute appreciably to physiologic and pathophysiologic responses in brain and other organs (Climino *et al.*, 2008). Emerging data indicate that PGE_2 plays a central role in neurodegenerative diseases. PGE_2 signaling is mediated by interactions with four distinct G

protein-coupled receptors, EP1-4, which are differentially expressed on neuronal and glial cells throughout the CNS (Climino *et al.*, 2008). EP2 activation has been shown to mediate microglia-induced paracrine neurotoxicity as well as to suppress the internalization of aggregated neurotoxic peptides in microglia (Jin *et al.*, 2007). PGE_2 is produced at high levels in the injured CNS, where it is generally considered a cytotoxic mediator of inflammation. LPS upregulates the expression of COX-2 and increase the release of PGE_2 in cultured microglia (Dutta *et al.*, 2008). Double labeling using immunohistochemistry identified that activated microglia rather than inlact resting microglia are the main intracellular locations of COX-2 expression (Dutta *et al.*, 2008; Sui *et al.*, 2009). In vivo pharmacological inhibition of COX-2 activity protects nigral dopaminergic neuronal loss and decreases microglial activation induced by intracerebral LPS injection, supporting the role of COX-2 in the pathogenesis of neuroinflammation-mediated neurodegeneration (Hunter *et al.*, 2007; Li *et al.*, 2008; Sui *et al.*, 2009). Furthermore, there is *in-vitro* and *in-vivo* evidence that microsomal prostaglandin E synthase (mPGES-1) and COX-2 through concerted de-novo synthesis necessitate PGE_2 production in activated microglia. Activation of cultured spinal microglia via TLR4 produces PGE_2 and causes NO release from these cells, showing that COX-PGE_2 pathway is regulated by p38 and iNOS (Matsui *et al.*, 2010). These findings emphasize that p38 in spinal microglia is a key player among inflammatory mediators, such as PGE_2 and NO.

2.8 Microglial Cells

The adult human brain contains several trillions of neurons supported by at least twice as much neuroglial cell. There are at least three different types of glial or supporting cells: oligodendrocytes, microglial and astrocytes. The microglia cells are the equivalent of monocyes/macrophages in the central nervous system. Microglia cells represent 10% of the cells in the adult central nervous system (CNS) and are morphologically characterized by small somas and ramified processes. Following activation in response to infection, or during inflammation that occurs as part of the pathogenesis of diseases such as multiple sclerosis or as a result of CNS injury, local microglia cells undergo morphological changes that include shortening of cellular processes and enlargement of their somas. Microglia cells also respond to 'foreign' material such as aggregated amyloid-β (El Khoury *et al.*, 1996; Akiyama *et al.*, 2000; McGeer and McGeer, 2001). Parenchymal microglia cells are mycloid progenitor cells that can differentiate into macrophage-like or dendritic-like cells when stimulated with macrophage colony-stimulating factor (M-CSF) and therefore acquire antigen-presenting

properties (Minghetti, 2005). Activated microglia cells up-regulate the expression of cell-surface proteins (MHC class II molecules, CD11b and scavenger receptors) and produce cytokines (tumor-necrosis factor (TNF), interleukin-6 (IL-6) and IL-1) and chemokines (CXC-chemokine ligand 8 (CXCL8) and CC-chemokine ligand 3 (CCL3). In response to amyloid-β deposition in Alzheimer's disease, microglia cells express different cell-surface receptors and can differentiate into cells with varying properties. For example, they can gain phagocytic properties by expressing cell-surface scavenger receptor molecules or neurotoxic properties by increasing the production of reactive oxygen species (ROS). *In-vitro*, fibrillar amyloid-β, alone or with other activators, can stimulate the production of neurotoxic ROS by inducing the expression of NADPH oxidase and inducible nitric-oxide synthase (iNOS) by microglia cells and macrophages (Van Muiswinkel *et al.*, 1999; Ishii, *et al.*, 2000). Amyloid-β can also indirectly stimulate iNOS expression by neuronal cells and subsequent nitric oxide (NO)-mediated neuronal-cell apoptosis in response to microglial-cell-secreted TNF-α (Heneka *et al.*, 1998; Combs *et al.*, 2001). Both lipopolysaccharide (LPS) and amyloid-β-activated microglia cells cause neuronal-cell death in hippocampal sections. Nevertheless, there is no clear evidence for microglia cell neurotoxicity in vivo. It has been proposed that the clinical symptoms that occur as part of Alzheimer's disease pathogenesis are due to a gradual increase of amyloid-β levels above a threshold that is no longer controlled by endogenous microglia cell clearance. However, it is possible that dysfunction of microglia cells could also have a pathological role at early phases of the disease (Streit, 2004). There are several studies of Alzheimer's disease showing that microglia-cell activation can lead to amyloid-β clearance supporting the concept that an important immunotherapeutic avenue is through microglia-cell activation in a manner that leads to amyloid-β removal without toxicity (Nicoll *et al.*, 2003; Akiyama and McGeer, 2004). During and after phagocytosis of amyloid-β, microglia cells might express cell-surface MHC class II molecules and has been observed for amyloid-β-fibril-associated microglia cells from patients with Alzheimer's disease (Akiyama *et al.*, 2000; McGeer and McGeer, 2001). Furthermore, compared with control brain tissue, microglia cells from post mortem samples taken from patients with Alzheimer's disease have increased expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, IL-12 and TNF-α (McGeer and McGeer, 2001).

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2.9 T Cells in Neuroinflammation

T cells are required for an effective immune response against a wide range of pathogens and for the generation of immunological memory. T cell activation can be divided into two phases: an antigen-specific signal delivered through the T cell antigen receptor, and a costimulatory signal delivered through accessory molecules on the T cell surface. Following activation, T cells differentiate to acquire distinct effector functions depending on the costimulatory signal, cytokine environment, and the pathogen itself. Although CD28 has been identified as the dominant costimulatory molecule, several other molecules have been described as having a costimulatory function e.g CD54 or ICAM1. Both have been shown to be readily resident on human naïve CD4+ T cells. A controlled study comparing the participation of both in T cell differentiation, and with no added cytokines, showed that co-stimulation through either CD3+CD28 or CD3+ICAM-1 induced differentiation to T effector and T memory cells, but costimulation through CD3+ICAM-1 also induced differentiation to Treg cells whereas costimulation through CD3+CD28 did not (Kohlmeier and Benedict, 2003).

The response to antigen stimulation *in-vivo* of naïve T cells is clonal expansion and differentiation into various populations of effector T cells. Eventually, these population of effector T cells contract in number leaving only a small population of long lived memory cells with significantly greater frequency of specificity for that particular antigen (Sprent and Surh, 2002). Memory T cells are qualitatively superior to naive cells when that particular antigen is encountered again in that they can respond to much lower concentrations of antigen, are less dependent on costimulatory signals and display effector functions more rapidly following activation than do naive T-cells (Veiga-Fernandes *et al.*, 2000). Development of naive T-cells to the various effector states including memory T-cell states is dependent upon signals received during activation and differentiation. It is not yet clear how many sets of signalling conditions can contribute to the varied differentiation outcomes available to naive T cells. Early activation of naive T cells requires two distinct signals (Frauwirth and Thompson, 2002). The first is mediated by interaction of the T-cell receptor (TCR) with its cognate antigen in the context of major histocompatibility complex. A naive T cell that receives only signal one, will enter a state of anergy or undergo apoptosis (Powell, *et al*., 1998). The second, or costimulatory, signal is delivered through accessory molecules on the T-cell surface and is antigen-independent. The TCR and various costimulatory signals differentially contribute to T-cell proliferation and the acquisition of effector functions, and also correlates with the onset of memory marker expression and increased cell division

(Grogan *et al.*, 2001; Bonnevier and Mueller, 2002). CD28 is the prototypic costimulatory molecule, and its ligands, B7.1 (CD80) and B7.2 (CD86), are expressed on professional antigen-presenting cells (Riley *et al.*, 2002). Costimulation through CD28 synergizes with signalling through the TCR, driving T-cell activation by enhancing gene expression, increasing proliferation and interleukin-2 (IL-2) production, providing protection from signal-1-induced apoptosis, and effectively promoting the progression of T cells from naive to effector and memory populations of both the Th1 and Th2 phenotypes. In addition to CD28's role in antigen-presenting cell adhesion, stimulation through leucocyte function-associated antigen-1 (LFA-1, CD11a, CD18) also provides a costimulatory signal for T-cell activation (Kohlmeier *et al.*, 2006). Costimulation of naive human CD4+ T cells through LFA-1 can provide an initial burst of proliferation and IL-2 production but fails to enhance the expansion of cell numbers or to promote cell viability (Palmer *et al.*, 2001). The inability of costimulation through LFA-1 to cause human T cells to function in the same manner as CD28, makes LFA-1 an excellent control for studies. The role of resident intercellular adhesion molecule-1 (ICAM-1, CD54) in T-cell activation is considerably less well characterized. ICAM-1 is expressed at low levels on resting and naive T cells, and is up-regulated following activation or in response to pro-inflammatory mediators (Roebuck and Finnegan, 1999).

Infiltration of lymphocytes into the CNS during neurodegenerative diseases is well established and the molecular mechanisms underlying their recruitment into the CNS has also been well documented (Peterson and Fujinami, 2007; Rezai-Zadeh 2009; Engelhardt, 2010; Sorescila, 2011; Fumagalli, 2011). However, the controversy of scientific evidence for the role of lymphocytes during neurodegeneration has raged unabated for more than a half century. It is now evident that after infiltration into the CNS and recognition of cognate antigen/MHC, peripherally activated lymphocytes can initiate inflammatory response in the CNS which can be either neuroprotective or neurotoxic (Engelhardt and Ransohoff, 2005; Engelhardt, 2010). The pathogenic role of T cells has been demonstrated in neurodegenerative diseases causing neuronal death (Fee, 2003; Appel, 2009; Brochard, 2009; Huang, 2009). The extent of the CNS injury during neurodegeneration has been correlated with the increase in T cells infiltration into the CNS suggesting the greater the infiltration, the greater the neuronal injury (Popovich *et al.*, 1997). It has been suggested that these infiltrating T cells can also mediate cell death and demyelination in neurodegenerative diseases, affecting other effector cells including microglia and/or macrophages (Popovich *et al.*, 1996). The adoptive transfer of T cells from spinal cord injury model mice and EAE-

induced mice to healthy recipients result in development of paralytic disease which further supports the pathogenic role of T cells (Popovich *et al*., 1996). It has been shown that during neurodegeneration and brain injury, both T cells and B cells are activated which is referred to as auto-reactive T cells or B cells (Wang, 1992; Olsson, 1993). The number of auto-reactive T cells is increased in neurodegeneration and CNS trauma and they predominantly release IFN-γ and TNF-α (Wang, 1992; Popovich *et al*., 1996; Kil, 1999). Moreover, these cytokines released by these auto-reactive cells can exacerbate ischaemia and excitotoxicity in the brain during neurodegeneration (Viviani, 2004). Studies have also demonstrated that TNF-α induces cell death via apoptotic pathways and its concentration was also found to be elevated during neurodegenerative diseases (Mogi, 2000). In addition, activated CD4+ T cells express Fas-ligand (FasL), which has been reported to induce cell death via apoptosis in neurodegenerative diseases (Dittel, 2000). These Fas and FasL are type I and II transmembrane receptors belonging to TNF/nerve growth factor and TNF families' protein respectively (Nagata, 1995). The up-regulation of Fas and their ligands have been demonstrated in the CNS during neurodegenerative disease resulting in apoptotic cell death (Sabelko-Downes, 1999). In addition, CD8+ T cells or cytotoxic T lymphocytes (CTL) are proposed to be involved in direct killing of neurons in a MHC-I dependent manner (Medana, 2001). The induction of MHC-I expression in neurons via IFN-γ has been documented and it has also been reported that the cytotoxicity of CTL in these neurons is mediated via either FasL-mediated neuronal apoptosis or perforin-dependent lysis of neurons (Neumann, 1995; Rensing-Ehl, 1996; Medana, 2000). Moreover, both CD4+ T cells and CD8+ T cells have been reported to be equally neurotoxic and mediated via direct cell contact mechanism involving FasL, LFA-1 and CD40 (Giuliani, 2003).

Despite the proposed role of T cells in neurodegeneration, there is growing evidence for a beneficial or neuroprotective role of lymphocytes in neurodegenerative diseases (Moalem, 2000; Beer, 2008). Adoptive transfer of auto-reactive T cells from EAE induced mice to healthy recipient induces pathology (Popovich, 1996). However, when these cells are transferred to the mice with partial optic nerve crush, a model for secondary neurodegeneration, they were found to be beneficial (Moalem, 1999).

2.10 Components of the Neuroinflammatory Cascade

Nitric oxide and iNOS plays a central role in microglia cell modulation of neurodegeneration. Microglia cells being primary immune cells in the CNS have similar actions to that of peripheral macrophages (Kreutzberg, 1996). Sustained and uncontrolled activation of

microglia can lead to an excess production of various factors that contribute to neuronal injury, most notably, nitric oxide, pro-inflammatory cytokines (IL-1β, TNF-α) (Gibbons and Dragunow, 2006), reactive oxygen species (ROS) (Wang *et al*., 2006) and glutamate (Takeuchi *et al*., 2006). On activation, microglia may produce excessive levels of nitric oxide via the increased expression of inducible nitric oxide synthase (iNOS) (Brown, 2007) and these events can lead to a disruption of neuronal mitochondrial electron transport chain function (Stewart and Heales, 2003). In particular, nitric oxide selectively inhibits mitochondrial respiration at cytochrome C oxidase (complex IV), resulting in a disruption of neuronal ATP synthesis and an increased generation of ROS (Moncada and Bolanos, 2006). Furthermore, excessive NO production may also be detrimental as it is capable of inducing protein modifications, in particular S-nitrosylation and nitration (Zhang *et al*., 2006). Therefore, an uncontrolled activation of iNOS in glial cells constitutes a critical event in inflammatory-mediated neurodegeneration. In addition to iNOS, the activation of NADPH oxidase (Mander and Brown, 2005), mediates production of superoxide anion radical, which reacts with NO leading to the generation of neurotoxic peroxynitrite ($ONOO^-$) (Bal- Price *et al*., 2002), a highly reactive intermediate that has been observed to inhibit mitochondrial respiration, induce caspase dependent neuronal apoptosis, and also induce glutamate release resulting in excitotoxicity and neuronal death (Bal-Price *et al*., 2002; Brown and Bal-Price, 2003). In addition to these short lived reactive intermediates, activated microglia also produce longer lived cytokines which act to enhance the expression of iNOS and increase nitric oxide production, as well as to stimulate the release of additional cytokines that activate neuronal death signaling cascades. Microglia cytokines have been reported to induce the expression of low affinity receptor CD23 in glial cells, a protein known to mediate iNOS induction in macrophages (Dugas *et al*., 1998), resulting in iNOS induction and subsequent increase in nitric oxide production (Hunot *et al*., 1999). Additionally, microglia cytokine production may play a deleterious role via their binding to specific cell surface receptors expressed in neurons that activate pro-apoptotic pathways. TNF-α has been shown to bind to the tumor necrosis factor receptor-1 (TNFR1), triggering caspase-8 activation via Fas-associated protein with a death domain (FADD). This leads to subsequent cleavage of caspase-3 which results in neuronal apoptosis (MacEwan, 2002; Taylor *et al*., 2005).

2.11 Intracellular Signaling and Regulation of Neuroinflammation

The regulation of inflammatory cytokine production and iNOS expression in activated microglia is under the control of intra-microglia mitogen activated protein kinase (MAPK) signaling pathway and the NF-κB signaling cascade. MAP kinases, which include extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK1/2/3), and p38 kinase (p38αβγδ), are important in the transduction of extracellular signals into cellular responses. When activated these kinases phosphorylate both cytosolic and nuclear target proteins resulting in the activation of transcription factors (i.e. STAT-1/2/3, NF-κB, CREB, c-jun) that ultimately regulate gene expression (Chang and Karin, 2001). The increased expression of both iNOS and cytokines in microglia is partly regulated by signaling through the MAPK pathway (Bhat *et al.*, 1998; Culbert *et al.*, 2006). For instance, activation of microglia cells with IFN-γ and LPS has been shown to lead to increases in iNOS and TNF-α expression via the ERK and p38 MAPK cascades (Bhat *et al.*, 1998). Furthermore, JNK1 (Pawate and Bhat, 2006) and ERK (Marcus *et al.*, 2003) have been shown to modulate iNOS induction in TNF-α/IL-1β-activated astrocytes, suggesting that the MAPK pathways plays a pivotal role in both LPS- and cytokine-induced production of pro-inflammatory molecule.

Various transcription factors, including NF-κB, activator protein-1 (AP-1), and the signal transducer and activator of transcription-1 (STAT-1) have been shown to be involved in pro-inflammatory responses in astrocytes and microglia cells. In terms of neuroinflammation, NF-κB activation has also been suggested to mediate iNOS induction and thus nitric oxide production (Bhat *et al.*, 2002; Davis *et al.*, 2005) and cytokine expression (Jana *et al.*, 2002; Nakajima *et al.*, 2006) in microglia cells. Furthermore, its activation is also implicated in cyclooxygenase-2 (COX-2) expression in activated astrocytes (Dai *et al.*, 2006), a molecule which mediates prostaglandin formation and seems to play a significant role in neuroinflammatory processes (Minghetti, 2004). Inappropriate regulation of AP-1 also enhances the expression of pro-inflammatory genes such as iNOS, TNF-α, IL-1β and COX-2 in activated microglial cells (Kang *et al.*, 2004; Bae *et al.*, 2006) and STAT-1 is involved in controlling iNOS expression (Dell'Albani *et al.*, 2001). With regards to the latter, suppression of STAT-1 phosphorylation has been shown to inhibit the expression of inflammatory molecules in astrocytes (Choi *et al.*, 2005).

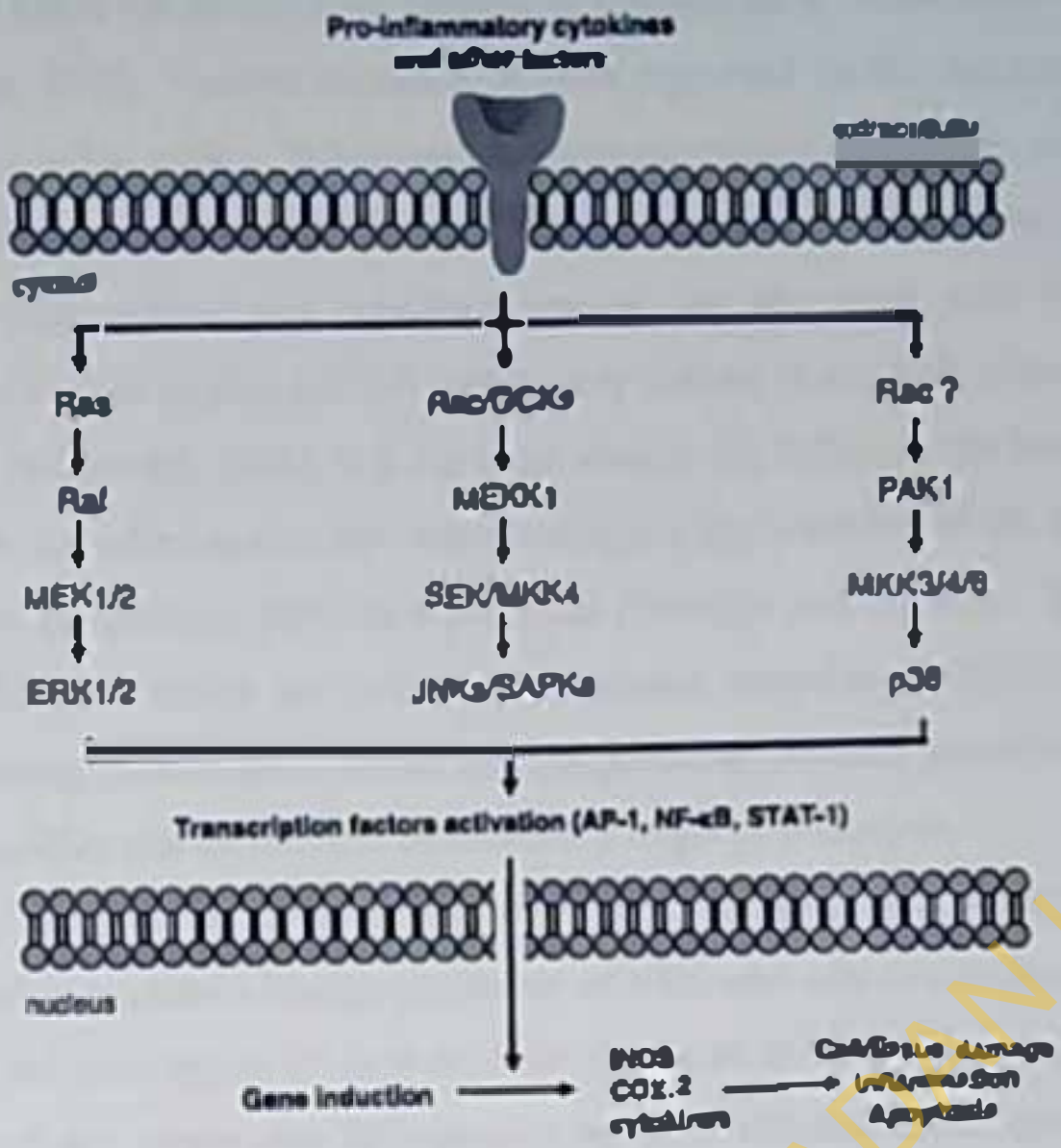

Figure 2.2: Potential involvement of MAPK in neuroinflammation. Activation of MAPK signaling leads to the induction of proinflammatory transcription factors (STAT-1, NF-κB), which in turn leads to an increase in the expression of inflammatory molecules such as iNOS, cytokines and COX-2 (Spencer *et al.*, 2012).

There is significant interplay between these signaling pathways, transcription factors and the production of inflammatory molecules and/or reactive oxygen species in glial cells (Bhat *et al.*, 2002; Koistinaho and Koistinaho, 2002; Whitton, 2007) and how these interactions play out in response to toxins and/or nutrients is pivotal in determining the neuroinflammatory response in the brain (Figure 2.2).

2.12 Neurodegenerative Diseases

Neurodegenerative diseases are huge and accounts for at least 15% of the global burden of diseases (Cruz *et al.*, 2006). Although efforts are geared towards reducing the burden, the lack of complete understanding of the underlying biology and disoriented search for reliable therapeutics might account for the yearly increase of cases of neurodegenerative diseases. The progressive loss of neurons in the CNS and the associated functional deficit of the affected region are unabated in neurodegenerative diseases.

Cell death either via apoptosis or necrosis or both accounts for the loss of neurons (DeLegge and Smoke, 2008). Various reasons have been suggested for the degeneration of neurons of the CNS including ageing, inflammation, stress and trauma and genetic predisposition (Amor, 2010; Collier, *et al.*, 2011; Tollervey, 2011). A number of studies have shown a strong link between inflammation and neurodegeneration but the exact role for inflammation in neurodegenerative process has not been clearly defined (Campbell, 2004; Lucas *et al.*, 2006; DeLegge and Smoke, 2008). It is not clear whether the inflammation causes the death of the neurons or the inflammatory infiltrate are simply a manifestation of the disease process but a number of possibilities have been proposed (Peterson and Fujinami, 2007; Shresta *et al.*, 2014). The possibilities are inflammation causing neurodegeneration or vice versa, other factor causing inflammation and/or neurodegeneration or both processes occurs as a cycle which amplifies one another and inflammation might be protective.

The key features of CNS inflammation are microglia cell activation, production of inflammatory mediators locally, expression of MHC and adhesion molecules, release of free-radicals and recruitment of immune cells (Lucas *et al.*, 2006). During neurodegeneration which probably comes after inflammation has been initiated, either the recruited peripheral immune cells, such as T cells sustain inflammation in the CNS or CNS resident immune competent cells such as microglia as well as neurons, astrocytes and oligodendrocytes, release inflammatory mediators to sustain the inflammatory process including recruiting more lymphocytes and immune cells to the CNS (Block and Hong, 2005; Kivisäkk, 2009). Some other neurodegenerative process might be initiated and sustained by infiltrating peripheral immune cells as seen in neurodegerative disease of autoimmune origin. Most commonly, inflammation starts within subarachnoid space which disseminates to other regions of the brain (Kivisäkk, 2009). During inflammation of the CNS, endothelial cells of the blood brain barrier (BBB) express various selectins and adhesion molecules that increase the migration of lymphocytes from the systemic circulation to the perivascular spaces of the brain (Engelhardt and Wolburg, 2004; Reboldi, 2009). Further, activated lymphocytes also express various receptors including chemokines receptors, integrins and selectins that help to interact with their respective ligands expressed on the surface of endothelial cells during neuroinflammation (Goverman, 2009; Engelhardt, 2010). Activated lymphocytes and cells of the CNS including microglia, astrocytes, neurons and oligodendrocytes release various pro-inflammatory cytokines such as IL-1, TNF-α, IL-23, INF-γ and chemokines including various neurotrophic factors which can contribute to the outcome of the CNS inflammation (Neumann, 2001; Kerschensteiner, 2009). There are several neurodegenerative diseases

including AD, MS, PD and stroke in which lymphocytes are actively involved and believed to be a key player in the initiation of CNS inflammation and probably progression of the disease.

2.13 Alzheimer's Disease (AD)

Alzheimers disease (AD) is characterized by death of cells in the hippocampus and frontal cortex secondary to chronic inflammation. Alzheimer's disease (AD) is a progressive neurodegenerative disease that mostly affects patients in their later stage of life (Isik, 2010). Typical symptoms of AD are loss of cognitive functions including emotion, learning and memory processing skills leading to dementia (Mattson, 2004; Jalbert *et al.*, 2008). Gradual progressive short-term memory failure, orientation problems and word-finding difficulties are characteristic symptoms for which patients often seek medical advice. In many cases this combination of symptoms marks the beginning of clinical Alzheimer's disease (AD). While most patients present for diagnosis are at 65 years of age and above, evidence suggests that the pathological processes underlying this devastating neurodegenerative disease start years, if not decades, before a clinical diagnosis can be made (Jack *et al.*, 2013).

The current paradigm suggests that the deposition of amyloid-β (Aβ) peptides marks the first detectable stages of the disease. Aβ is generated constantly through the sequential action of two aspartyl proteases, γ-secretase and β-secretase, which cleave amyloid precursor protein (APP) (Querfurth and LaFerla, 2010). The amount of Aβ in the cerebral tissue is tightly controlled, as the processing, secretion and degradation of Aβ and its removal from brain parenchyma are all highly regulated processes.

The tissue concentration of Aβ seems to be critical for maintenance of the peptide's structure, and a rise in tissue concentration is the likely cause of misfolding and aggregation. While overproduction of Aβ, caused by mutations in the genes encoding APP and the presenilins PS1 and PS2, accounts for the hereditary form of AD (Bertram *et al.*, 2010), impaired clearance mechanisms are thought to be responsible for the majority of sporadic, non-hereditary cases of AD (Mawuenyega *et al.*, 2010) (Figure 2.3). Aggregation of Aβ transits the monomeric Aβ peptide into larger oligomeric, fibrillar and aggregated species which are recognized by various receptors that mediate the endocytosis and phagocytosis of the various Aβ forms. Aβ oligomers are considered to be the most neurotoxic form when added directly to neuronal cultures (Walsh *et al.*, 2002). However, *in vivo* there is a dynamic continuum of Aβ aggregation forms, and the observed toxicity and degeneration could be mediated partly via pro-inflammatory cytokines derived from activated microglia. Of note, the same receptors

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that sense pathogen-associated molecular patterns such as bacterial lipopolysaccharide and viral surface proteins are also triggered by Aβ aggregates. It might thus be that misfolded Aβ could represent a conserved molecular pattern for which the innate immune system has evolved immunological signaling receptors. Indeed, several microbes, including bacteria and fungi, express surface amyloids, also called 'curli fibers', that carry out specific and essential functions for the microorganisms (Hammer *et al.*, 2007; Epstein *et al.*, 2008). Interestingly, Congo red, which still is used to stain Aβ deposits in postmortem brains from patients with AD, was originally also used as a stain for bacterial amyloid fibrils. Thus, it is not far-fetched to hypothesize that the detection of amyloid by pattern-recognition receptors such as Toll-like receptors (TLRs), CD36 and others on cells of the immune system in the brain could have evolved as a host response to microbial challenges. While many of the inflammatory mediators released upon the activation of such cells might aid in the function of the brain and initially support the clearance of pathogenic Aβ, others might directly compromise neuronal function and survival and adult neurogenesis (Monje *et al.*, 2003). In contrast to an immune response to microbes, which is terminated once the stimulating pathogen has been removed, sustained elevation of Aβ and continuous Aβ aggregation does not allow the resolution of inflammation but instead fuels a chronic reaction of the innate immune system. Over time, chronic neuroinflammation causes distinct changes in the brain, which probably contribute to the degeneration of neurons and, in turn, functional decline.

The pathological impression of AD is characterized by the deposition of amyloid-beta (Aβ) protein plaques in the brain parenchyma and accumulation of tau proteins within neurons (Krause and Muller, 2010). These protein plaques are thought to interfere with synaptic transmission and neuron-neuron communication leading to neuronal death (Ang, 2010; Alzheimer's-Association, 2011). Further, high levels of tau proteins within neurons form tangles and block transportation of nutrients or other vital cellular factors throughout the cell which has been suggested to be one of the reasons for cell death in AD (Ballatore *et al.*, 2007; Iqbal, 2010; Alzheimer's-Association, 2011). In AD, amyloid-beta plaques and tau proteins are considered to be crucial in the pathology as the resultant inflammatory process might be in response to the accumulating plaques and tangles. The inflammatory responses in AD can be characterized by the up-regulation of cytokines and chemokines along with activation of microglia (Akiyama *et al.*, 2000). The activated microglia clusters can be seen near amyloid-beta deposition site and these cells also express high levels of MHC-II, cytokines and chemokines contributing to disease progression (Griffin, 1998; Streit *et al.*, 1999).

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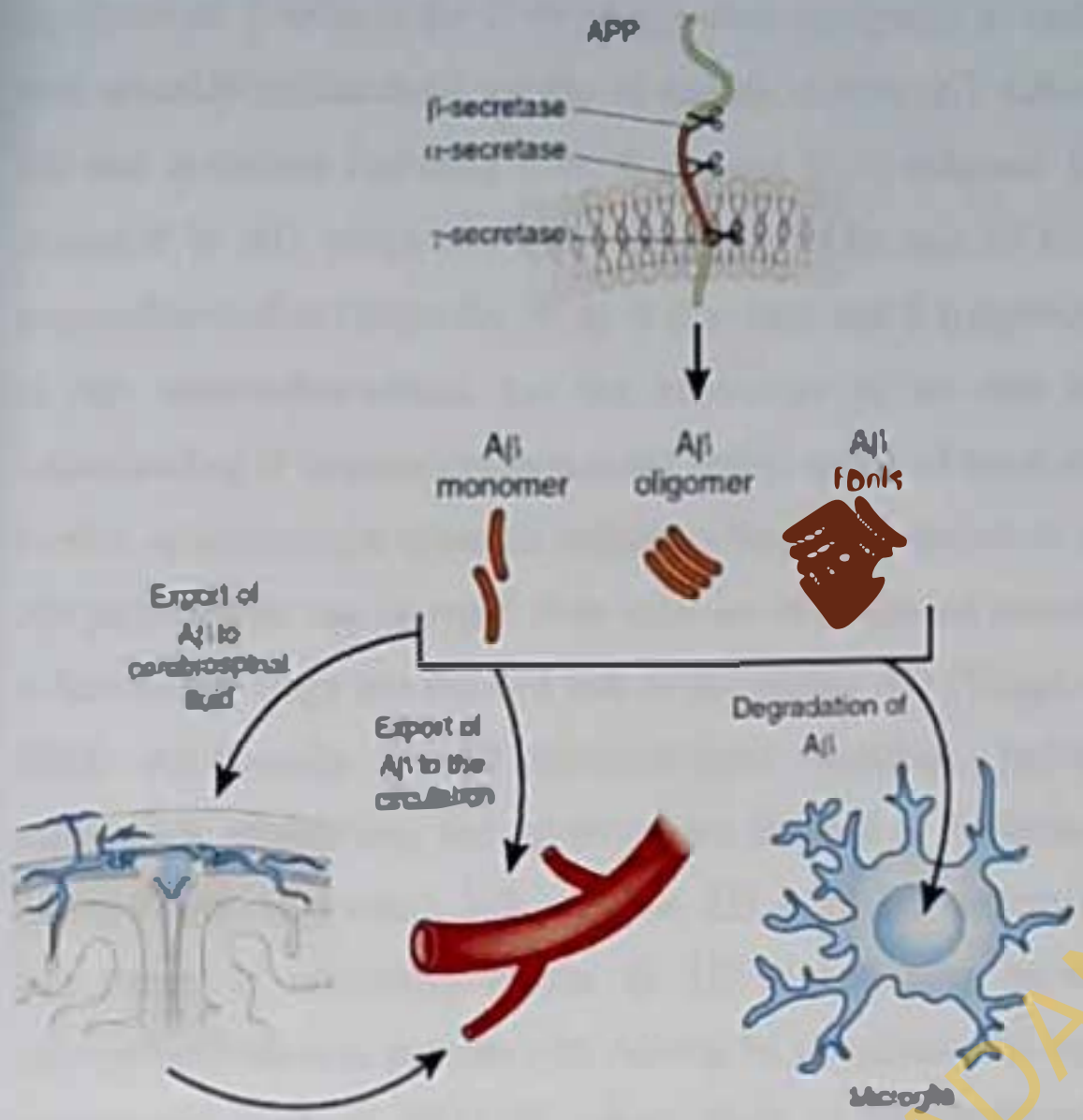

Figure 2.3: Clearance of Aβ. The Aβ precursor APP undergoes processing by proteases α-, β-, γ- secretase. Aβ monomers can assemble into Aβ oligomers and fibrils (middle). Aβ can then redistribute into the extracellular fluid as well as, directly or indirectly, into the blood circulation (bottom left). Furthermore, microglia cells take up Aβ and degrade it (bottom right) and can thereby contribute to the clearance of Aβ (Hencka *et al.*, 2015).

These microglia cells are also involved in clearing of amyloid-beta and this function has been shown to be enhanced in the presence of TGF-β (Wyss-Coray, 2001; 2006). In addition, reactive astrocytes also clusters at sites of amyloid-beta deposition and also expressing various cytokines, growth factors, adhesion molecules and prostaglandins which have been suggested to be involved in inhibition of microglia ability to clear amyloid beta (Mrak, 1996; DeWitt, 1998; Hampel, 2005). The analysis of brain autopsy has also revealed that there is a significant increase in inflammatory markers as well as an increase in complement activation and lysis of neurites in AD subjects when compared to non-demented subjects, which also strongly suggests the involvement of inflammation in AD (Webster, 1997). There are evidences for suggestion that it is the inflammatory response that leads to the recruitment of lymphocytes from the systemic circulation into the brain. Also, T cells have been detected in the brain of AD patients (Togo *et al.*, 2002). Soresella, (2010; 2011) reported the up-

regulation of T cells in the CNS of AD when compared to healthy controls. These studies have revealed an increased activity of various subsets of T cells such as Th-17 and Th-9 in AD and cytokines including IL-9, IL-21 and IL-23 released from these T cells are also increased in AD which has been suggested to be one of the factors in AD-associated neuroinflammation (Saresella, 2010). It is evident that T lymphocytes are present and involve in AD neuroinflammation, but the knowledge of its role is still emerging, a better understanding of this phenomenon could help in search of novel drug targets.

Further epidemiologic evidence indicating that inflammation is an important contributor to AD pathogenesis has emerged from outcome of prolonged treatment with nonsteroidal anti-inflammatory drugs and reduced risk of developing AD (Weggen *et al.*, 2001; Sastre *et al.*, 2003). Additionally, several immunological mediators, including complement factors, eicosanoids, chemokines and cytokines, are elevated in the brain and cerebrospinal fluid of patients with AD, which indicates that AD is an inflammatory process. The pathogenic importance of neuroinflammation in AD is becoming increasingly evident. Ongoing neuroinflnmmation in patients with AD can be visualized with ligands for positron emission tomography, such as PK11195, which binds to the peripheral benzodiazepine receptor expressed on activated microglia cells (Cagnin *et al*, 2001). Of note, analysis of PK11195 binding in patients suffering from mild cognitive impainment, which represents a clinical precursor phase of AD, has helped to identify those patients who will probably develop full AD within a certain time period (Yasuno *et al.*, 2012). Moreover, genetic and experimental data have further changed the perception of neuroinflammation in AD as a contributor to AD pathogenesis rather than an extraneous reaction (Harold *et al.*, 2013; Karch *et al.*, 2014).

The deposition of Aβ in the brain commences decades before clinical memory decline becomes evident and before the diagnosis of AD (Jack *et al.*, 2013). Hence, the thought that Aβ deposits can activate microglia, the principal effector cells of the immune system in the brain, early in disease pathogenesis is significant. Microglia form a lattice throughout the brain and express many immunological receptors, such as TLR2, TLR4 and TLR6, as well as their co-receptors, including CD36, CD14 and CD47 (Weggen *et al.*, 2001; Liu *et al.*, 2011). These receptors often act together and thereby augment the response to Aβ exposure. For example, recognition of fibrillar Aβ by CD36 triggers the formation of a TLR4-TLR6 heterodimer that results in signaling via the transcription factor NFκB in transfected HEK293 human embryonic kidney cells and in microglia (Stewart *et al.*, 2010). Deletion of MyD88, the shared signaling adaptor for cytokines of the interleukin 1β (IL-1β) family and TLRs, improves amyloid pathology in the APP/PS1 mouse model of AD (mice that express

transgenes encoding mutant APP and PS1) but results in only minor improvements in cognitive activity (Lim *et al*., 2011; 2012). The microglia activation of the mitogen-activated protein kinase p38, production of reactive oxygen species and phagocytosis of fibrillar Aβ might also depend on the specific interaction of TLR2, TLR4 and CD14 because neutralizing antibodies to these receptors attenuate or even block their respective functions in microglia-like BV2 cells (Reed-Geaghan *et al*., 2009). Microglia cells can also be activated by Aβ oligomers before they form deposits in a process that requires the scavenger receptor SR-A and the activated potassium channel KCa3.1 (Maezawa *et al*., 2011). Stimulation by Aβ oligomers occurs at low nanomolar concentrations similar to those observed after stimulation with lipopolysaccharide. Hence, focal activation of microglia and astroglia by Aβ can precede the deposition of aggregated Aβ in APP models of AD, and this may begin earlier than previously anticipated (Heneka *et al*., 2005; Wright *et al*., 2013).

Evidence from neuropathological evaluation and imaging by positron emission tomography with the amyloid dye PiB suggest that the first activation of the immune system takes place within the limbic system and in particular in the entorhinal cortex and hippocampus (Sojkova and Resnick, 2011). It is conceivable that such activation could also result from the other factors acting as DAMPs, including ATP, chromogranin A or double-stranded DNA, leaking from damaged or degenerating neurons (Davalos *et al*., 2005). A complex formed by two members of the diverse family of S100 proteins, MRP8 (S100A9) and MRP14 (S100A8), has been found to be increased in the brain and cerebrospinal fluid of human patients with AD (Reed-Geaghan *et al*., 2009; Maezawa *et al*., 2011) . Heterodimers of MRP8 and MRP14 act as DAMPs through the activation of TLR4. Moreover, MRP14-mediated inflammatory stimuli are responsible for upregulation of the β-site APP–cleaving enzyme BACE1, which is the rate-limiting enzyme of APP processing. These data support the hypothesis that inflammatory molecules act as part of a vicious cycle by contributing to the generation of Aβ. Chronic activation of microglia in AD might also lead to microglia demise and subsequent replacement through proliferation. One hypothesis is that the microglia which proliferate in a microenvironment of immunological activation develop a gene-expression pattern different from that of microglia that resided in the affected brain area before such activation. Over time, such newly generated, divergent microglia could sustain a chronic type of neuroinflammation in AD. While this is an attractive hypothesis, the possibility that peripheral cells of the immune system are recruited to the brain and contribute to the clearance of Aβ cannot be completely excluded. Indeed, there is some evidence that cells of the myeloid lineage are attracted from the periphery to the site of plaque formation in a

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manner that is dependent on the chemokine receptor CCR2 (El Khoury *et al.*, 2007). Notably, loss of CCR2 results in gene dose-dependent aggravation of amyloid pathology in Tg2576 mice (which overexpress a mutant form of APP), suggesting that the burden of Aβ clearance is shared between central cells of the immune system and peripheral cells of the immune system under the experimental conditions used in this model (El Khoury *et al.*, 2007). Preliminary studies have found that the aging brain shows extensive upregulation of genes associated with the innate immune system (Cribbs *et al.*, 2012). In neurodegenerative diseases such as AD, an altered gene-expression pattern could be greatly exacerbated, depending on the phase of the disease.

2.13.1 Signal-Transduction Pathways and Inflammatory Mediators

The activation of receptors of the innate immune system by microbial PAMPs in microglia induces a range of signaling pathways that lead to an orchestrated response to the pathogens sensed. It is possible that microglia may not be able to distinguish between an invading pathogen and oligomeric or fibrillar Aβ. In fact, many of the signal-transduction pathways that are elicited by such neurodegenerative stimuli are also activated during host defense against pathogens. The immune system responds to microbes by mounting a proteolytic cascade that regulates the production of highly proinflammatory cytokines of the IL-1β family. These cytokines, including IL-1β and IL-18, are leaderless proteins that are expressed as biologically inactive precursor forms. Activated caspase-1 or caspase-8 proteolytically activates cytokines of the IL-1β family and mediates their release into the cytosol. Activation of caspase-1 itself is controlled by large multimolecular signaling complexes called 'inflammasomes' (Latz *et al.*, 2013). Inflammasomes consist of sensor molecules of the NLR (Nod-like receptor) family or PYHIN (pyrin and HIN domain–containing) family; these bind to the inflammasome adaptor ASC, which in turn multimerizes and activate caspase-1. The inflammasome sensor NLRP3 is important for mediating neuroinflammation, as it can sense a range of aggregated substances, including Aβ aggregates (Halle *et al.*, 2008). Evidence abounds that brains from patients with AD have a greater abundance of active caspase-1 than do those of age-matched control subjects. In addition, APP/PS1 mice that are deficient in NLRP3, caspase-1 or ASC are largely protected from AD (Halle *et al.*, 2008; Latz *et al.*, 2013). Lack of NLRP3 decreases the Aβ-induced formation of IL-1β in the brain and improves the clearance of Aβ by microglia in APP/PS1 mice. NLRP3-deficient APP/PS1 mice show almost normal cognitive performance (Yasuno *et al.*, 2012). Moreover, NLRP3-

deficient APP/PS1 mice are completely protected from Aβ-induced suppression of synaptic plasticity (a measure of the ability of neurons to modulate their response to stimulation by changes at the synapse). Synaptic plasticity is particularly sensitive to IL-1β, as this cytokine is able to disrupt the formation of dendritic spines (the structures on a neuron's dendrite that receive input from a synapse) mediated by brain-derived neurotrophic factor (BDNF) and tropomyosin-related kinase (TRKB), and thus memory consolidation, by activating p38 (Tong *et al.*, 2012). Of particular interest, the NLRP3 inflammasome is also an important contributor to normal age-related systemic inflammatory responses as well as brain inflammation. Ablation of NLRP3 in aged mice protects them from age-related cognitive decline even in the absence of experimental brain amyloidosis (i.e., without expression of the transgenes encoding APP and PS1). Such studies suggest that NLRP3 is a critical determinant for the development of low-grade sterile inflammatory responses during aging (Youm *et al.*, 2013).

The inflammatory response in the brain can maintain a dangerous feed-forward loop. It has been demonstrated that stimulation of the immune system in response to Aβ and proinflammatory cytokines impairs microglia clearance of Aβ and neuronal debris (Heneka *et al.*, 2013). At the same time, activation of the immune system may compromise the microglia generation of neurotrophic factors. Together, phenotypic changes in microglia contribute to impaired cognitive performance. In line with the hypothesis that proinflammatory molecules such as the IL-1β family of cytokines or factors that simulate TLRs can impair the clearance function of microglia are findings showing that disrupting IRAK4, an essential kinase downstream of TLRs and receptors for IL-1β cytokines, improves the clearance of Aβ and shifts microglia cells from a proinflammatory phenotype toward an anti-inflammatory phenotype (Cameron *et al.*, 2012).

Other proinflammatory factors may also affect the pathogenesis of AD. For example, increased levels of p40, a subunit of IL-23, can be detected in cerebrospinal fluid from patients with AD, which suggests that the IL-12–and–IL-23 signaling pathway is activated (Vom Berg *et al.*, 2012). Indeed, genetic ablation of p40 itself (or the components p35 and p19) leads to a decreased cerebral Aβ load in APP/PS1 mice and ameliorates behavioral deficits. Likewise, intracerebroventricular administration of p40-neutralizing antibodies lowers the concentration of soluble Aβ peptides and improves spatial memory. Notably, in the brain, microglia are the sole source of IL-12 and IL-23, and genetic ablation of the IL-12 or 23 pathway does not alter the processing of APP. Since the receptor for IL-23 has high expression on astrocytes, microglial p40 may stimulate the astroglial uptake of Aβ.

Astrocytes can also interfere indirectly with microglial removal of Aβ by releasing lipidated apolipoprotein E, which is important for microglial phagocytosis of Aβ (Tervel *et al.*, 2011). Other factors, such as small-molecule mediators, can also influence neurodegenerative processes by influencing the inflammatory state. For example, the release of nitric oxide (NO), the presence of free radicals and the secondary formation of peroxynitrite are well-described features of inflammatory processes in various tissues. During AD, the inducible isoform of NO synthase (iNOS) is expressed by neurons and glial cells in response to proinflammatory cytokines (Vodovotz *et al.*, 1996). Enhanced expression of iNOS during an inflammatory response can increase the local production of NO.

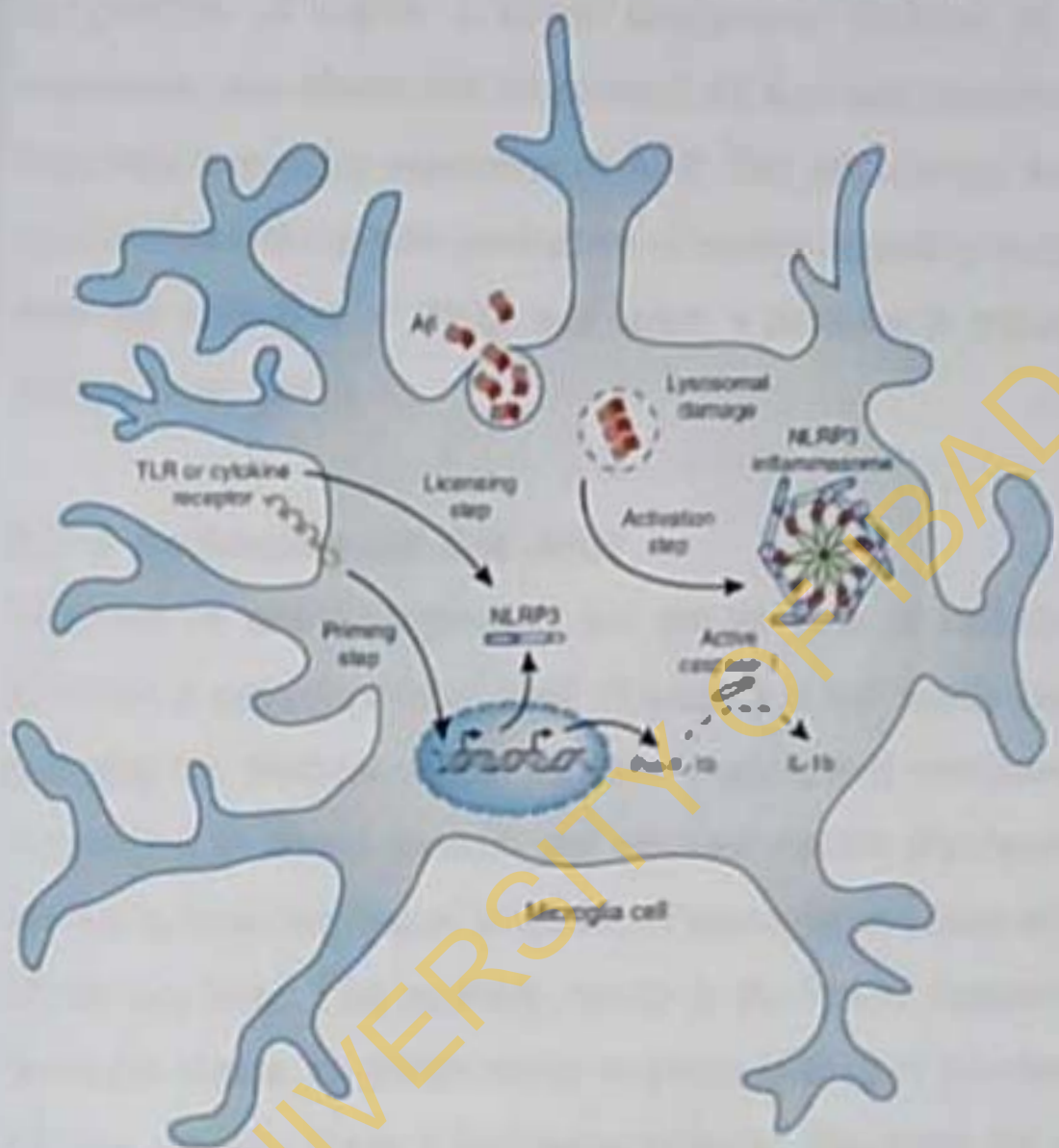

Figure 2.4: Inflammasomes and the production of active IL-1β. The activation of microglia with TLR agonist or cytokines leads to the transcriptional induction of genes encoding components of the NLRP3 inflammasome and pro-IL-1β (priming step). Additional signals including deubiquitination of NLRP3 are further required for activation of NLRP3 (licensing step). Aβ can induce lysosomal damage that leads to assembly of the NLRP3 inflammasome (activation step) and the activation of caspase-1. Active caspase-1 leads to the processing of IL-1β and release of the bioactive form of IL-1β (Heneka *et al.*, 2015).

While NO has been suggested to be involved in neurodegenerative processes, including the inhibition of mitochondrial respiration, axonal and synaptic damage, and the induction of neuronal apoptosis, the Aβ peptide itself represents a direct target of modification by NO and peroxynitrite. In fact, the Tyr10 of Aβ becomes nitrated by iNOS33 in human AD and in AD models.

iNOS-mediated nitration of Aβ enhances the peptide's propensity to aggregate and to form seeding cores of Aβ plaques. Notably, nitrated Aβ is more potent in suppressing synaptic plasticity than is non-nitrated Aβ, and both genetic ablation and pharmacological inhibition of iNOS protects mice from spatial memory dysfunction. Furthermore, global genetic upregulation of hsp70, a major endogenous inhibitor of NF-κB signaling and iNOS expression, also diminishes the cerebral Aβ load and improves the cognitive performance of mice with transgenic expression of APP. This effect might be mediated by the upregulation of microglia and astroglia production of insulin-degrading enzyme (IDE) but might also arise from the inhibition of iNOS, and hence, a decrease in nitrated Aβ (Hoshino *et al.*, 2011; Heneka *et al.*, 2015).

2.13.2 Dysfunction and Cell Death

The start of neurodegeneration and precise time of initiation of molecular mechanisms involved in pathogenesis or death of neurons is still highly debated. Microglia is constantly scanning the dendritic spines for their integrity and continuously involved in shaping and remodeling its neural contacts and neuronal circuits (Paolicelli *et al.*, 2011). Activation of microglia, however, causes retraction of microglia processes and is accompanied by swelling of the cell body. This probably results in the loss of monitoring of neuronal synapses by microglia during an inflammatory response leading to neuronal changes and disruption of relevant circuits. There is increasing evidence supporting the hypothesis that inflammatory mediators affect neuronal functioning long before structural damage and cell death. Indeed, several cytokines, including IL-1β, IL-18, IFN-λ and TNF, have been shown to suppress long-term potentiation in the hippocampus (Yirmiya and Goshen, 2011; Lynch, 2014). Moreover exposure of hippocampal slices to Aβ aggregates suppresses long-term potentiation only in the presence of NOS2, which suggests that at least at some stage NO exerts a negative influence on neuronal integrity and function (Wang *et al.*, 2004). In support of the notion that inflammatory signals can cause neuronal dysfunction, lack of NLRP3 relieves the suppression of long-term potentiation in APP/PS1 mice (Heneka *et al.*, 2013). Targeting inflammatory cytokines by systemic treatment of APP/PS1 mice with MW01-2-151SRM

(also called MW-151; an experimental therapeutic agent that attenuates the production of proinflammatory cytokines by activated microglia) results in diminished microglia and astroglia reactivity, protection from loss of key synaptic proteins and overall improved synaptic plasticity (Bachstetter *et al.*, 2012). Microglia neurotoxicity mediated by proinflammatory cytokines may be partially influenced by surrounding astrocytes. Astrocytes release acidic fibroblast growth factor, which substantially increases microglia-mediated neuronal death via activation of the receptor FGFR1IIb(Lee *et al.*, 2011). In a similar way, the chemokine receptor CX3CR1 on microglia has been found to protect against cognitive deficits in a mouse model of AD (Cho *et al.*, 2011).

The induction of neurodegeneration by soluble inflammatory mediators may not be the only mechanisms by which microglia contribute to the killing of neurons. For example, it has been found that activated microglia can destroy functional neurons by direct phagocytosis (Neniskyte, 2001). This microglia cannibalism requires stimulation by Aβ; this enhances the presence of phosphatidylserine on neuronal processes, which leads to increased uptake by microglia. Notably, this so-called neuronal 'phagoptosis' occurs without obvious signs of neuronal necrosis or apoptosis. One hypothesis is that the mechanisms described above may exist in parallel but operate in specific disease phases and/or depend on the state of activation of the innate immune system.

2.13.3 Inducing Tau Pathology

Deposition of Aβ represents the mechanism initiating AD (Jack *et al.*, 2013). The link between the mechanisms initiated by deposition of Aβ and formation of intraneural neurofibrillary tangles (NFTs) another hallmark of the AD remains unclear. NFTs are self associated hyperphosphorylated tau proteins. The normal function of tau proteins is in stabilization of microtubules, but the association of hyperphosphorylated tau proteins can subsequently cause various cellular changes that lead to neuronal degeneration from inside the cells. There are quite some evidence linking microglia-driven neuroinflammatory response in AD to NFT formation and tau pathology. For example, lipopolysaccharide-induced systemic inflammation can increase tau pathology (hyperphosphorylation and tangling) through a mechanism involving the cyclin-dependent kinase CDK5 (Kitazawa *et al.*, 2005). Similar findings have been obtained with various mouse models of systemic inflammation (Lee, 2010; Bhaskar *et al.* 2010; Sy *et al.*, 2011). However, such a peripheral

challenge might not be required, as local microglia-driven responses can be sufficient to drive tau pathology. Indeed, the activation of microglia precedes NFT formation in young PS19 mice, which transgenically express mutant tau with substitution of serine for the proline at position 301 (a mouse model of tau pathology) (Yoshiyama *et al.*, 2007). Furthermore, initiating immunosuppression in PS19 mice as early as 2 months of age leads to diminished tau pathology and greater lifespan. Such results are consistent with data showing that activation of microglia induces phosphorylation of tau in primary mouse neurons and that this action probably requires activation of the receptor for IL-1β and signal transduction via p38 (Gorlovoy *et al.*, 2009; Bhaskar *et al.*, 2010). Interestingly, expression of CX3CR1 on microglia seems to restrict this pathological mechanism, as knockdown of CX3CR1 increases the phosphorylation and aggregation of tau even further, most probably due to increased release of IL-1β (Bhaskar *et al.*, 2010). Support for the proposal of an NFT-driving role for cytokines has been provided by experiments demonstrating that an acute increase in IL-1β in aged 3xTg-AD mice (which transgenically express three mutations associated with familial AD) further increases tau pathology (Ghosh *et al.*, 2013). Together these data provide evidence in support of the hypothesis that activation of the innate immune system represents an important and accessible link between Aβ and tau pathology in AD.

2.13.4 Phagocytic Clearance of Aβ

One of the main cell types responsible for removing cellular debris and aggregated proteins from brain parenchyma is microglia. Microglia contributes to the clearance of Aβ by phagocytosis and the degradation of Aβ and by their release of enzymes that are able to degrade Aβ in the extracellular space, such as IDE. Both mechanisms may be compromised by genetic predisposition, exogenous factors or changes in brain metabolism and neurotransmitter profiles. The phagocytic clearance function of microglia is greatly impaired in response to degeneration of the locus ceruleus (LC) (Heneka *et al.*, 2010). This small midbrain nucleus, located at the tectum of the fourth ventricle, is the chief source of norepinephrine in the human brain. Degeneration of the LC, which seems to be an early phenomenon in AD, enhances the inflammatory response to the deposition of Aβ and compromises microglia phagocytosis due to decreased norepinephrine levels in LC projection areas (Hoshino *et al.*, 2011). It is likely that sustained exposure to proinflammatory cytokines or some form of damage-associated molecular patterns could account for the attenuated

microglia phagocytosis. That hypothesis is further supported by findings demonstrating that genetic deficiency in MRP14 increases microglia phagocytosis of Aβ (Kummer *et al*., 2012). Several surface receptors have been shown to mediate phagocytic clearance of Aβ, including TLR2, TLR4, TLR6, CD14 and CD36 (Grommes *et al*., 2008). A role for the tyrosine phosphatase CD45 has also been demonstrated (Zhu *et al*., 2011). Apart from receptors on the microglia, the phagocytic uptake of Aβ may also be modulated by neuronal exosomes. This is largely dependent on the form by which the Aβ aggregate is delivered. For example, neuronal exosomes bind Aβ and promote its phagocytic clearance in a phosphatidylserine-dependent way (Yuyama *et al*., 2012).

2.13.5 Genetic Associations

Surprisingly, several genome-wide association studies of sporadic cases of AD have identified a set of genes that suggest a pathogenic role for inflammatory processes in AD (Karch *et al* 2014). The identified gene variants associated with a risk of developing AD include the gene encoding complement receptor CR1 (Harold *et al*., 2013), the genes encoding MS4A6A and MS4A4AE (Hollingworth *et al*. 2011) (membrane-spanning proteins expressed on myeloid cells) (Liang and Tedder, 2001) and the gene encoding CD33 (Siglec-3) (a myeloid-cell-surface receptor) (Hollingworth *et al*. 2011). CD33 is a transmembrane protein that contains on immune receptor tyrosine-based inhibitory motif: these motifs are usually involved in the inhibition and control of cellular responses. In keeping with this, activation of CD33 has been shown to suppress the production of proinflammatory cytokines by monocytes (Lajaunias *et al*., 2005). The strongest AD-associated mutation in the locus encoding CD33 is the single-nucleotide polymorphism rs3865444, which has now been linked to an increase in imaging by positron emission tomography with PiB, indicative of increased individual deposition of Aβ in mutant carriers (Bradshaw *et al*., 2013). Furthermore, circulating monocytes (close relatives of brain-resident microglia) that carry the rs3865444 mutation of the gene encoding CD33 have a decreased capacity to ingest fluorescent dextran and Aβ by phagocytosis. Patients with AD show increased expression of CD33 by microglia, which inhibits microglia removal of Aβ *in vitro* and *in vivo* (Griciuc *et al*., 2013). Together these findings suggest that genetic factors confer an increased risk of sporadic AD by compromising otherwise beneficial microglia clearance functions. Further support for the importance of immune system-related genetic factors for AD stems from two independent studies that have associated rare variants in TREM2, a triggering receptor on myeloid cells, with an increased risk for the development of AD (Lee *et al*., 2011; Cho *et al*.,

2011). TREM2 is a surface receptor that is responsible for initiating immune responses of macrophages and dendritic cells by forming a receptor signaling complex with the kinase-binding protein TYROBP. In the brain, TYROBP is expressed by microglia cells, and increased TYROBP expression at sites of Aβ deposition in mice with transgenic APP expression has been reported (Frank *et al.*, 2008; Melchior *et al.*, 2010). In this particular location, TREM2 might also be involved in the phagocytic clearance of cellular debris and in the downregulation of inflammatory signals in response to TLR ligation (Hamerman *et al.*, 2006). The ability of TREM2 to mediate activation of cells of the immune system and phagocytosis without increasing proinflammatory cytokine production suggests that this receptor is involved in the physiological clearance of Aβ (Bouchon *et al.*, 2001). The upregulation of TREM2 at Aβ plaque sites may represent an attempt to enhance the endogenous clearance capacity. In this context it should be mentioned that the long-known and most frequent risk variants—hetero- or homozygosity for the gene encoding apolipoprotein E4—may also be involved in the dysregulation of phagocytosis and inflammatory responses. In addition to alterations in the aforementioned genes, single-nucleotide polymorphisms in other genes found to be associated with AD by genome-wide association studies probably encode molecules involved in the regulation of immune responses, although their precise mechanisms of interaction has yet to be defined (Karch *et al.*, 2014).

2.13.6 Microglia-Targeting Therapies

As impaired microglial clearance has been identified as a disease-promoting factor, several attempts have been made to positively influence this factor by pharmacological, vaccine-based or gene-therapy strategies. For example, galantamine, a drug approved for the treatment of AD, is an acetylcholinesterase inhibitor that engages the nicotinic acetylcholine receptor α7 and can thereby increase the microglia uptake of Aβ (Takata *et al.*, 2010). Likewise, the nuclear hormone receptor PPAR-γ represents another factor that can be targeted by existing drugs, such as the thiazolidinedione class of antidiabetics (Gorlovoy, *et al.*, 2009). The treatment of APP/PS1 mice with PPAR-γ agonists rapidly increases the removal of Aβ by microglia and, presumably, astrocytes (Mandrekar-Colucci *et al.*, 2012). The mechanisms involved may include direct upregulation of microglial CD36 expression and lipidation of apolipoprotein E dependent on transcription factors of the LXR family (Yamanaka, 2012; Mandrekar-Colucci *et al.*, 2012).

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Furthermore, several nonsteroidal anti-inflammatory drugs that have been proven epidemiologically to reduce the risk of AD are potent activators of PPAR-γ, an effect that might be the molecular mechanism behind their efficacy (Heneka *et al.*, 2015). Another strategy for increasing the removal of Aβ is passive or active vaccination against Aβ. However, thus far this approach has been limited by the development of concomitant inflammation. The development of humanized antibodies to Aβ of the immunoglobulin G4 subtype might overcome this risk of ligation of Fc receptors; as such antibodies could increase the uptake of Aβ oligomers by microglia with less neurotoxicity (Adolfsson *et al.*, 1979; Ghosh *et al.*, 2013). Furthermore, the time point of intervention might be critical for the success of these approaches. So far, most interventional studies have been started in patients with substantial clinical signs of AD, a time point at which the disease process can potentially not be halted anymore. At present, interventional trials in asymptomatic patients at risk for the development of AD are under way. In addition, conformation-specific antibodies that recognize toxic soluble Aβ oligomers rather than all forms of Aβ are being tested for efficacy. The outcomes of these ongoing clinical trials will show whether passive or active Aβ immunotherapy can prevent or delay the progression of AD.

Beyond their phagocytic uptake of aggregated proteins and neuronal debris, microglia cells can also dismantle extracellular proteinaceous debris by releasing proteases such as IDE. This mechanism has important effects *in vivo*, as demonstrated by the finding that a twofold increase in IDE expression is sufficient to prevent the deposition of Aβ plaques in an AD model of transgenic expression of APP (Leissring *et al.*, 2003). While the function of IDE has been studied, much less is known about the regulation of microglia release of IDE. Cholesterol-lowering statins are yet another class of drugs that have received attention in this context, as they can reduce the risk of developing AD. Statins stimulate unconventional secretion of IDE by exosomes and could thereby substantially contribute to the extracellular degradation of Aβ (Tamboli *et al.*, 2010). However, this type of extracellular degradation is less efficient once microglia cells face post-translationally modified forms of Aβ, as phosphorylation of Aβ at Ser8 restricts its proteolytic clearance by IDE and other proteases (Kumar *et al.*, 2012). Therefore, it is conceivable that the effectiveness of statins is influenced by the stage of the disease.

2.14 Flavonoids and Neurodegeneration

A vast majority of drug treatments of neurodegenerative disorders treat the symptoms rather than preventing the underlying degeneration of neurons. Consequently there is a desire to

develop novel therapies capable of preventing the progressive loss of specific neuronal populations that underlie pathology in these diseases (Legos *et al.*, 2002; Narayan *et al.*, 2002). Flavonoids have been shown to be effective in protecting against both age-related cognitive and motor decline in vivo (Joseph *et al.*, 1999; Vauzour *et al.*, 2007; Williams *et al.*, 2008). This neuroprotective potential may be due to a number of physiological functions attributable to flavonoids, including their antioxidant properties (Bastianetto *et al.*, 2000), their interactions with intracellular signaling pathways, the regulation of cell survival/apoptotic genes and mitochondrial function (Williams *et al.*, 2004; Spencer *et al.*, 2009a; Spencer, 2009b). For example, flavonoids and their *in-vivo* metabolites have been shown to modulate signaling through tyrosine kinase, phosphoinositide 3-kinase (PI3 kinase), protein kinase C (PKC) and mitogen activated protein kinase (MAP kinase) pathways (Spencer, 2009a). These signaling cascades are known to critically control inflammatory processes in the brain, including the activation of microglia in response to cytokines and the induction of iNOS and nitric oxide production (Bhat *et al.*, 1998; Kaminska *et al.*, 2009; Wen *et al.*, 2011). As a consequence, flavonoids have been suggested as novel therapeutic agents for the reduction of the deleterious effects of neuroinflammation in the brain and thus also as potential preventive drugs for neurodegenerative disease development.

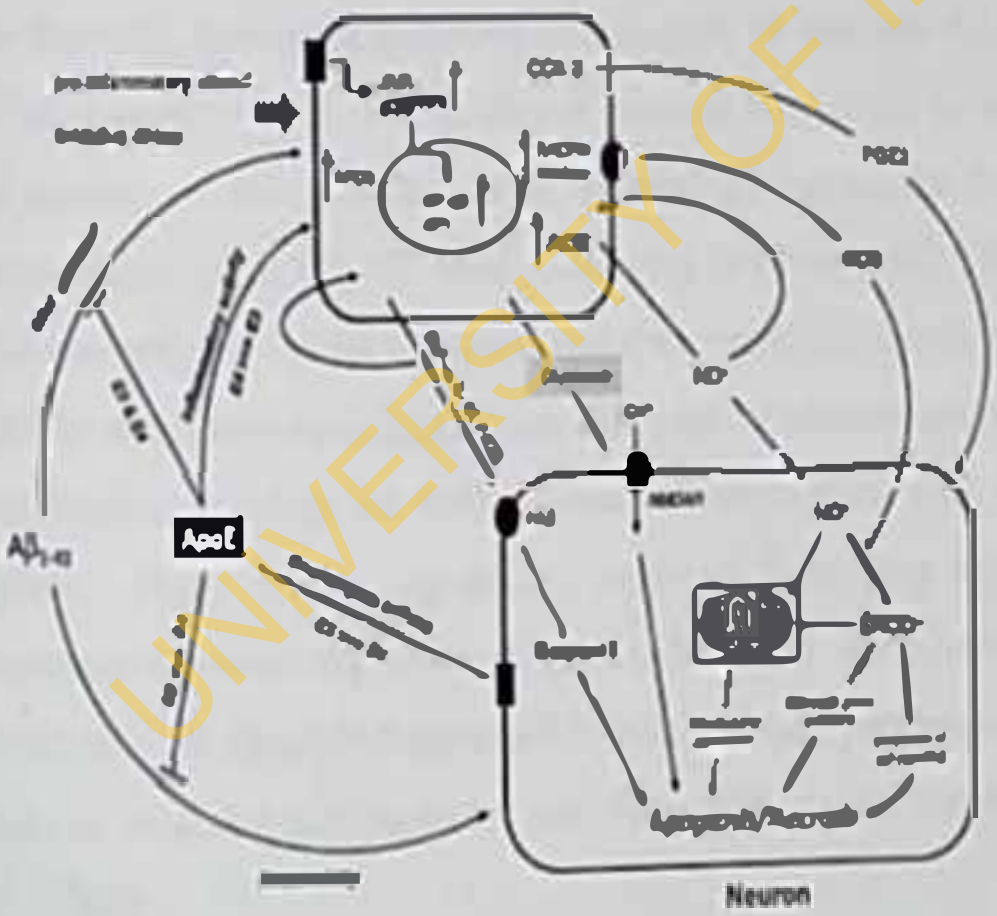

Figure 2.5: Activated glial cells in neuroinflammatory-induced neurodegeneration (Spencer *et al.*, 2012).

develop novel therapies capable of preventing the progressive loss of specific neuronal populations that underlie pathology in these diseases (Legos *et al.*, 2002; Narayan *et al.*, 2002). Flavonoids have been shown to be effective in protecting against both age-related cognitive and motor decline in vivo (Joseph *et al.*, 1999; Vauzour *et al.*, 2007; Williams *et al.*, 2008). This neuroprotective potential may be due to a number of physiological functions attributable to flavonoids, including their antioxidant properties (Bastianetto *et al.*, 2000), their interactions with intracellular signaling pathways, the regulation of cell survival/apoptotic genes and mitochondrial function (Williams *et al.*, 2004; Spencer *et al.*, 2009a; Spencer, 2009b). For example, flavonoids and their *in-vivo* metabolites have been shown to modulate signaling through tyrosine kinase, phosphoinositide 3-kinase (PI3 kinase), protein kinase C (PKC) and mitogen activated protein kinase (MAP kinase) pathways (Spencer, 2009a). These signaling cascades are known to critically control inflammatory processes in the brain, including the activation of microglia in response to cytokines and the induction of iNOS and nitric oxide production (Bhat *et al.*, 1998; Kominsky *et al.*, 2009; Wen *et al.*, 2011). As a consequence, flavonoids have been suggested as novel therapeutic agents for the reduction of the deleterious effects of neuroinflammation in the brain and thus also as potential preventive drugs for neurodegenerative disease development.

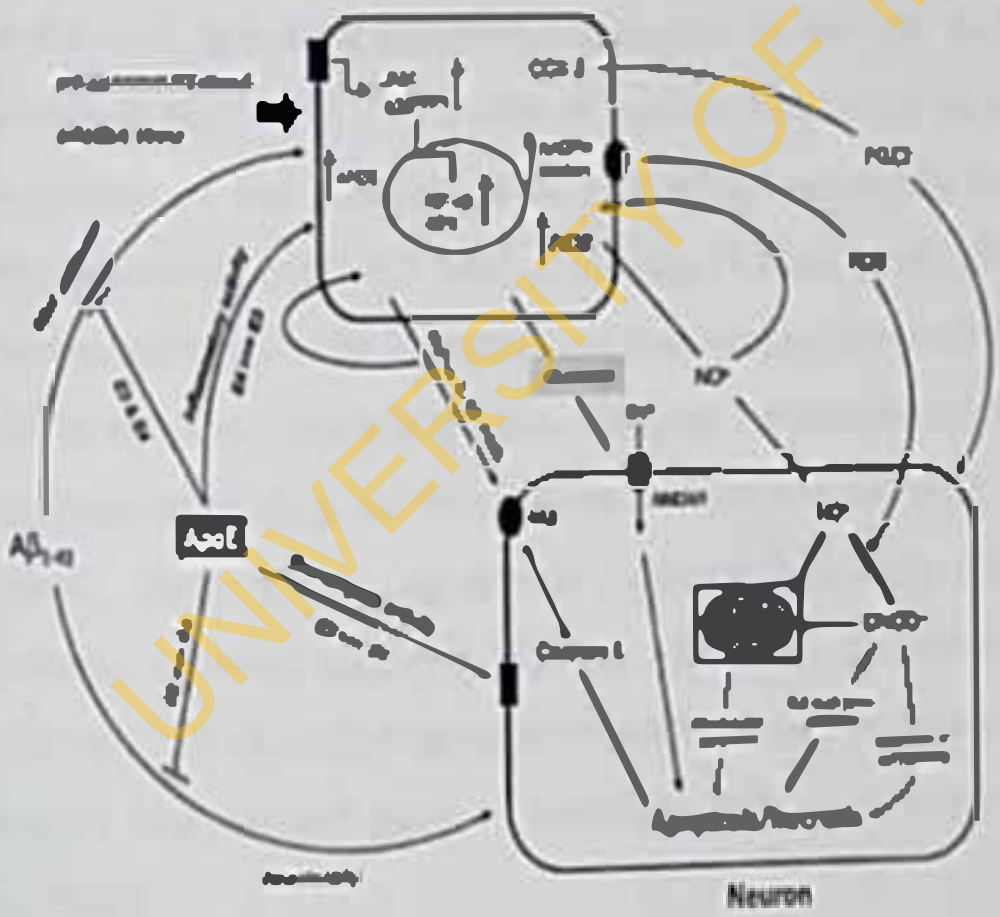

Figure 2.5: Activated glial cells in neuroinflammatory-induced neurodegeneration (Spencer *et al.*, 2012).

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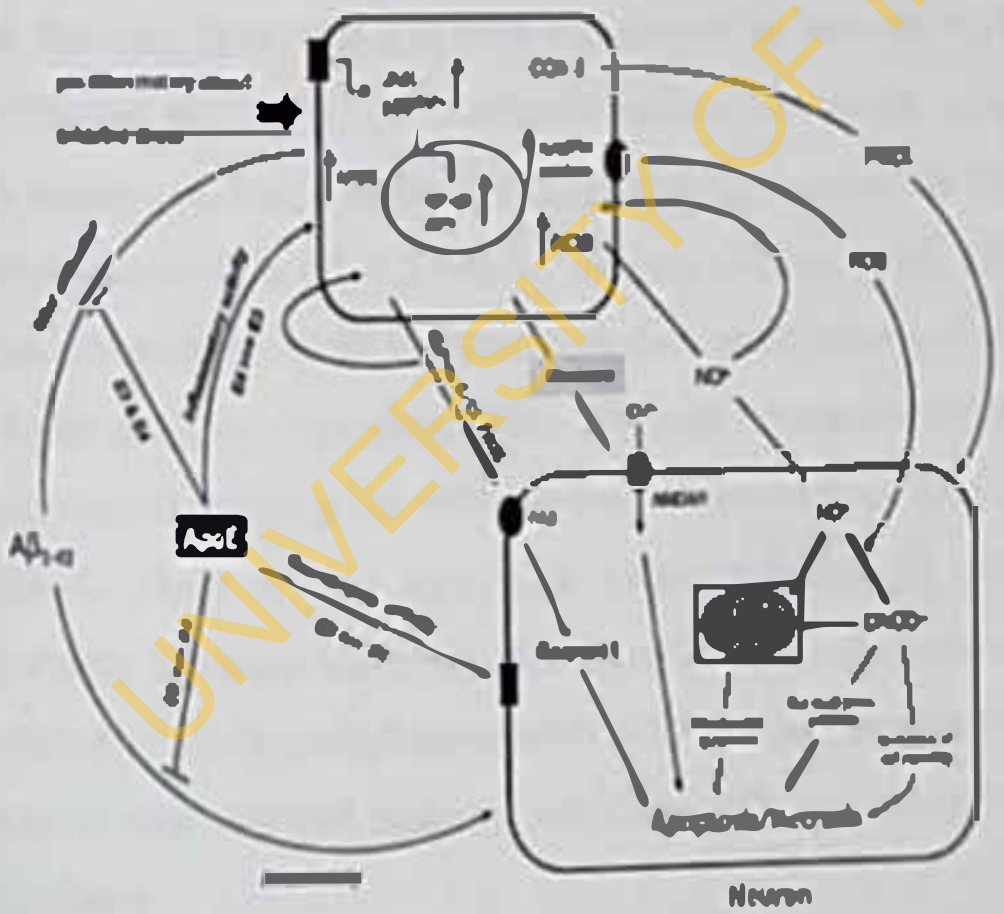
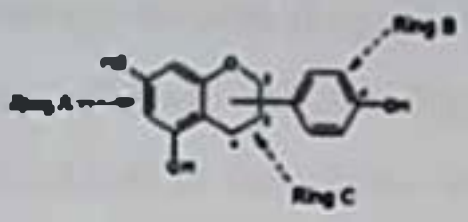
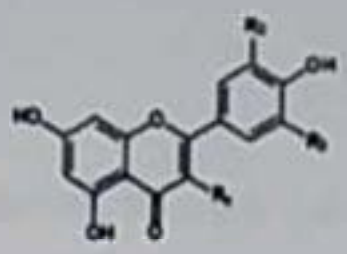


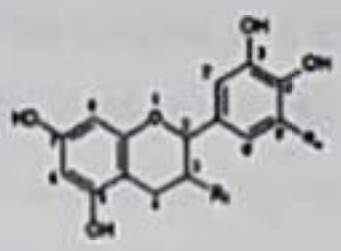
Figure 2.5: Activated glial cells in neuroinflammatory-induced neurodegeneration (Spencer *et al.*, 2012).

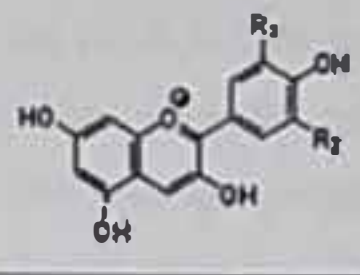
The interaction of flavonoid with signaling pathways involved in neurodegeneration was in the past linked to their direct (i.e. classical) antioxidant effects (Rice-Evans *et al.*, 1996). However, data now suggest that their actions on the brain are more likely to be mediated by their ability to protect vulnerable neurons, enhance existing neuronal function, stimulate neuronal regeneration and induce neurogenesis (Spencer, 2009b, 2010). Indeed, it has become evident that flavonoids are able to exert neuroprotective actions (at low, physiological concentrations) via their interactions with critical neuronal/glial intracellular signaling pathways pivotal in controlling neuronal resistance to neurotoxins, including oxidants ('indirect' antioxidant nature) (Levites *et al.*, 2001) and inflammatory mediators (Spencer, 2009a), or through their chelation of transition metal ions such as iron (Levites *et al.*, 2002; Mandel *et al.*, 2005, 2006). Interestingly, flavonoids have close structural homology to specific inhibitors of cell signaling cascades, such as the PD98059, a MAPK inhibitor and the LY294002, a phosphatidylinositol-3 kinase (PI3) inhibitor. In the context of neuroinflammation, the MAPK inhibitor PD98059 has been shown to effectively block iNOS expression and nitric oxide production in activated microglia cells (Bhat *et al.*, 1998), suggesting that flavonoids may also be capable of such anti-inflammatory activity through actions on this signaling pathway. Furthermore, LY294002 was modeled on the structure of the flavonol, quercetin and both compounds fit into the ATP binding pocket of the enzyme (Vlahos *et al.*, 1994). The ability of various flavonoids to modulate PI3-kinase is related to the number of, and substitution of, hydroxylgroups on the flavonoid B-ring and the degree of unsaturation of the C2–C3 bond in the ring (Vlahos *et al.*, 1994).

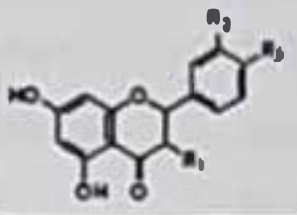
Consequently, it can be hypothesized that interactions with PI3 and other signaling pathways may be structure-dependent. Thus, different flavonoids are likely to express different cellular outcomes depending on their degree of interaction with either receptors or downstream kinases. There is also significant evidence indicating that flavonoids interact with, and modulate neuronal signaling (Spencer, 2009a,b). For example, epicatechin and its in vivo metabolite 30-O-methyl-epicatechin elicit strong protective effects against oxidized LDL-induced neuronal cell death by inhibiting JNK, c-jun and caspase-3 activation (Schroeter *et al.*, 2001).











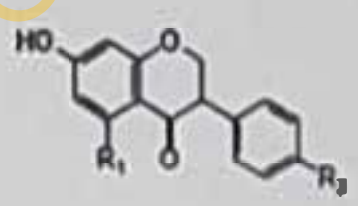


Figure 2.6: Flavonoids structures. The major differences between the individual groups reside in the hydroxylation pattern of the structure, the degree of saturation of the c-ring and the substitution in the 3-position (A) general structure of flavonoids (B) structure of flavonols and flavones (C) structure of flavanols, also reffered as flavan-3-ols (D) structure of anthocyanidins (E) structure of flavanones and flavanonols (F) structure of isoflavones (Spencer *et al.*, 2012).

2.15 *Moringa oleifera*

2.15.1 Botanical description

Moringa oleifera (Lam.) is a plant indigenous to south Asia, mainly in the sub Himalayan tracts of India, Pakistan, Bangladesh and Afghanistan. It is now widely grown and has been naturalized in many countries of the world including Nigeria. The exponential growth in use of herbal medicines in many official systems of medicine as remedies for diverse conditions is well documented (Burkill, 1985; Ganatra *et al.*, 2012).

Moringa oleifera is a perennial, evergreen tree that grows up to 20ft (6.1 m) tall, with a straight trunk and corky whitish bark. The tree has tuberous taproot, brittle stem and pale green compound tripinnate leaves 30-60 cm (11.8 to 23.6 in) in length and many small leaflets. The lateral leaflets are elliptic in shape while the terminal ones are obovate and slightly larger than the lateral ones. The fruit pods are pendulous, green turning greenish brown, triangular and split lengthwise into 3 parts when dry. The pods are 1 to 4 ft (30-120 cm) long and 1.8 cm (0.7 in) wide and tapering at both ends. The pods contain about 10 to 20 seeds embedded in the fleshy pith (Patel *et al.*, 2010).

Kingdom	Plantae
Sub kingdom	Tracheobionta
Super Division	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Capparales
Family	Moringaceae
Genus	*Moringa*
Species	*oleifera*
Current name	Horseradish
Common names	Tree of life, drumstick tree, "mother's best friend"
Yoruba-	*Ewe ile, ewe igbale,* or *idagbo monoye*
Vernacular names	*Gawara, habiwal hausa, konamorade,* or *rini moka (Fulani), bagarmar* *moka, bagarmar masar, barambo, komukin zaila, shipka hali, shukn halinka, rimin macaro, rimin turawa, zogali,* or *zogalla-gauli (Hausa)* and *odudu oyibo, okochi egbu, okwe oli, okwe oyibo, okugbora ile, ukwe, ikwe beke (Ibo)*

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2.15.2 Nutritional value

The *Moringa oleifera* leaves are highly nutritious, being a significant source of beta-carotene, Vitamin C, protein, iron, and potassium. The leaves are commonly dried and crushed into a powder, and used in soups and sauces. Amino acids in green leafy vegetables vary considerably, and many that are staples, are low in the sulphur bearing amino acids methionine and cystine (Gassenschmidt *et al.*, 1995). The Bureau of plant industry, in its report, stated that weight per weight, Moringa leaves have the calcium equivalent of four glasses of milk, the vitamin C content of seven oranges, potassium of three bananas, three times the iron of spinach, four times the amount of vitamin A in carrots, and two times the protein in milk. The Moringa seeds yield 38-40% edible oil (called ben oil from the high concentration of behenic acid contained in the oil). The refined oil is clear, odoriess, and resists rancidity at least as well as any other botanical oil.

2.15.3 Chemical Constituents of *Moringa oleifera* leaves

Faizi *et al.* (1995) reported the isolation of two nitrile glycosides from the ethanol extracts of *Moringa oleifera* leaves, niazirin and niazirinin and three mustard oil glycosides, 4-[(4'-O-acetylalpha-L-rhamnosyloxy) benzyl]isothiocyanate, niaziminin A, and niaziminin B. Six new and three synthetically known glycosides (Faizi *et al.*, 1995) were also isolated from the leaves of *Moringa oleifera*, from the ethanolic extract. Most of these compounds, bearing thiocarbamate, carbamate or nitrile groups, are fully acetylated glycosides, which are very rare in nature. Bennet *et al.* (2003) isolated 4-(alpha-l-rhamnopyranosyloxy)-benzylglucosinolate and three monoacetyl isomers of this glucosinolate from the ethanolic extract of the leaves. The leaves also contains quercetin-3-O-glucoside and quercetin-3-O-(6"-malonyl-glucoside), and lower amounts of kaempferol-3-O -glucoside and kaempferol-3-O-(6"-malonyl-glucoside), 3-caffeoylquinic acid and 5-caffeoylquinic acid. Manguro and Lemmen (2007) reported the isolation of five flavonol glycosides characterised as kaempferide 3-O-(2",3"-diacetylglucoside), kaempferide 3-O-(2"-Ogalloylrhamnoside), kaempferide 3-O-(2"-O-galloylrutinoside)-7-O-alpha-rhamnoside, kaempferol 3-O-[beta-glucosyl-(1 → 2)]-[alpha-rhamnosyl-(1 → 6)]-beta-glucoside-7-Oalpharhamnoside and kaempferol 3-O-[alpha-rhamnosyl-(1 → 2)]-[alpha-rhamnosyl-(1 →4)]-betaglucoside-7-O-alpha-rhamnoside together with benzoic acid 4-O-beta-glucoside, benzoic acid 4-O-alpha-rhamnosyl-(1 → 2)-beta-glucoside and benzaldehyde 4-O-beta-glucoside from methanolic extract of *Moringa oleifera* leaves. Also obtained from the same extract were known compounds, kaempferol 3-O-alpha-rhamnoside, kaempferol, syringic acid, gallic acid, rutin

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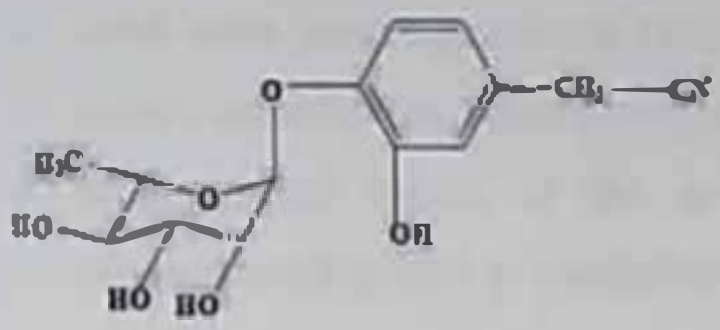
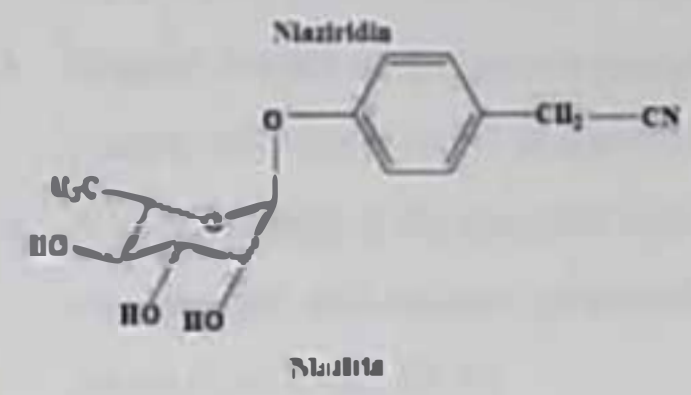
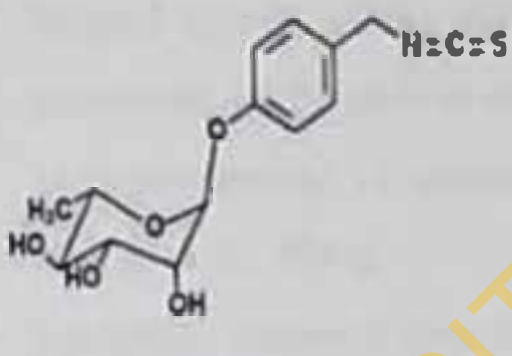
and quercetin 3-O-beta-glucoside. Their structures were determined using spectroscopic methods as well as comparison with data from known compounds. Hueih- Min Chen *et al.* (2007) using GC-MS isolated 44 compounds from the leaves. Singh *et al.* (2009) reponed presence of gallic acid, chlorogenic acid, ellagic acid, ferulic acid, kaempferol, quercetin and vanillin from the aqueous extracts of leaves, fruits and seeds of *Moringa oliefera*. All compounds were analyzed by HPLC and MS/MS techniques.



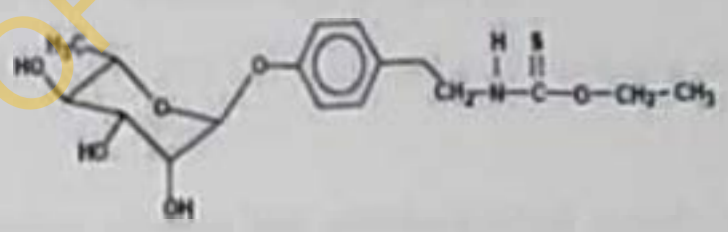
Niaziridin



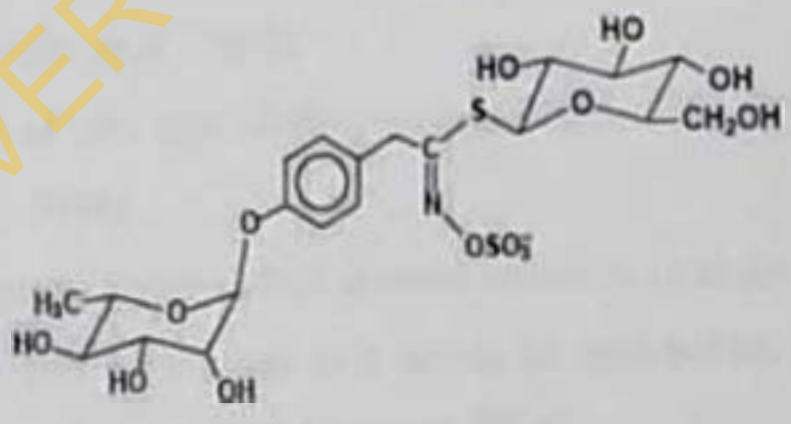
Niazirin



4-(α-L-rhamnopyranosyloxy) benzyl isothiocyanate



Niaziminin



4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate

Figure 2.7: Structure of some of phytoconstituents from *Moringa oleifera* (Mishra *et al.* 2011

2.15.4 Some of the Pharmacological Actions of *Moringa oleifera*

- Methanol extract of leaf and root caused significant reduction in thermal hyperalgesia, and mechanical allodynia in complete Freund's adjuvant induced arthritis in rats. (Manaheji *et al.*, 2011).
- Ethanol extract of the leaves has arbotifacient activity in rats treated with 175 mg/Kg from ten days of post mating period (Sethi *et al.*, 1988).
- Fresh leave juice, aqueous and ethanol extracts have bacteriocidal and bacteriostatic activity against some human pathogenic bacteria (Alam *et al.*, 2009).
- Hydro-ethanol extract of the dried pod ameliorates DMBA induced renal carcinogenesis in mice by mechanisms related to its antioxidant properties (Paliwal *et al.*, 2011).
- Ethanol extract of the leaves changes some CVS parameters favorably in a manner comparable with atenolol in adrenalin induced rats (AIR) (Ara *et al.*, 2008).
- Winter samples of the stem and stalk have higher calcium and phenol compounds, and also stonger anti-oxidant properties compared to the summer samples: except the leaves (Tsai *et al.*, 2011).
- Ethanol extract of the leaves shows hepatoprotective ability on the histology of paracetamol induced liver damage in rats (Busaimoh *et al.*, 2011).
- Methanol extract of leaves stimulate both cellular and humoral immune response (Sudha *et al.*, 2010).
- Lipophilic methanol extract of *Moringa oleifera* has antiplasmodial activity against chloroquine sensitive strain of plasmodium falciparum with a mean IC_{50} value >50µg/ml (Kohler *et al.*, 2002).
- Water extract of *Moringa oleifera* seed has larvicidal activity against *Aedes aegypti* (Fereira *et al.*, 2009).
- Leave powder of *Moringa oleifera* boost immunity in about 80% of 263 HIV patients and confirms that 43% usage in a survey on metabolism and transport to improve clinical outcome (Moncra and Maponga, 2010).
- *Moringa oleifera* leaves is used for 24 medicinal purposes in Uganda and it contains tannins, steroids and triterpenoids, flavonoids, saponins, anthraquinones, alkaloids and reducing sugars (Kasolo *et al.*, 2010).
- Ethanolic extract of *Moringa oleifera* leaves prevented ovariectomy induced bone loss to a level comparable with estradiol (Sanganna, *et al.*, 2010).

- Finely powdered dried seed kernel of *Moringa oleifera* showed significant improvement in symptoms score and severity of asthmatic attacks (Agrawal and Mehta, 2008).
- Fully acetylated thiocarbamate glycoside isolated from *Moringa oleifera* leaves showed hypotensive activity (Faizi *et al.*, 1995).
- *Moringa oleifera* leave extract increase efficacy of chemotherapy in human with adenocarcinoma of the pancreas by inhibiting growth and apoptosis.
- *Moringa oleifera* have broad activities like diuretic (Morton, 1991), purgative, antifungal (Terras *et al.*, 1995), antimicrobial (Spiliotis and Lalas 1998), antibacterial (Doughari *et al.*, 2007).
- Anti-inflammatory, antitumor, antioxidant, anti-aging, estrogenic, anti-progestational, hypoglycemic, anti-hyperthyroidism (Tahiliani and Kar 2000)
- Anti-ulcer (Pal *et al.*, 1995), hypocholesterolemic, antispasmodic, antihypertensive, relieving headaches and migraines, convulsion (Patel *et al.*, 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 List of Materials

Tween80
Diazepam
Pentobarbitone
Pentylenetetrazole
Strychnine
Picrotoxin
Lipopolysaccharide
FBS
RPMI 1640
Glutamine
Trypsin
EDTA
PBS
MTT kit
Methanol
DMSO
Sulphanilamide
NNED
DCFDA assay kit
ELISA kits
Lysis buffer
PMSF
Nuclear Extraction kit
Rabbit anti-iNOS
Rabbit anti-COX2
Goat anti-rabbit IgG
Rabbit anti-actin antibody
DMEM
Luciferin-luciferase bioluminescent assay kit
MAP Kinase Multi-Target
Sandwich ELISA kit
Penicillin and streptomycin
Anti-CD3 (OKT3)
Anti-CD11a (HB202)
Anti-CD54 (R6.5D6)
Trypan blue
CFSE
Annexin V
7AAD
CCR7

Y-maze
Open field apparatus
Hole board
Elevated plus maze
Observation chambers
Freezers
Microscope
Micro plate readers
96 Well plates
24 well plates
6 well plates
T 75 culture flask
Heamocytometer

3.2 Plant collection and authentification

The leaves of *Moringa oleifera* was collected at the domestic garden at Ojoo, Ibadan, Nigeria in August, 2010. It was identified and authenticated by Mr. O. Oshinyemi, a plant taxonomist at Forest Herbarium Ibadan (FHI), Forestry Research Institute of Nigeria (FRIN), Ibadan where a voucher specimen (Number FHI 109601) was deposited

3.3 Plant Extraction

The leaves were air-dried and powdered. Five hundred grams (500 g) of plant powder were macerated in 50 % ethanol for 72 hours. The extract was filtered and solvent removed using rotary evaporator under reduced temperature and pressure (BUCHI Rotavapor R-205). Percentage yield were calculated and plant extract stored in at 4 °C till needed for analyses.

3.4 Phytochemical Analysis

The qualitative determination of the phytochemical constituents was performed at the Department of Pharmacognosy, University of Ibadan, Ibadan. It was conducted using the standard methods described by Farnsworth (1989); Sofowora (1993).

3.4.1 Determination of alkaloids

Wagner's test: Crude ethanol extract (2 mg) was acidified with 1.5 % v/v of hydrochloric acid and a few drops of Wagner's reagent (iodine in potassium iodide) was added. A yellow or brown ppt. indicates the presence of alkaloids.

3.4.2 Determination of saponin

Foam Test: The extract (0.5 g portions) was shaken with 2 mL of water. Foam produced which persisted for ten minutes indicated the presence of saponins.

3.4.3 Determination of tannins and phenolic compounds

Ferric chloride test: To 1 mL aliquot of each of the extract 3-4 drops of neutral 5% ferric chloride so-lution was added. Formation of dark green colour indicated the presence of phenols.

3.4.4 Determination of anthraquinones

Test for combined anthraquinones: One gram of powdered extract was boiled with 2 mL of 10 % hydrochloric acid for 5 minutes. The mixture was filtered while hot and filtrate was allowed to cool. The cooled filtrate was partitioned against equal volume of chloroform and the chloroform layer was transferred into a test tube using a pipette. Equal volume of 10% ammonia solution was added into the chloroform layer, shaken and allowed to separate. The separated aqueous layer was observed for any colour change, delicate rose pink colour showed the presence of an anthraquinone.

3.4.5 Determination of cardenolide (cardiac glycoside)

Keller-Killiani's test: 1 mL of of the extract was mixed with 5 mL of 70% alcohol for 2 minutes. This was filtered and to the filtrates was added 10 mL of water and 0.5 mL of lead acetate. This was filtered and the filtrate was shaken with 5 mL of chloroform. The chloroform layers were separated in a porcelain dish and the solvent removed by evaporation. This was cooled and dissolved in 3 mL glacial acid containing 2 drops of 5 % ferric chloride solution. The solution was carefully transferred to the surface of 2 mL concentrated sulphuric acid. A reddish brown layer formed at the junction of the two liquids and the upper layer which slowly became bluish green and darkening with standing indicated the presence of cardiac glycosides.

3.4.6 Test for Coumarins

Crude extract (1 g) was placed in a test tube and covered with filter paper moistened with dilute sodium hydroxide (NaOH), then heated on water bath for a few minutes. The filter paper was examined under UV light, yellow fluorescence indicated the presence of coumarins

3.5 Animals

Male mice, weighing between 20-25 g purchased from the Central Animal House, University of Ibadan, Ibadan, Nigeria, were used to evaluate the acute toxicity of ethanol extract of *Moringa oleifera* leaves (EMOL). The animals were kept in a well ventilated environment with free access to food (rodent pellets from Ladokun Feeds) and water *ad libitum*

3.6 Acute Toxicity Study

The method described by Lorke (1983) was used to determine the LD_{50} of ethanol extract. Initial dose finding procedure involved administering 10, 100 and 1000 mg/Kg of extract orally using canula to three groups of three mice each. The treated animals were monitored for 24 h mortality and general behavior. From the results of the above step, 4 different doses of (800, 1600, 3200 and 6400 mg/Kg) were chosen and administered p. o. respectively to 4 groups of one mouse per group. The treated animals were monitored for 24 h. The LD_{50} was then calculated as the geometric mean of the lowest dose showing death and the highest dose showing no death.

3.7 Preparation of working solution of ethanol extract of *Moringa oleifera* leaves (EMOL)

The dark brown coloured EMOL (1 g) was weighed out and dissolved in 5 mL of 5 % tween 80. The 5 % tween 80 used as vehicle was prepared by adding 0.5 mL of tween 80 to 9.5 mL of distilled water. Using appropriate formula and calculation, dilutions were made from the working solution prepared such that ≤ 1 mL was administered orally to the animals.

3.8 Distribution of animals and administration of extract

Animals were randomly distributed into groups and orally administered either EMOL or vehicle. Control mice were given 10 mL/Kg 5% Tween 80 (Oyemitan *et al.*, 2008).

3.9 Behavioral Studies

3.9.1 Animals

Animals were randomly divided into six groups of five each. Male mice, weighing between 18-22 g were used to evaluate the effect of ethanol extract of *Moringa oleifera* leaves (EMOL) on behavior. The mice were provided food and water *ad libitum*.

3.9.2 Distribution of animals and administration of extract

Thirty mice were randomly distributed into six (6) groups of five (5) each. Animals in 4 out of the 6 groups were treated with 250 mg/Kg, 500 mg/Kg, 1000 mg/Kg and 2000 mg/Kg EMOL orally. The remaining groups received either 10 mL/Kg 5% Tween 80 (Oyemitan *et*

al., 2008) or 3 mg/Kg diazepam (Oyemitan *et al.*, 2008) to serve as control and standard respectively.

3.9.3 Novelty Induced Behavior (NIB)

NIB was assessed by the method described by Ajayi and Ukponmwan (1994) with some modifications in an open field. The open field is a rectangular arena composed of a hardboard floor (36 × 36 cm^2) with a surrounding wall 30 cm high made of white painted wood. The floor is divided into squares of 9 cm^2. The mice were allowed 6 - 10 minutes epochs during which locomotion, rearing and grooming were observed and scored.

This allowed for characterization of drug-induced alterations. The mice were then returned to their home cages. Each test session involved allowing the mice to acclimatize to the testing environment (a quiet well ventilated room) for 30 mins. All behavioural testing was carried out between 9 nm and 2 pm. The extract was administered to the mice before placing in the open field arena. One hour after administration each mouse was introduced into the arena and frequency of grooming (the number of body cleaning with paws picking of the body and pubis with mouth and face washing actions) and rearing frequency (number of times animal stands on its hind legs or with its forearm against the wall of cage or in free air) was scored for 30 mins. The procedure was repeated for all the mice in the different groups. There are six groups of five mice each. The groups are vehicle (10 ml/Kg; 5% Tween 80), 250. 500, 1000 and 2000 mg/Kg EMOL, and diazepam (3 mg/Kg). After each session, the floor of the apparatus was wiped with 70 % ethanol and dried thoroughly to remove traces of previous path.

3.9.4 Exploratory Activity (Head Dip)

The hole board test was used to assay potential sedative effects. The hole board is a wooden box. 40 × 40 cm, with sixteen holes with (diameter 3 cm) evenly spaced on the floor (Hui *et al.*, 2001). One hour after oral administration each mouse was placed at the centre of the board and the number of head dips into the holes scored over a 5 min period. Results obtained were expressed as mean total number of head dips (Lister, 1987). The procedure was repeated for all the mice in the different groups. There are six groups of five mice each. The groups are vehicle (10 ml/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and diazepam (3 mg/Kg). After each trial, the floor of the apparatus was wiped with 70% ethanol and dried thoroughly to remove traces of previous path.

3.9.5 Learning and memory (Y-maze)

Y-maze was used to assess the effect of the extract on short term memory. The Y-maze is composed of three equally spaced arms (120°; 41 cm long × 15 cm high × 5 cm wide). The parameters assessed are arm entries (locomotor activity) and spontaneous alternation performance (memory). One hour after oral administration each mouse was placed in one of the arm compartments and allowed to move freely for 5 min. Entry was defined as when body except tail of a mouse completely enters into an arm compartment. The sequence of entry was recorded manually. Alternation is defined as entry into all three arms consecutively. The arms were labeled A, B, and C, thus consecutive entries is ABC, BCA, and CAB.

Percentage alternations was calculated as

$$\left\{\frac{\textit{Actual alternation}}{\textit{Maximum alternation}}\right\} \times 100$$

Where the maximum number of spontaneous alternations was then calculated as

$$[\textit{Total number of arms entered}] - 2$$

The procedure was repeated for all the mice in the different groups. The groups are vehicle (10 ml/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and diazepam (3 mg/Kg). The apparatus was cleaned after each animal session to eliminate odour from previous animal (Brocco *et al.*, 2002).

3.9.6 Anxiolytic test (Elevated plus maze)

The elevated plus maze model (Handley and Mithani 1984; Pellow *et. al.* 1985) was used to assess anti-anxiety effect. Lister (1987) validated the use of the elevated plus maze in testing anxiolytic effect in mice. The mice were assessed for the aversion of the open space and height. The elevated plus maze with two open and two closed arms was used. The plus used is made of wood with open arms 30 × 5 × 15 cm and closed arm 30 × 5 × 15 cm. The arms extend from the central platform (5 × 5 cm). The open arms, the central platform, and the floor of the closed arms are painted black. The apparatus is mounted on a wooden base raising it by 38.5 cm above the floor. Also the open arms have a slight ledge 4mm high to prevent mice from slipping and falling off the edge. One hour after oral administration each mouse was placed in turn at the centre facing one of the closed arms and assessed for 5 mins. The following behavior was scored: open arm entries, closed arm entries, time spent in open

arm and time spent in closed arm. The index of open arm avoidance was interpreted as level of anxiety (Trullas and Skolnick, 1993) and calculated as

$$\left[100 - \frac{(\%\text{ time on open arm} + \%\text{ entries into open arms})}{2}\right]$$

The procedure was repeated for all the mice in the different groups. There are six groups of five miee each. The groups are vehicle (10 mL/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and diazepam (1 mg/Kg). After each mouse assessment the lingering olfactory cues was cleaned using 70% ethyl alcohol. The doses used fall in the range that do not affect motor coordination (Reddy and Kulkami, 1997).

3.9.7 Sedative test (Pentobarbitone-induced sleeping time)

The pentobarbitone-induced hypnosis test that measures onset and duration of sleep was used to assess sedative activity. Pentobabitone is an ultra-short acting barbiturate type hypnotic. It induces sedation or hypnosis in animals by potentiating the GABA mediated post synaptic inhibition through an allosteric modification of GABA receptors. Substances that have CNS depressant activity either decrease the time for onset of sleep or prolong duration of sleep or both (Trevor and Way 2007).

The protocol for pentobarbitone induced sleeping time was according to method of Turner (1965). The mice were divided randomly into six groups of five mice each. The groups are vehicle (10 ml/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and diazepam (3 mg/Kg). One hour ofter oral administration, pentobarbitone 40 mg/Kg, i.p., (Sigma Chemicals USA) was administered to each mouse to induce sleep. Each mouse was observed for latent period (time between pentobarbitone administration to loss of righting reflex) and duration of sleep (time between loss and recovery of righting reflex).

3.10 Anticonvulsant

3.10.1 Animals

Male mice, weighing between 18-22 g were used to evaluate the effect of ethanol extract of *Moringa oleifera* leaves (EMOL) on convulsion. The mice were provided food and water *ad libtum*.

3.10.2 Pentylenetetrazole (PTZ)-induced convulsion

PTZ (85 mg/Kg; s.c.) was used to induce clonic-tonic convulsion in mice (Swinyard *et al.* 1989). The mice were divided into six groups of ten (10) each. The groups are vehicle (10

arm and time spent in closed arm. The index of open arm avoidance was interpreted as level of anxiety (Triullas and Skolnick, 1993) and calculated as

$$\left[100 - \frac{(\%\text{ time on open arm} + \%\text{ entries into open arms})}{2}\right]$$

The procedure was repeated for all the mice in the different groups. There are six groups of five mice each. The groups are vehicle (10 ml/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and diazepam (1 mg/Kg). After each mouse assessment the lingering olfactory cues was cleaned using 70% ethyl alcohol. The doses used fall in the range that do not affect motor coordination (Reddy and Kulkami, 1997).

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mL/Kg; 5% Tween 80). 250, 500, 1000 and 2000 mg/Kg EMOL, and phenobarbitone (40 mg/Kg). One hour after administration (p.o) the convulsant were used to challenge the animal. The percentage survival was recorded for each group.

3.10.3 Picrotoxin-induced convulsion

Picrotoxin (14 mg/Kg; i.p.) (Gupta *et al.*, 1999) was used to induce limbic seizures followed by status epilepticus in mice. The mice were divided into six groups of ten each. The groups are vehicle (10 mL/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and phenobarbitone (40 mg/Kg). One hour after administration (p.o) the convulsant were used to challenge the animal. The percentage survival was recorded for each group.

3.10.4 Strychnine-induced convulsion

Strychnine (2 mg/Kg; i.p.) (Aguilar-Santamaria and Tortoriello, 1996) used to induce seizures. The mice were divided into six groups of ten each. The groups are vehicle (10 mL/Kg; 5% Tween 80), 250, 500, 1000 and 2000 mg/Kg EMOL, and phenobarbitone (40 mg/Kg). One hour after administration (p.o) the convulsant were used to challenge the animal. The percentage survival was recorded for each group.

3.11 LPS-induced cognitive deficit in mice

3.11.1 Animals

Thirty male Swiss albino mice weighing 20-25 g were obtained from the Central Laboratory Animal House of the College of Medicine, University of Ibadan, Ibadan, Nigeria. Animals were maintained in accordance with the University of Ibadan Ethical Committee guidelines for the care and use of laboratory animals.

3.11.2 Distribution of animals and administration of extract

The mice were randomly divided into five groups of six animals each and housed in separate cages. All animals had free access to food and water. The experimental animals were pretreated orally with EMOL at 100, 200, and 400 mg/Kg or normal saline (10 ml/Kg) once daily for seven consecutive days. Thereafter, 250 µg/Kg lipopolysaccharide (LPS) was administered intraperitoneally to the three groups that received EMOL and one of the groups that received normal saline for another seven days to induce memory impairment. Twenty-four hours after last administration the animals were subjected to Y-maze and object recognition tests.

3.11.3 Y-maze (Spatial Memory test)

Y-maze was used to assess the effect of the extract on short term memory. The Y-maze is composed of three equally spaced arms (120°; 41 cm long × 15 cm high × 5 cm wide). The parameters assessed are arm entries (locomotor activity) and spontaneous alternation performance (memory). Twenty four hours after oral administration each mouse was placed in one of the arm compartments and allowed to move freely for 5 min. Entry was defined as when body except tail of a mouse completely enters into an arm compartment. The sequence of entry was recorded manually. Alternation is defined as entry into all three arms consecutively. The arms were labeled A, B, and C, thus consecutive entries is ABC, BCA, and CAB. Where the maximum number of spontaneous alternations was then calculated as

$$[Total\ number\ of\ arms\ entered] - 2$$

Percentage alternations was calculated as

$$\left[\frac{Actual\ alternation}{Maximum\ alternation}\right] \times 100$$

The procedure was repeated for all the mice in the different groups. The apparatus was cleaned after each animal session to eliminate odour from previous animal (Brocco *et al.*, 2002).

3.11.4 Object recognition task (Cognitive memory test)

Mice were allowed to habituate to the open field box used for the object recognition task for 2 days prior to the test (ie for about 5 minutes on the last 2 days of administration). On the test day, each mouse was allowed a training session of 5 minutes with two identical objects (small plastic toys) placed in opposite direction in the open field. Time spent exploring each object during training session was recorded. The mouse was then returned to its cage. After 1h, one of the objects was replaced with novel object and the mouse returned for the test session. The test session last for 5 minutes and time spent exploring the familiar and novel object was recorded. After each session,the arena and objects were cleaned thoroughly with 10 % ethanol to ensure that behavior of the mice was not guided by previous mouse odor cues.

3.12 *In-vitro* antineuroinflammatory mechanism

3.12.1 Cell Culture

Murine microglia cell line BV-2 obtained from Interlab Cell Line Collection, Banca Biologica Cell Factoty, Genoa, Italy (ICLC ATL03001) was cultured in RPMI 1640 (Gibco) supplemented with 10 % FBS (Sigma), 2 mM glutamine (Sigma). Cells were split 1:5 when they reached confluence using trypsin/EDTA solution in PBS. Cultures were grown at 37 °C in 5 % CO_2 until 80 % confluence.

3.12.2 MTT assay for cell viability

The viability of BV cells after treatment with crude extract and fractions was determined by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay. The yellow compound MTT is reduced by mitochondrial dehydrogenases to the water-insoluble blue compound formazan, depending on the viability of cells. BV-2 were cultured in 96-well plates for 48 hours, and then pretreated for 30 minutes with or without extract (100, 150 and 200 µg/mL) followed by incubation with LPS 100 ng/mL for 24 hours. Twenty microlitres (20 µL) MTT solution (Sigma) (5 mg/mL) was added to each well. The 96 well plate were incubated for 4 hours at 37°C in a CO_2 -incubator. One hundred and eighty microliters (180 µL) of medium was removed from each well without disturbing the cell clusters and replaced with methanol/DMSO solution (50:50). The preparations were mixed thoroughly on a plate shaker with the cell containing formazan crystals. After all of the crystals were dissolved, the absorbance was read at 540 nm with a microplate reader.

3.12.3 Determination of Nitrite production by BV-2 cells

Quantification of nitrite accumulation in BV-2cells was carried out as described earlier (Olajide *et al.*, 2013). Cells were seeded in 96-well plates (2×10^5/200 mL/well), cultured for 48 hours, and then incubated with or without LPS (100 ng/mL) in the absence or presence of extract (100, 150, 200 µg/mL) for 24 h. As a parameter of NO synthesis, nitrite concentration was assessed in the supernatant of BV-2 cells by the Griess reaction with a commercially available kit (Promega, Southampton, UK). Absorbance was measured at 540 nm using a Tecan F50 microplate reader. Nitrite concentrations in the supernatants were determined by comparison with a sodium nitrite standard curve. Experiments were performed at least three times and in triplicate.

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3.12.4 Determination of PGE_2

PGE_2 production was carried out as earlier described (Olajide *et al.*, 2013). Briefly, cultured BV-2 cells were pretreated for 30 min with or without extract (100, 150, 200 µg/mL) followed by incubation with LPS 100 ng for 24 h.After the incubation period, supernatants were collected, centrifuged at 1200 rpm for 5min and levels of PGE_2 in the medium were measured by enzyme immunoassay (EIA) (Arbor Assays, Michigan, USA) according to the manufacturer's instructions

3.12.5 Determination of pro-inflammatory cytokines production

BV-2 cells were seeded in 96-well plates (2×10^5/200 µL/well), cultured for 48 hours and incubated with or without LPS (100 ng/mL) in the absence or presence of extract (100, 150, 200 µg/mL) for 24 h. TNF-α and IL-6 concentrations in supernatants were assayed with a commercially available ELISA kit (BioLegend, UK) according to the manufacturer's instruction. Absorbance was measured in a plate reader at a wavelength of 450 nm. Experiments were performed at least three times and in triplicate.

3.12.6 Determination of reactive oxygen species (ROS) in BV-2 cells

The effect of LPS on intracellular ROS levels in BV2 cells was performed using the fluorescent 2', 7'-dichlorofluorescin diacetate (DCFDA)-cellular reactive oxygen species detection assay kit (Abcam). DCFDA is a permanent fluorogenic dye capable of being deacetylated into a non fluorescent compound after diffusion into a cell. ROS of different species (OH', O_2^{2-} etc) oxidize the deacetylated DCFDA to highly fluorescent DCF. BV2 microglia was incubated with 10µM DCFDA for 30 min at 37 °C. After removal of excess DCFDA, cells were washed and then pre-treated with extract (100, 150 and 200 µg/mL) for 30 min followed by stimulation with100 ng/mL LPS for 4 h at 37 °C. Intracellular production of ROS was measured by the fluorescence detection of dichlorofluorescein (DCF) as the oxidised product of DCFH on a microplate reader with an excitation wavelength of 485 nm and emission wavelength of 535 nm.

3.12.7 Immunoblotting

Following pre-treatment with quercetin, kaempferol or rutin and stimulation with LPS (100 ng/ml), cell lysates were prepared by washing cells with PBS, followed by addition of lysis buffer and phenylmethylsulfonyl fluoride (PMSF), and centrifugation for 10 min. Nuclear

extracts were prepared using EpiSeeker Nuclear Extraction Kit (Abcam), according to the manufacturer's instructions. Briefly, cells were washed with cold PBS, followed by the addition of 20 µL of pre-extraction buffer and incubation on ice for 10 min. Thereafter, cells were centrifuged at 12,000 rpm for 1 min. Supernatants were discarded, and 10 µL of extraction buffer was added to the pellet and incubated on ice for 15 min, followed by centrifugation at 13,500 rpm for 15 min at 4 °C. The resulting nuclear extracts in the supernatants were collected. 25 µg of protein was subjected to sodium dodecyl sulphate-polyacrylamide (SDS) gel electrophoresis. Proteins were then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA) for 2 h. Membranes were then blocked at room temperature for 1 h and then incubated with primary antibodies overnight at 4 °C. Primary antibodies used in the experiments were rabbit anti-iNOS (Santa Cruz, 1:500), rabbit anti-COX2 (Santa Cruz, 1:500). Blots were detected with Alexa Fluor 680 goat anti-rabbit IgG (Life technologies, UK) using the Licor Odyssey infrared imager. Equal protein loading was assessed using rabbit anti-actin antibody (Sigma, 1:1000).

3.13 *In-vitro* immunomodulatory mechanisms

3.13.1 Cell Culture (RAW 264.7 cell)

RAW 264.7 cells obtained from American Type Culture Collection (ATCC TIB 71) were cultured in Dulbecco's Modified Essential Medium (DMEM) with 4 mM L-glutamine and 4.5 g/L glucose (endotoxin level <0.005 endotoxin U/ml. BioWhittaker, Bioproducts Heidelberg, Germany) supplemented with 10% heat-inactivated FBS (Gibco-BRL Life Technologies). Cells were maintained at 37 °C in 5% CO_2 and used for experiments between passages 5 and 20. The human embryonic kidney cell line 293 (HEK293; DSMZ-German collection of microorganisms and cell cultures, ACC 305) was grown in DMEM (BioWhittaker, Bioproducts, Heidelberg, Germany) supplemented with 10% FCS (Biochrom KG, Berlin, Germany) and 2 mM glutamine (Merck, Munich, Germany). Cells were splitted into 1:10 when they reached approximately 85-90 % confluence using 0.05% trypsin/0.02% EDTA in PBS.

3.13.2 ATP assay for cell viability

Viability of RAW 264.7 cells was determined by the ATP assay. ATP plays a central role in energy exchange in biological systems, and is present in all metabolically active cells. Thus, levels of ATP can be used to determine the functional integrity of cells. Cells (2×10^5/mL)

were cultured for 48 h, and then incubated with LPS (100 ng/mL) or EMOL for 24 hrs. The concentration of ATP was measured through a sensitive luciferin-luciferase bioluminescent assay using a kit (Promega). After incubation, 100 µL of reconstituted substrate was added to the cells. Luminescence was then measured in Berthold Luminometer.

3.13.3 Multiplex ELISA p38, ERK1/2 and JNK MAP kinases

Investigation of the effects of compounds on phosphorylation of p38, ERK1/2 and JNK MAP kinases was carried out as earlier described by Olajide *et al.*, 2013, with slight modifications. RAW 264.7 cells were left untreated or treated with compounds (12.5 µM of both kaempferol and quercetin; and 25 µM of rutin) or LPS (100 ng/mL) for 24 h. At the end of the stimulation period, cells were washed with cold phosphatebuffered saline (PBS) and lysed with pre-formulated lysis buffer (Cell Signalling Technologies). Cell lysates were subjected to PathScans MAP Kinase Multi-Target Sandwich ELISA for phospho-p38, phospho-ERK1/2 and phospho-JNK, according to the manufacturer's instructions (Cell Signalling Technologies, Inc). Absorbance values were measured with a plate reader at 450 nm.

3.14 Reverse Phase Fractionation

3.14.1 HPLC-DAD Analysis

Hundred milligram of each fraction was dissolved in 50 mL methanol for HPLC-DAD analysis. The semi-preparative reversed-phase HPLC analysis was carried out on an Agilent 1260 Preparative HPLC system consisting of a preparative pump, degasser, autosampler and Diode Array Detector (DAD). Analyses of the samples (F20, F50, F80, F100) were conducted using a semi-preparative reversed-phase column, ACE 10 C18-HL column (150 × 10 mm, 10 µm; Hichrom Ltd) with a C18 guard column ACE3310110GD (10 × 10 mm, 10 µm, Hichrom Ltd). The mobile phase consisted of Solvent A (0.1% v/v TFA in water) and Solvent B (0.1% v/v of TFA in MeOH) at a flow rate of 3.00 mL/min. Gradient elution was employed starting at 30% B for 3 min, 30% - 100% B for 30 min, isocratic 100% B for 10 min, and finally 100% - 30% B for 2 min. At the end of this sequence, the column was equilibrated under the initial conditions for 2 min. The sample injection volume was 100 µL and the DAD detector was set to scan from 200 nm to 400 nm. Data were analyzed using the OpenLAB Chromatography Data System. According to the peaks on the chromatogram, F20 was further subjected to prep-HPLC repeatedly under the same conditions to yield

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isoquercetin (mg). The isolated pure compound was identified by comparing their proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) and MS with reported data.

3.15 T-cell Immunomodulatory mechanisms

3.15.1 Cell Culture (Jurkat cell)

The leukemic T cell line Jurkat E6.1 was purchased from American Type Culture Collection (TIB-152). Culture medium for all cell was RPMI 1640 (Mediatech, Herndon. VA), containing 10% FBS (Atlanta Biologicals, Norcross, GA), 50 U/mL each of penicillin and streptomycin (Life Technologies, Grand Island. NY), and 20 mM glutamine (Life Technologies).

3.15.2 Trypan blue cell exclusion assay

On the day of experiment the cells were counted and plated at 100,000 cells/well in a 96 well plate. 0.8 µL of vehicle DMSO and prepared concentration of extract in DMSO were added such that the concentration of DMSO does not exceed 0.4% of medium. The experiment was carefully planned with 10 minutes between each treatment to allow for counting using trypan blue exclusion dye on the microscope. The numbers of dead (dark blue stained) and viable (pan stained) cells in the grids of hemocytometer were recorded under an optical microscope (100× magnification; Olympus, Tokyo, Japan). The death rate Jurkat cells at the different concentrations were calculated as follows: cell death rate = dead cell count/ (dead cell count + viable cell count) × 100%.

3.15.3 Antibody (Abs) and chemicals

Anti-CD3 (OKT3), anti-CD11a (HB202), and anti-CD54 (R6.5D6) were purchased from American Type Culture Collection (Manassas, VA) and purified from serum-free hybridoma culture medium using protein G-Sepharose. Trypan blue. CFSE was purchased from Molecular Probes (Eugene, OR). Annexin V-phycoerythrin was purchased from Pharmingen. 7AAD, CCR7

3.15.4 CFSE Assay

Specified amount of cells resuspended in serum free RPMI contained Falcon tubes were labeled with 2.5 µM CSFE in the hood with the light turned off. The tube was wrapped in

isoquercetin (mg). The isolated pure compound was identified by comparing their proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) and MS with reported data.

3.15 T-cell immunomodulatory mechanisms

3.15.1 Cell Culture (Jurkat cell)

The leukemic T cell line Jurkat E6.1 was purchased from American Type Culture Collection (TIB-152). Culture medium for all cell was RPMI 1640 (Mediatech, Herndon, VA), containing 10% FBS (Atlanta Biologicals, Norcross, GA), 50 U/mL each of penicillin and streptomycin (Life Technologies, Grand Island, NY), and 20 mM glutamine (Life Technologies).

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3.15.4 CFSE Assay

Specified amount of cells resuspended in serum free RPMI contained Falcon tubes were labeled with 2.5 µM CSFE in the hood with the light turned off. The tube was wrapped in

aluminium foil and cells were allowed to absorb stain for about 15 minutes at the incubator at 37°C and 5% CO_2. Equal volume of complete RPMI was added before stained cells were resuspended in fresh complete RPMI and plated at 100,000 cells per well with the light off. The vehicle and extract was then added and placed in the incubator at 37°C and 5% CO_2. At the time points (day 1, 4, and 7) the cells were collected washed twice with ice cold PBS and resuspended in 100 µL 1X Annexin V buffer running on the Accurri.

3.15.5 Annexin V

At the time points (day 1, 4, and 7), cells were collected washed twice with ice cold PBS and stained for 15 minutes in the dark at room temperature with 1 µl Annexin V in 99 µl of 1X Annexin V buffer. The cells were resuspended in 100 µl 1X Annexin V buffer before running on Accurri.

3.15.6 7AAD

At the time points (day 1, 4, and 7), cells were collected and washed twice with ice cold PBS and stained for 15minutes in the dark at room temperature with 5 µl 7AAD in 95 µl of 1X Annexin V buffer. The cells were resuspended in 100 µl 1X Annexin V buffer before running on Accurri.

3.15.7 CCR7 assay

At the time points (day 1, 4, and 7), cells were collected and washed twice with ice cold PBS and stained for 15 minutes in the dark at room temperature with 1.5 µl Annexin V in 98.5 µl of 1X Annexin V buffer. The cells were resuspended in 100 µl 1X Annexin V buffer before running on Accurri.

3.15.8 Flow Cytometry

Flow cytometry was performed using an Accuri C6 (Accuri Cytometers, Ann Arbor, MI). Data analysis was performed using CFlow (Accuri) software. Dot plots and histogram representing 10,000 cells/event were prepared and analyzed.

3.16 Statistical Analysis

Data were analysed using Graph Pad Prism software version 5.00 and were expressed as mean ± S.E.M (standard error of mean). Statistical analysis of data was carried out using one way ANOVA, followed by Dunnet test for comparison between groups. P-values less than

aluminium foil and cells were allowed to absorb stain for about 15 minutes at the incubator at 37°C and 5% CO_2. Equal volume of complete RPMI was added before stained cells were resuspended in fresh complete RPMI and plated at 100,000 cells per well with the light off. The vehicle and extract was then added and placed in the incubator at 37°C and 5% CO_2. At the time points (day 1, 4, and 7) the cells were collected washed twice with ice cold PBS and resuspended in 100 µL 1X Annexin V buffer running on the Accurri.

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3.15.6 7AAD

At the time points (day 1, 4, and 7), cells were collected and washed twice with ice cold PBS and stained for 15minutes in the dark at room temperature with 5 µl 7AAD in 95 µl of 1X Annexin V buffer. The cells were resuspended in 100 µl 1X Annexin V buffer before running on Accurri.

3.15.7 CCR7 assay

At the time points (day 1, 4, and 7), cells were collected and washed twice with ice cold PBS and stained for 15 minutes in the dark at room temperature with 1.5 µl Annexin V in 98.5 µl of 1X Annexin V buffer. The cells were resuspended in 100 µl 1X Annexin V buffer before running on Accurri.

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3.16 Statistical Analysis

Data were analysed using Graph Pad Prism software version 5.00 and were expressed as mean ± S.E.M (standard error of mean). Statistical analysis of data was carried out using one way ANOVA, followed by Dunnet test for comparison between groups. P-values less than

0.05 ($p<0.05$) were considered to be statistically significant. OpenLAB Chromatography Data System was used to analyse data from the HPLC. Flow cytometry data were analysed using Flow Accurri software.

CHAPTER FOUR

RESULTS

4.1 Preliminary phytochemical screening

Results of the preliminary phytochemical screening revealed the presence of saponins, condensed tannins, cardiac glycoside, free anthraquinones, and coumarins.

Table 4.1: Evaluation of secondary metabolites present in EMOL

Phytochemicals	Result
Alkaloids	-
Saponins	+++
Condensed Tannins	+++
Free Anthraquinones	+++
Combined Anthraquinones	+
Cardiac Glycosides	+++
Cyanogenic Glycosides	-
Coumarins	++

+++ = abundant, ++ = present, + = trace, - = absent

4.2 Acute Toxicity Test

Acute toxicity studies gave the LD_{50} >5000 mg/Kg foroml route.

4.3 NEUROPHARMACOLOGICAL EFFECTS OF ETHANOL EXTRACT OF *Moringa oleifera* LEAVES

4.3.1 Novelty Induced Behavior (NIB)

The effect of ethanol extract of *Moringa oleifera* leaves (EMOL) on NIB is presented in figure 4.1. Administration of crude extract of ethanol extract of *Moringa oleifera* leaves (250-2000 mg/Kg. p.o.) showed a significant reduction [$F(5, 24) = 382$; $P < 0.0001$] in rearing when compared to control that received Tween 80. Treatment with EMOL resulted in a reduction [$F(5, 23) = 382$; $P < 0.0001$] of grooming relative to control mice. The most pronounced effect was at 2000 mg/Kg which resulted in 40.80 ± 6.54 and 13.80 ± 1.32 for rearing and grooming respectively (Figure: 4.1).

4.2 Acute Toxicity Test

Acute toxicity studies gave the LD_{50} >5000 mg/Kg for oral route.

4.3 NEUROPHARMACOLOGICAL EFFECTS OF ETHANOL EXTRACT OF *Moringa oleifera* LEAVES

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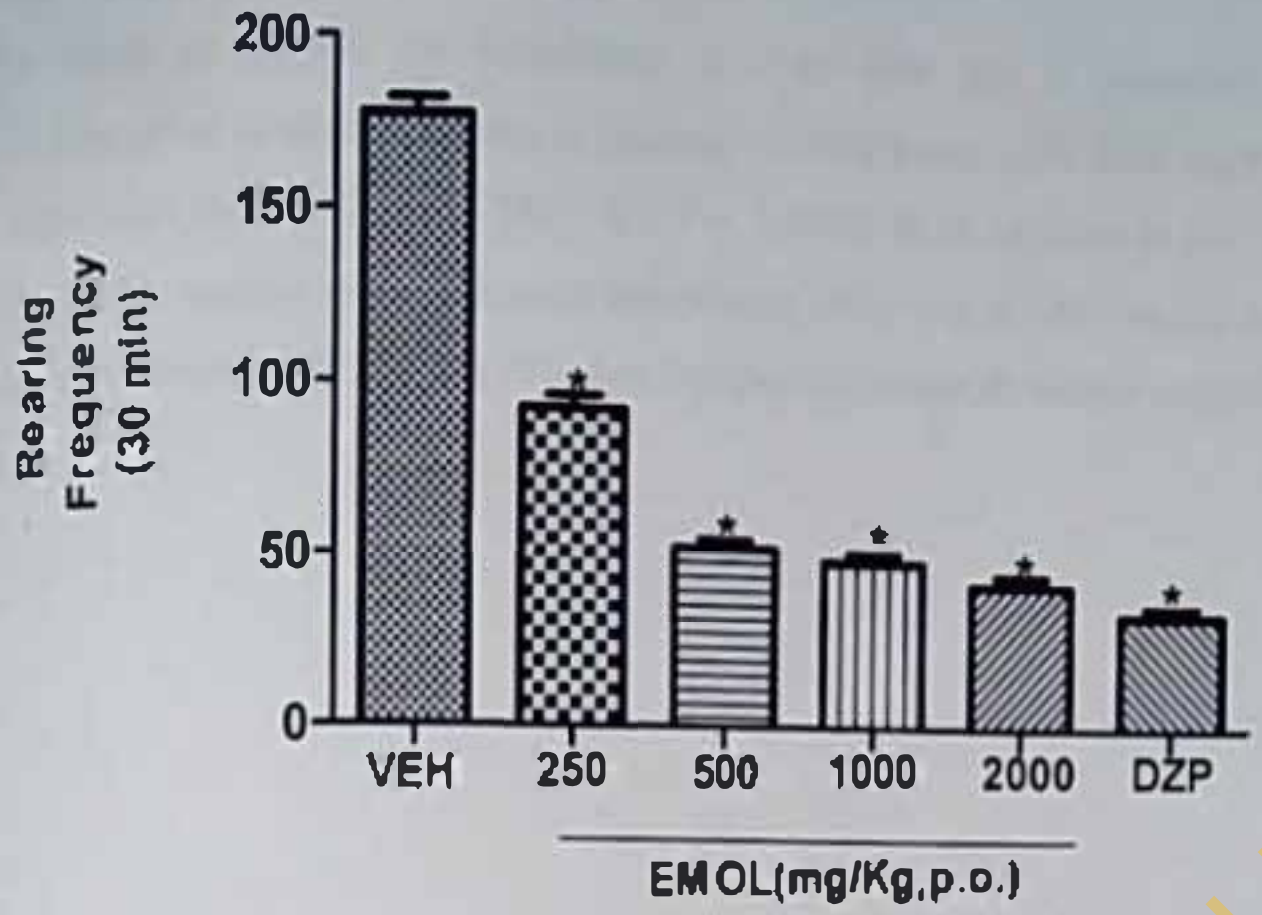


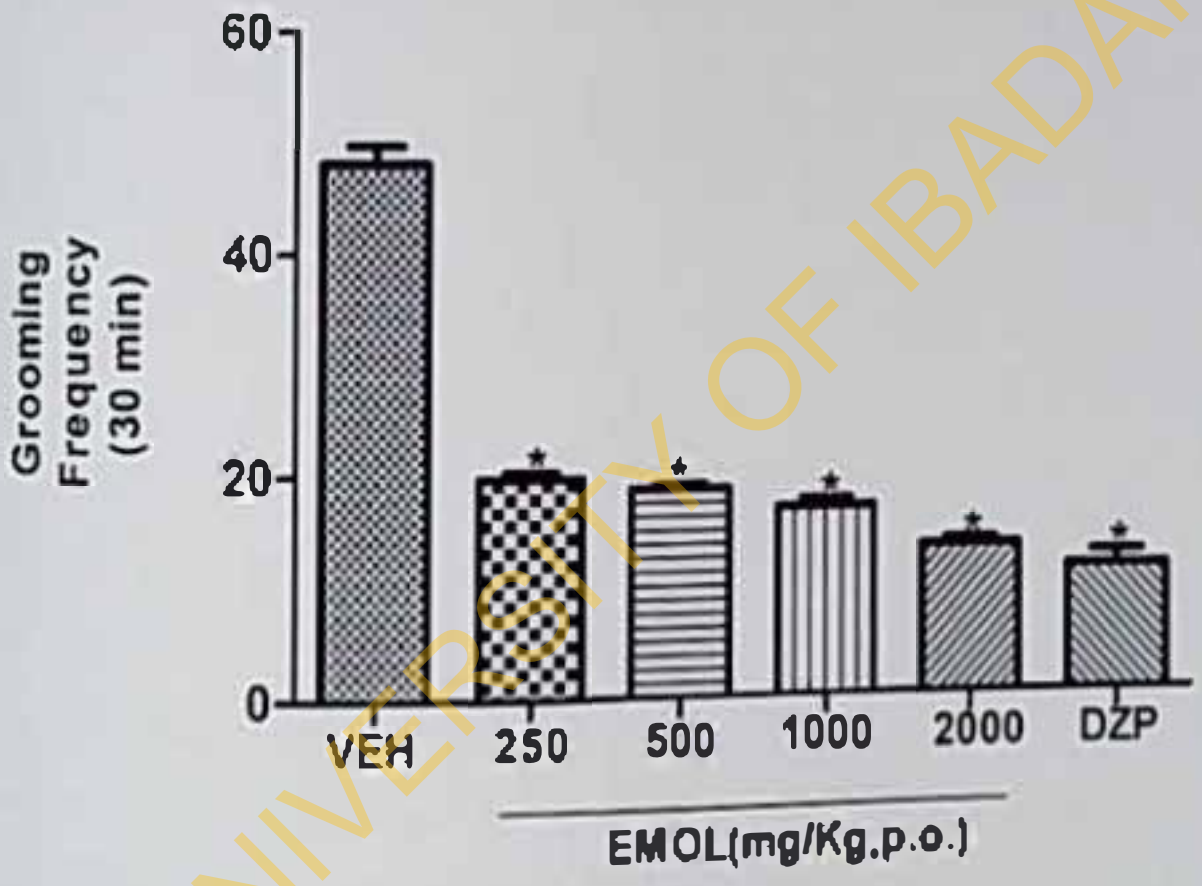


Figure 4.1: The effect of the ethanol extract of *Moringa oleifera* leaves on novelty induced rearing and grooming in open field test.

Bars represent mean values with standard error bars.

One way ANOVA followed by Dunnet's muhiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera* leaves

4.3.2 Locomotion in open field test

The effect of EMOL on locomotion in open field test is presented in figure 4.2. Administration of ethanol extract of *Moringa oleifera* leaves (250-2000 mg/Kg, p.o.) showed a significant reduction [F (5, 24) = 87; P < 0.0001] in locomotion in the open field when compared to control mice. The most pronounced effect was at 1000 mg/Kg which resulted in a lower locomotion (27.60 ± 4.93) when compared to tween 80 treated control mice.

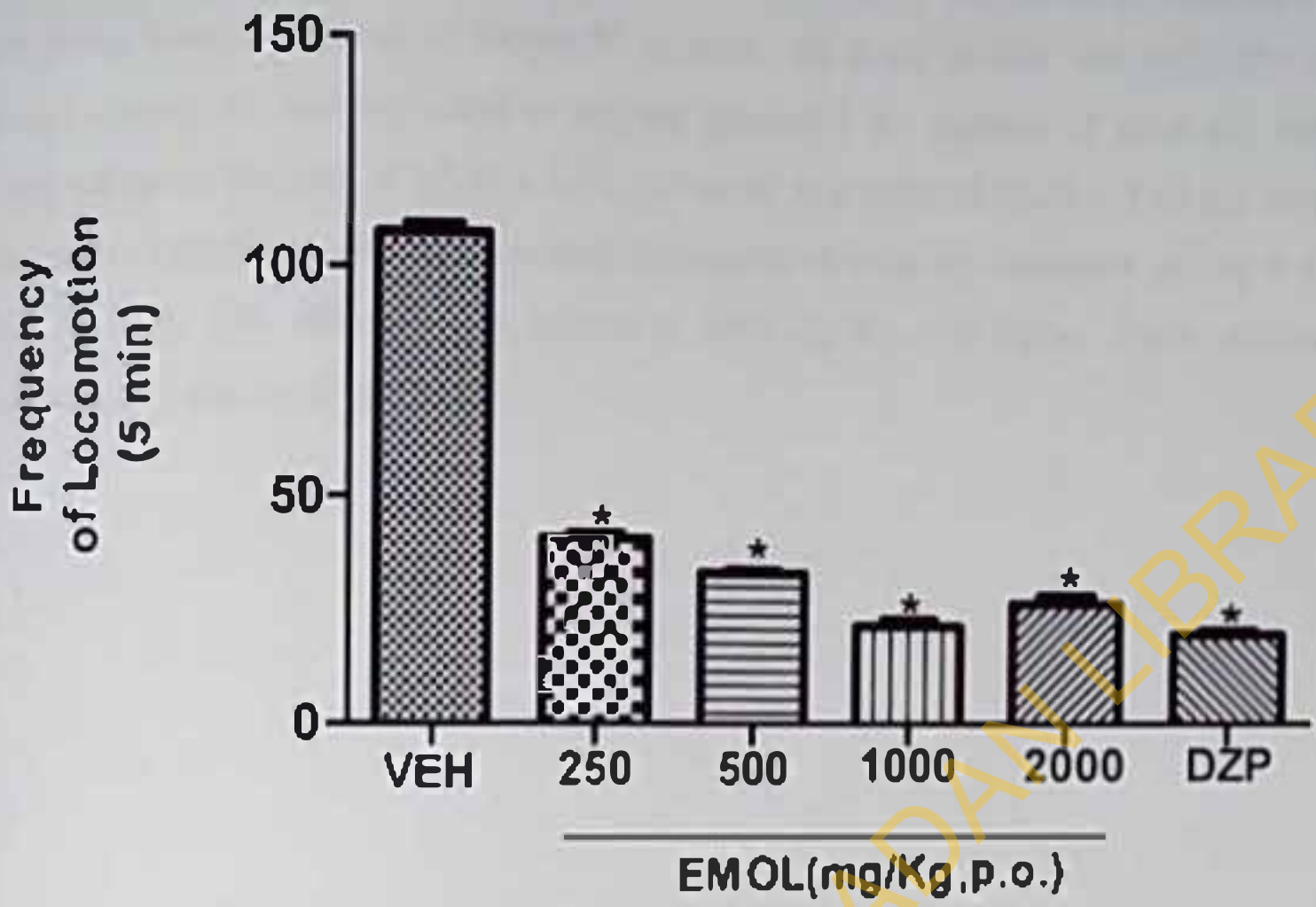


Figure 4.2: The effect of the ethnol extract of *Moringa oleifera* leaves on locomotion behavior in open field test.

Bars represent mean values with error bars.

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera* leaves

4.3.3 Exploratory activity in hole board test:

The administration of ethanol extract of *Moringa oleifera* leaves (250-2000 mg/Kg, p.o) showed a significant reduction [F (5, 24) = 49; P < 0.0017] in number of head dips on the hole board when compared to Tween 80 in mice. At doses of 250, 500 and 1000 mg/Kg, ethanol extract of *Moringa oleifera* slightly decreased the number of head dip responses giving values to the tune of 17.00 ± 2.65, compared to a value of 30.20 ± 2.62 for Tween 80. Diazepam (3 mg/Kg, p.o) also decreased the number of head dip responses, giving a value of 14.40 ± 2.42. The effect of the extract at 2000 mg/Kg was higher when compared to diazepam at 3 mg/Kg (Figure: 4.3).

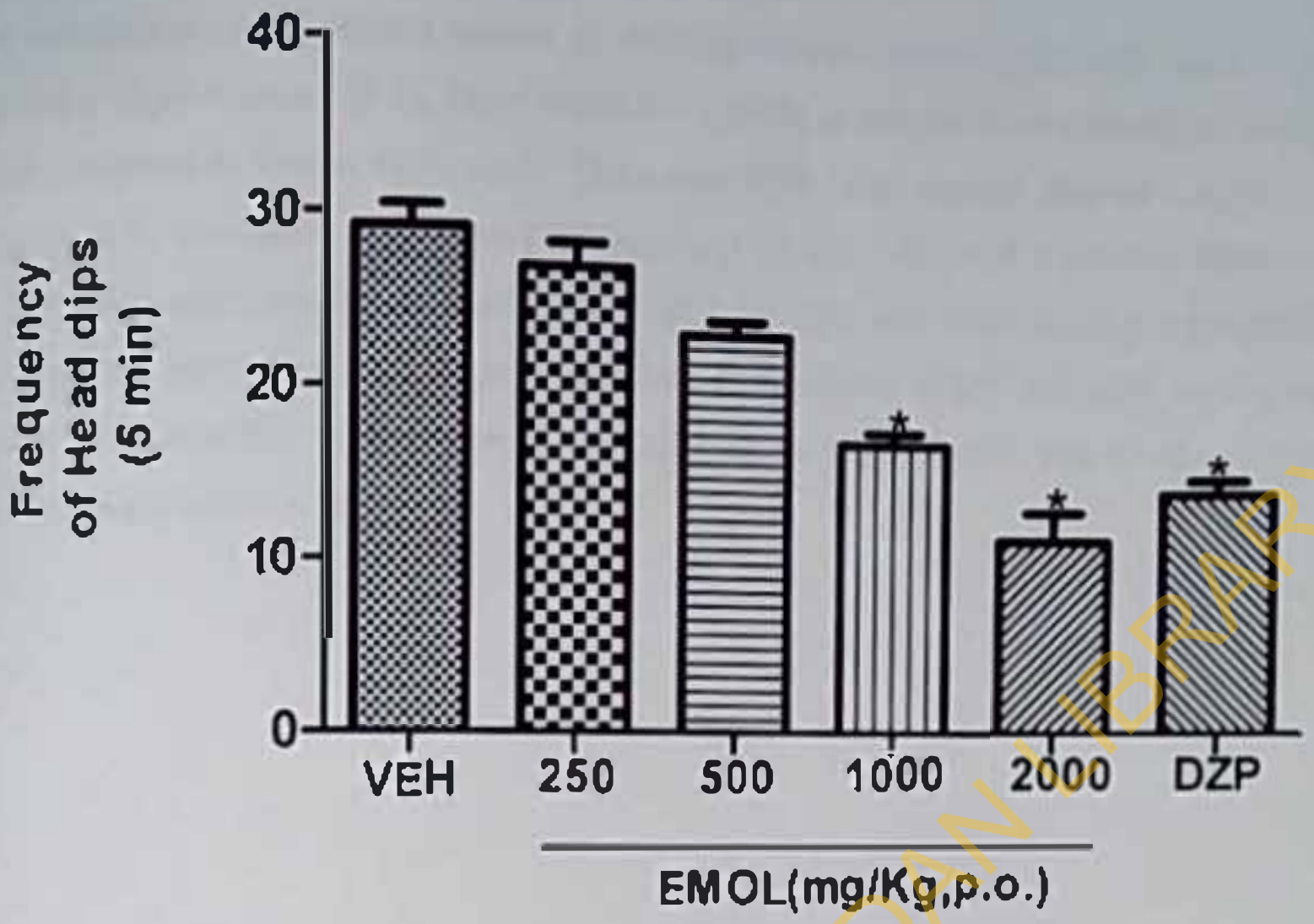


Figure 4.3: The effect of the ethanol extract of *Moringa oleifera* leaves on exploratory activity in hole board test.

Bars represent mean values with error bars.

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

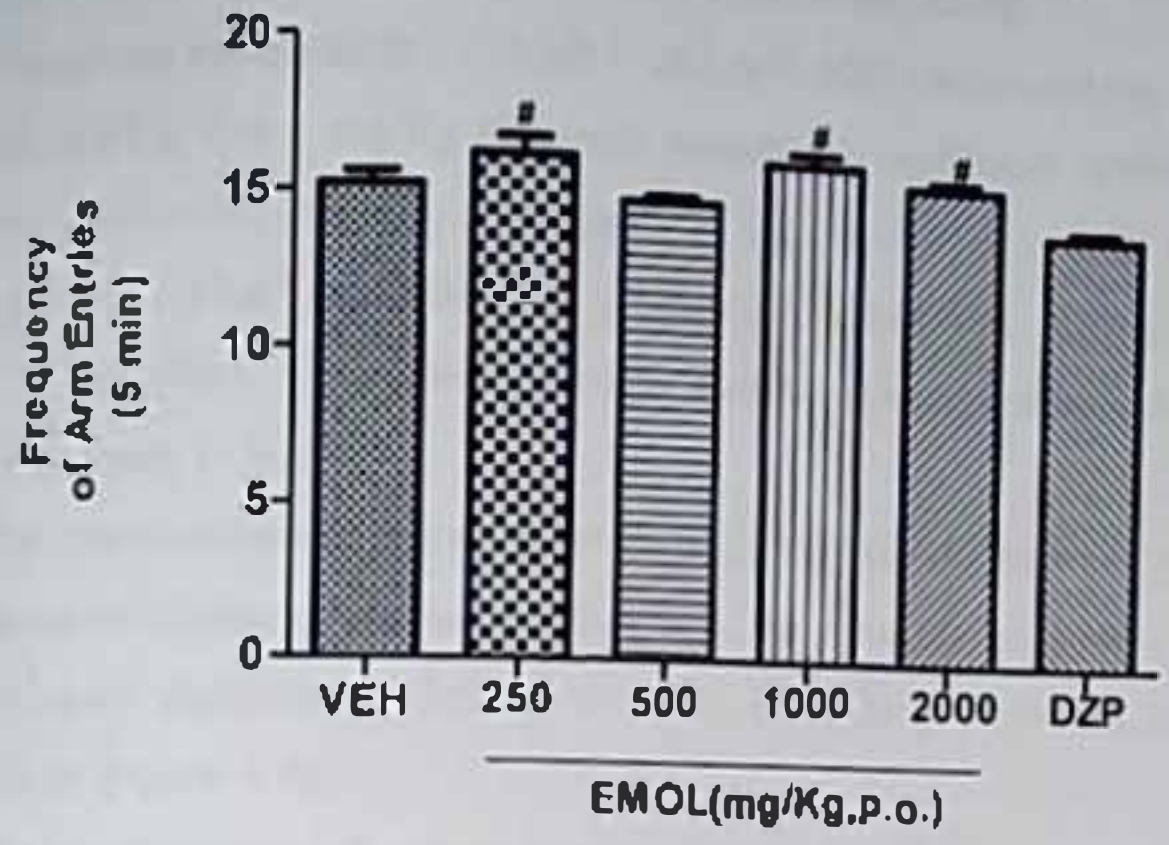
DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera*

4.3.4 Learning and memory measured by Y maze

The administration of ethanol extract of *Moringa oleifera* leaves (250-2000 mg/Kg. p.o) showed a slight decrease [F (5, 24) = 4.979; P = 0.0029] in number of arm entries in Y-maze when compared to Tween 80 in mice. The extract (250–2000 mg/Kg) showed a significant increase in % alternation at 250 and 2000 mg/Kg [F (5, 24) = 32.52; P < 0.0001]. Diazepam at 3 mg/Kg significantly decreased and EMOL at 250 and 2000 mg/Kg significantly increased the percentage alternation. The effect of the extract at 250 and 2000 mg/Kg was greater than the effect of diazepam (3 mg/Kg). Highest % alternation was 85.00 ± 4.07 at 2000 mg/Kg (Figure: 4.4).



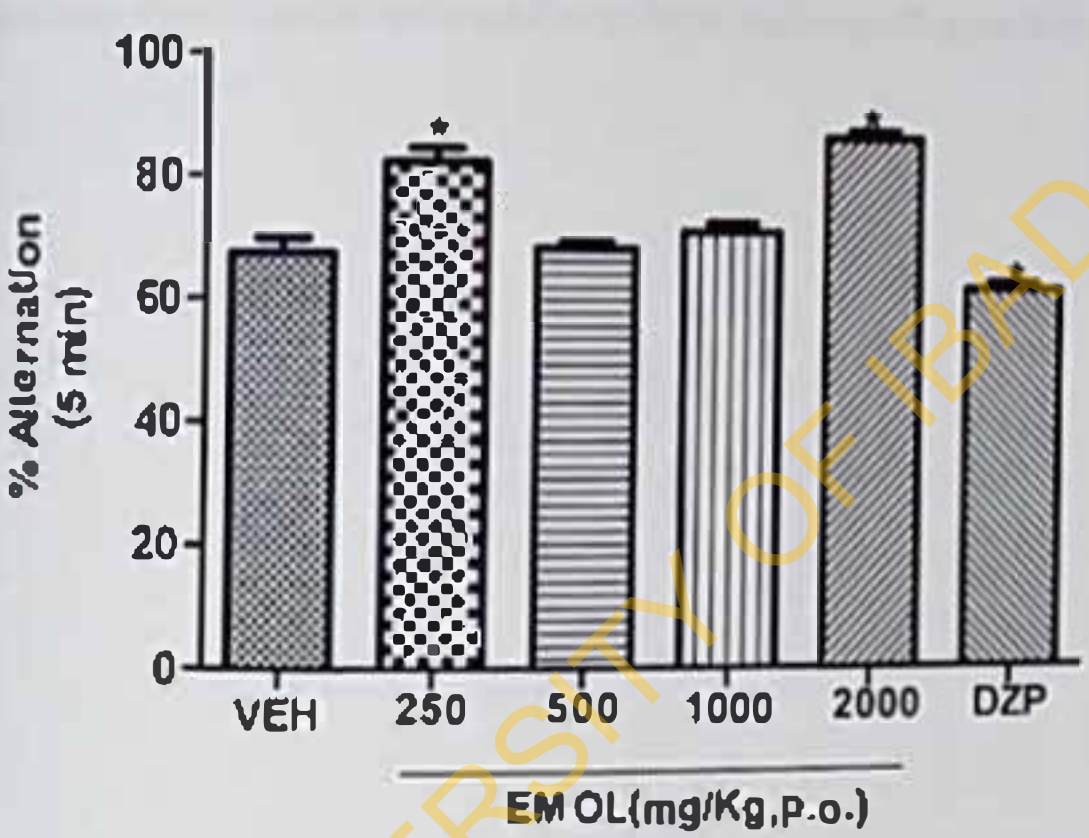


Figure 4.4: The effect of the ethanol extract of *Moringa oleifera* leaves on learning and memory in mice (% alternation and arm entries)

Bars represent mean values with error bars.

One way ANOVA followed by Dunnet's multiple comparison test.

#P < 0.05, indicate significant difference from diazepam.

* P < 0.05, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

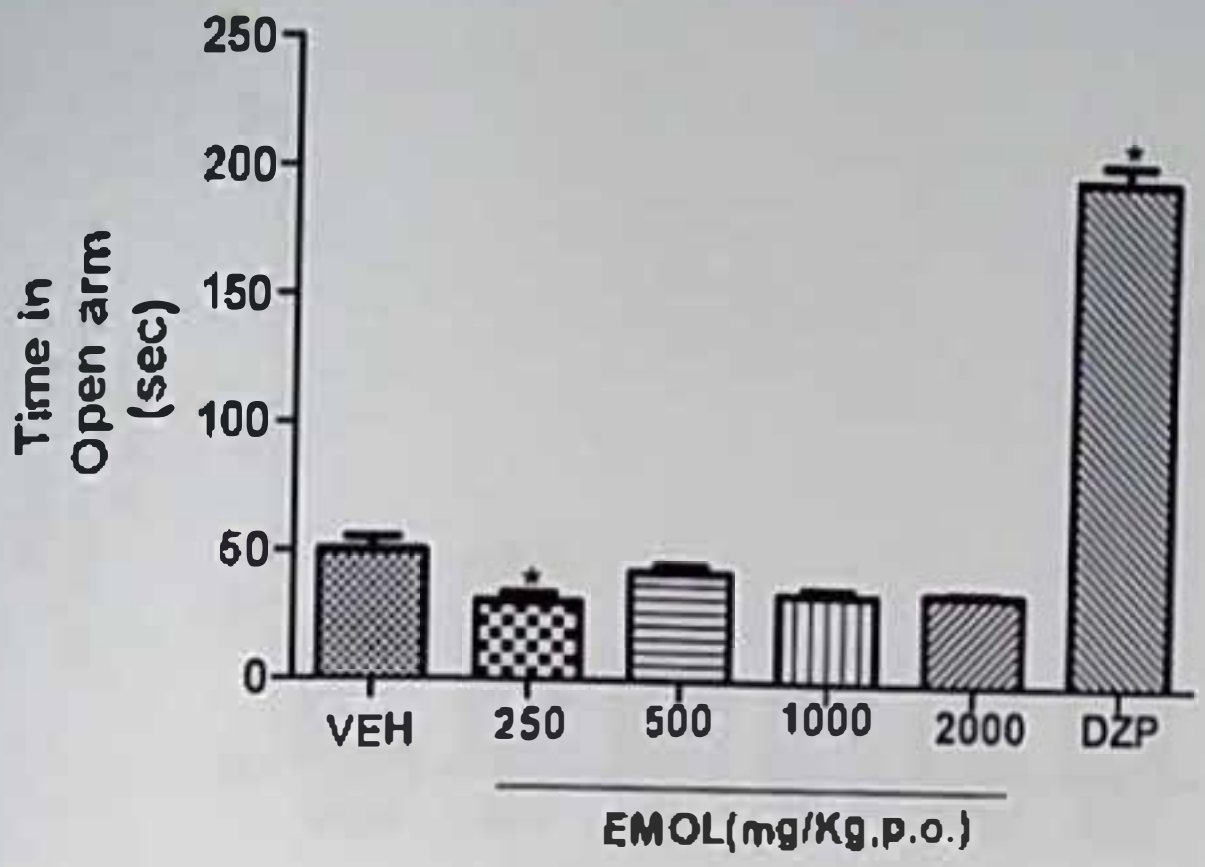
EMOL: Ethanol extract of *Moringa oleifera* leaves

4.3.5 Anxiety measured by Elevated Plus Maze (EPM)

The extract significantly [$F(5,24) = 280$, $p<0.0001$] decreased the time spent in open arm at 250 mg/Kg, and 1 mg/Kg diazepam produced a significant increase in time spent in open arm. Diazepam (1 mg/Kg) significantly [$F(5, 24) = 191.7$, $p<0.0001$] decreased time spent in open while EMOL at 2000 mg/Kg significantly increased time spent in close arm (Figure: 4.5). The effect of the extract at tested dose was almost the opposite when compared to that of diazepam (1 mg/Kg).

The administration of extract (250-2000 mg/Kg) significantly [$F(5, 24) = 69.26$, $p<0.0001$] increased number of entries into close arm, but it was decreased by diazepam. Also, 1 mg/Kg diazepam significantly [$F(5,24) = 192.6$, $p<0.0001$] increased the number of open arm entries (Figure: 4.6a).

The administration of extract increased the index of open arm avoidance, while 1 mg/Kg diazepam significantly reduced the index avoidance of open arm (Figure 4.6b)



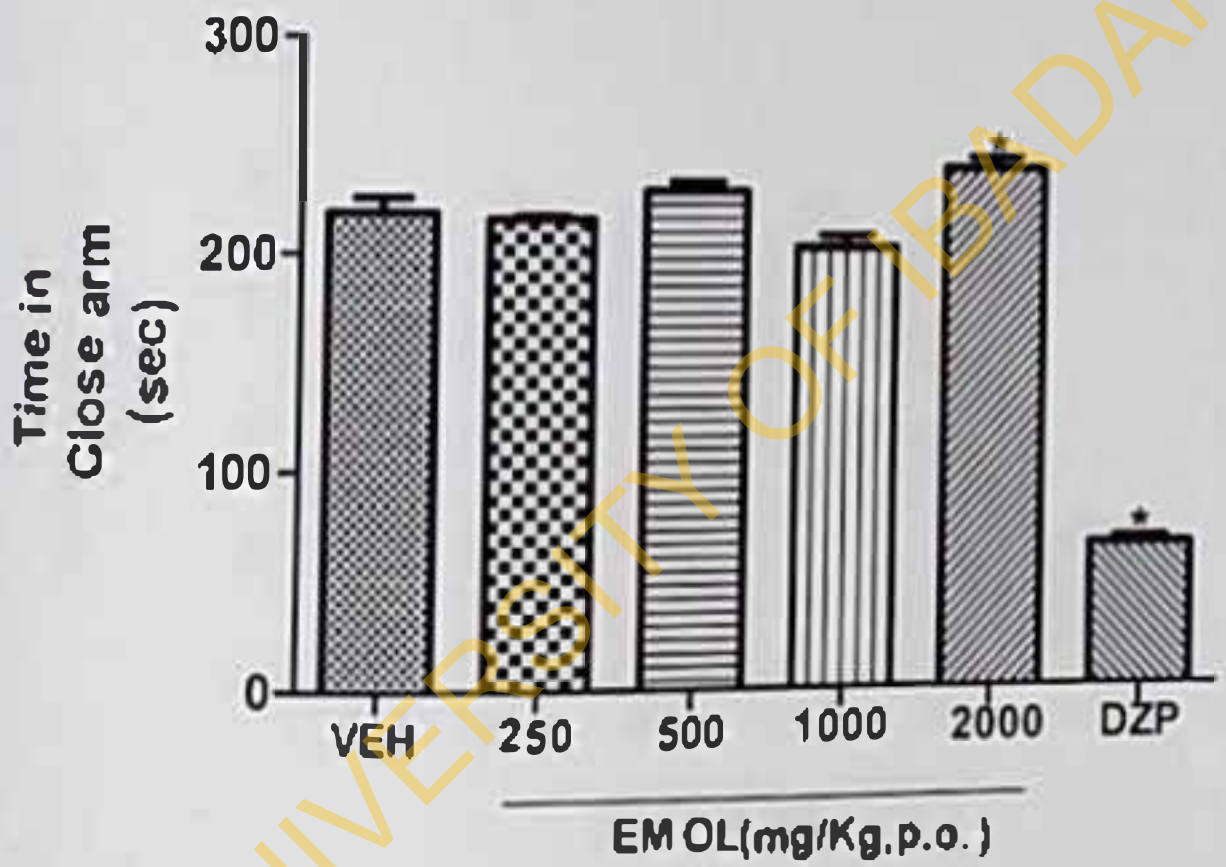


Figure 4.5: The effect of the ethanol extract of *Moringa oleifera* leaves on the elevated plus maze (time spent in open and close arm)

Bars represent mean values with error bars.

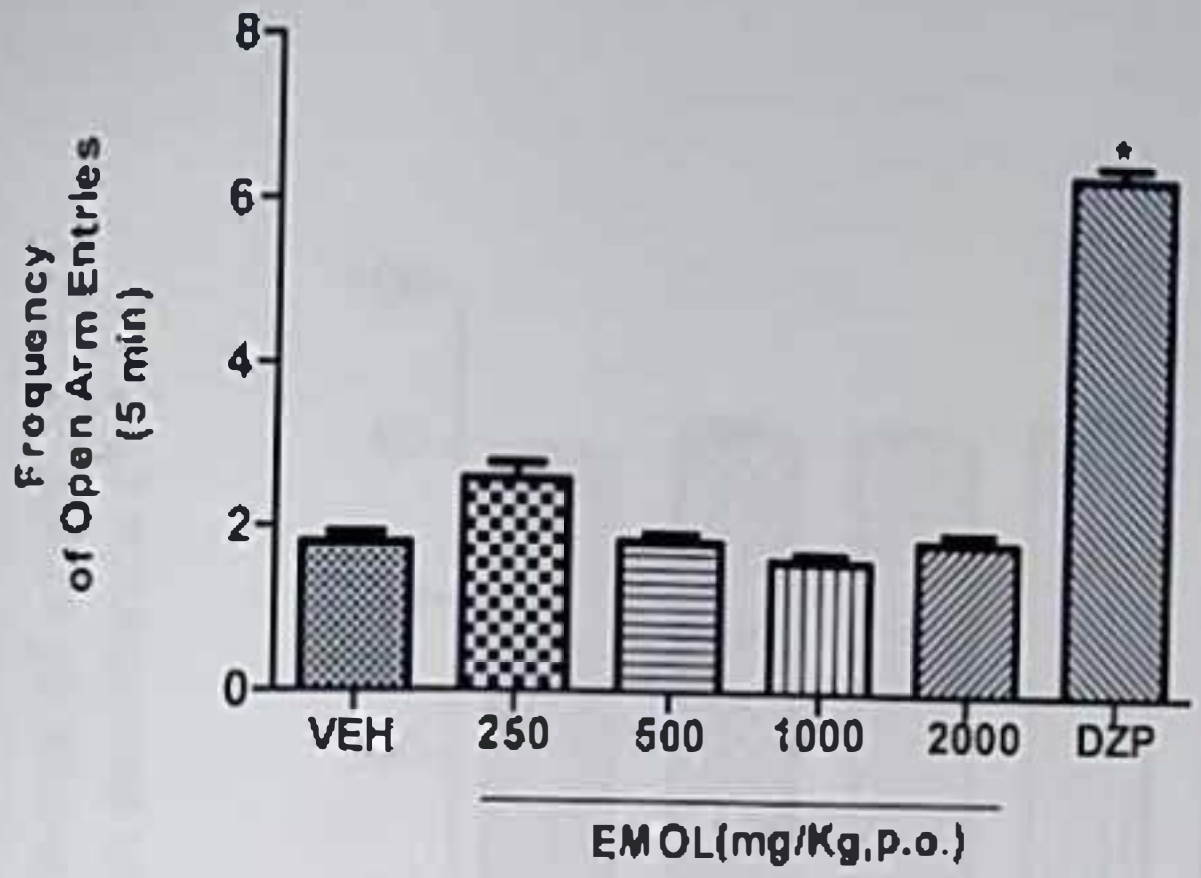
One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera* leaves



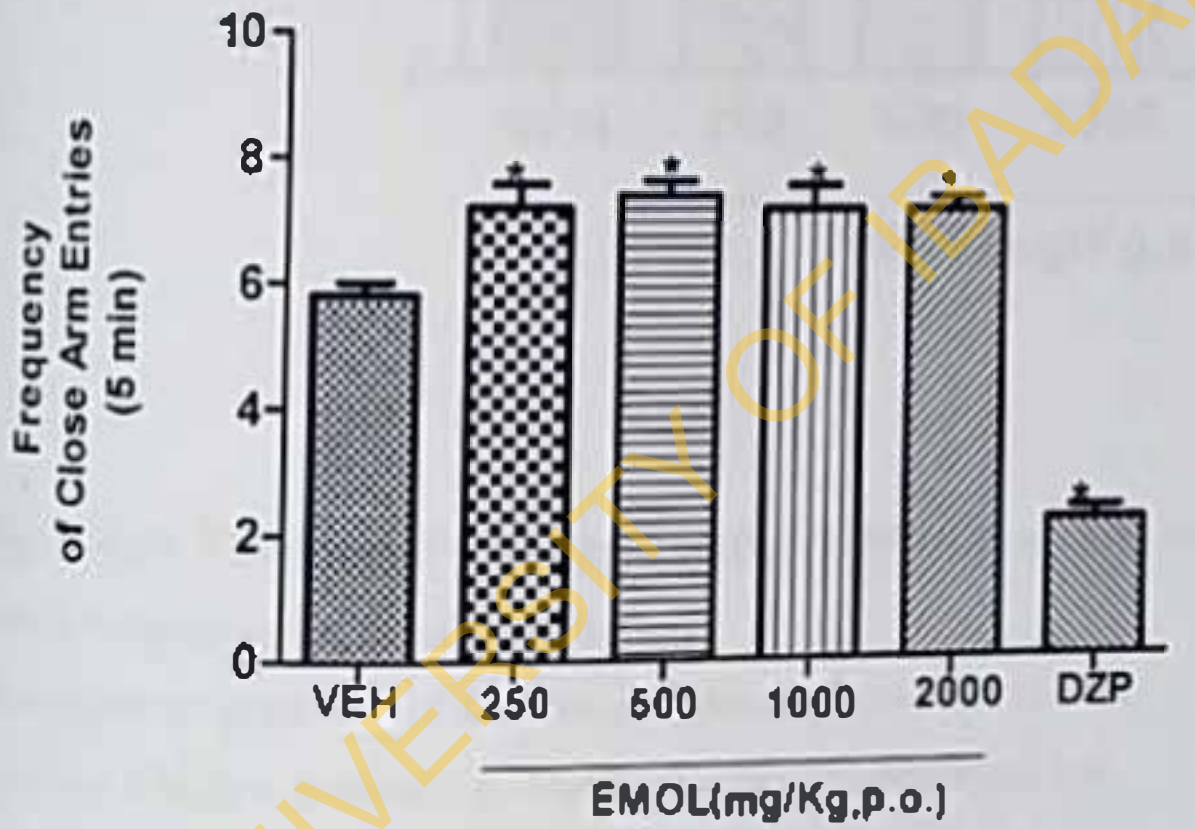


Figure 4.6a: The effect of the ethanol extract of *Moringa oleifera* leaves on elevated plus maze (arm entries)

Bars represent mean values with error bars.

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera* leaves

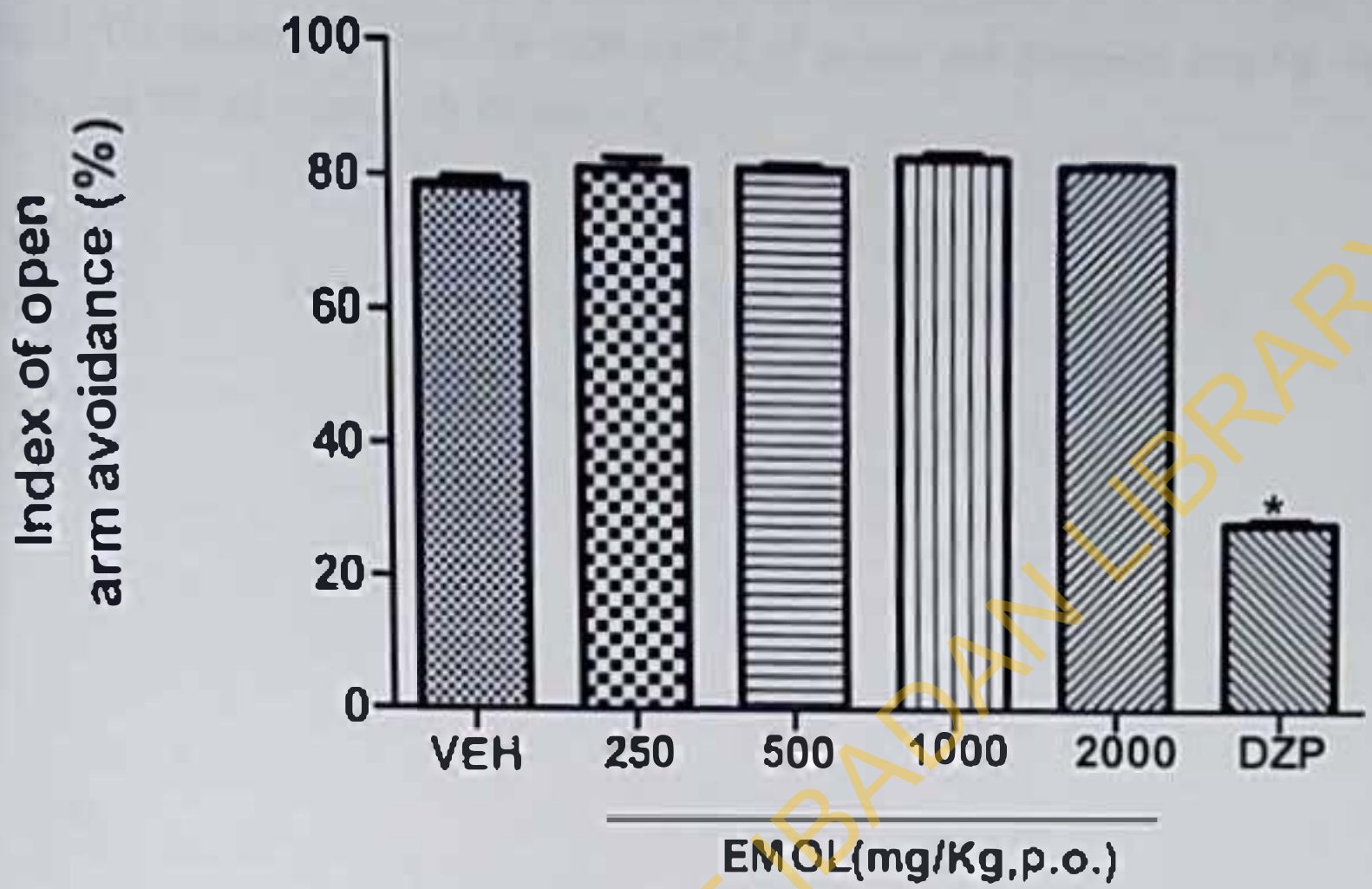


Figure 4.6b: The effect of the ethanol extract of *Moringa oleifera* leaves on index of open arm avoidance in elevated plus maze

Bars represent mean values of index of open arm avoidance (IOAA).

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera*

4.3.6 Pentobarbitone-induced sleeping time:

The extract (250-2000 mg/Kg) showed a significant dose-dependent reduction [F (5, 24) = 0.19; P < 0.0001] in sleep latency by pentobarbitone. Effect of the extract on latency to sleep at 2000 mg/Kg (1.40 ± 0.22) was comparable to diazepam 3 mg/Kg (0.81 ± 0.28) (Figure: 4.7). Sleep duration also showed a significant dose dependent increase [F (5, 20) = 266; P < 0.0001]. The duration of sleep for 2000 mg/Kg of extract and diazepam 3mg/Kg were 159±32 and 185±12 respectively (Figure: 4.8)

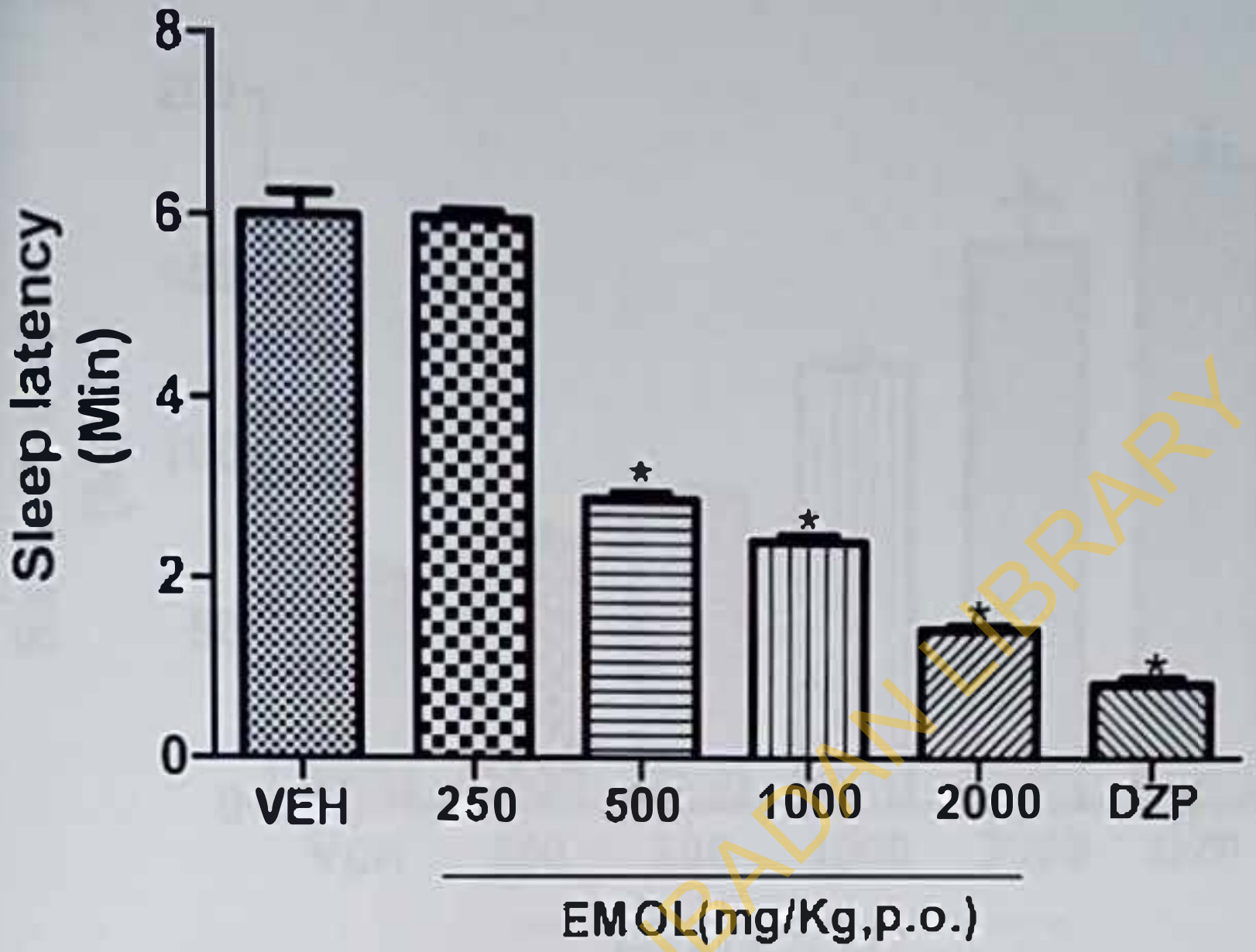


Figure 4.7: The effect of the ethanol extract of *Moringa oleifera* leaves on sleep latency in pentobarbitone sleep test.

Bars represent mean values with error bars.

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 mL/Kg)

EMOL: Ethanol extract of *Moringa oleifera* leaves

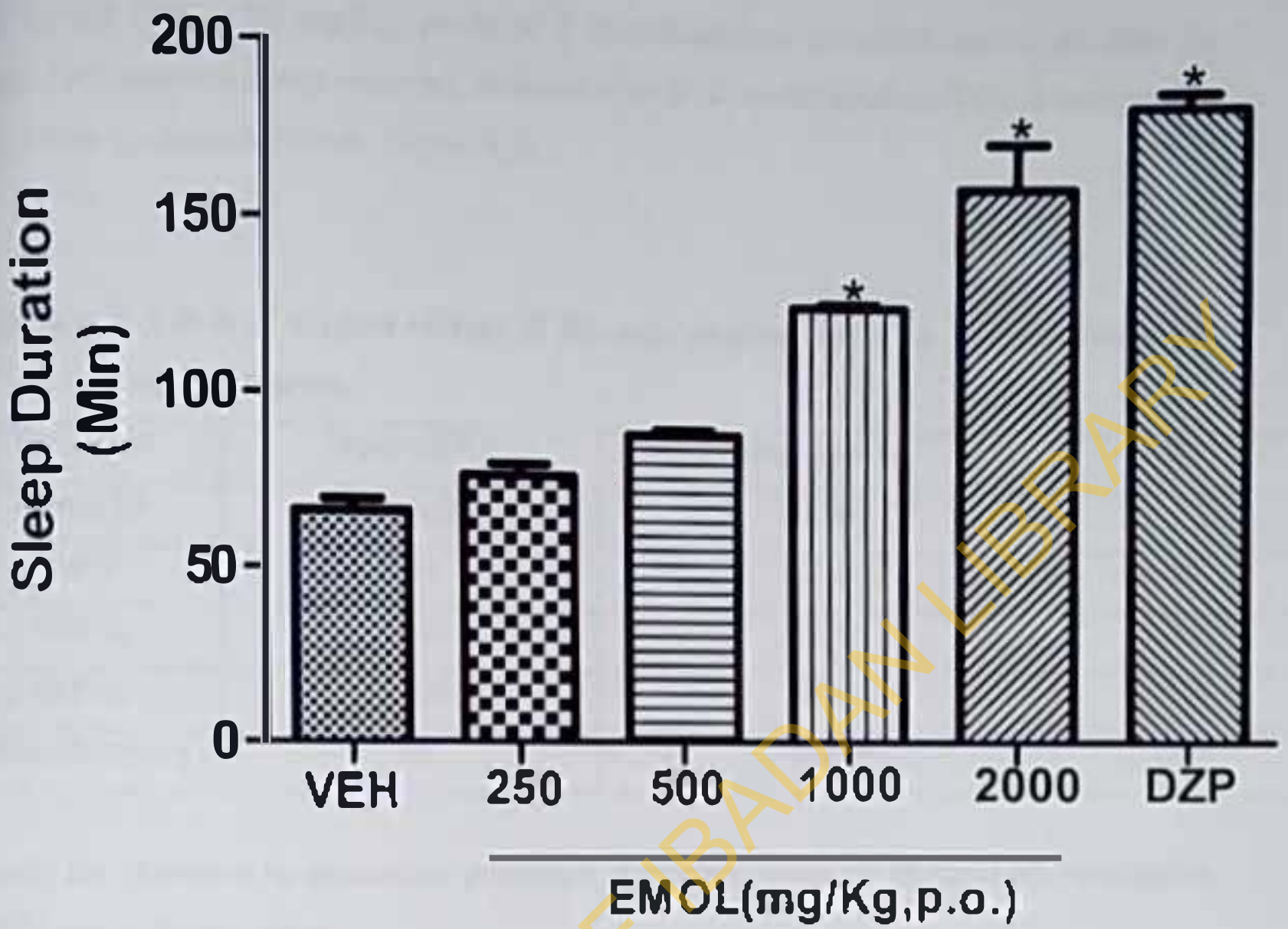


Figure 4.8: The effect of the ethanol extract of *Moringa oleifera* leaves on sleep duration in pentobarbitone sleep test.

Bars represent mean values with error bars.

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from control (vehicle).

DZP: Diazepam (3 mg/Kg)

VEH: 5% Tween 80 (10 ml/Kg)

EMOL: Ethanol extract of *Moringa oleifera* leaves

4.4 NEUROPROTECTIVE EFFECT OF *Moringa oleifera* IN CHEMICAL INDUCED CONVULSION

4.4.1 Pentylenetetrazole-induced convulsion:

The extract (250–2000 mg/Kg) produced a dose-dependent protection (survival) after 24 hours. 70% protection was recorded 24 hours after PTZ administration. This is comparable with 100% for phenobarbitone (Table: 4.2)

TABLE 4.2: Effect of ethanol extract of *Moringa oleifera* leaves on pentylenetetrazole induced seizure.

Treatment	Dose mg/Kg	24hrs survival	%
Tween 80	10 mL/Kg	0/10	-
EMOL	500	2/10	20
EMOL	1000	5/10	50
EMOL	2000	7/10	70*
Phenobarbitone	40	10/10	100*

Results are expressed as percentage protection of ethanol extract of *Moringa oleifera* leaves in PTZ induced convulsion

Test of significance was carried out using Chi square.

* Indicate significant difference from control $P < 0.0001$ (Chi square).

EMOL = ethanol extract of *Moringa oleifera* leaves; Control mice received Tween 80, n = 10 mice per group

4.4.2 Strychnine-induced convulsion

The extract did not protect mice against strychnine-induced convulsion

TABLE 4.3: Effect of ethanol extract of *Moringa oleifera* leaves on strychnine-induced seizure.

Treatment	Dose mg/kg	24hrs survival	%
Tween 80	10ml/kg	0/10	-
EMOL	500	0/10	-
EMOL	1000	0/10	-
EMOL	2000	0/10	-
Phenobarbitone	40	10/10	100

Results are expressed as percentage protection of ethanol extract of *Moringa oleifera* leaves in strychnine-induced convulsion

Test of significance was carried out using Chi square.

EMOL = ethanol extract of *Moringa. oleifera* leaves; Control mice received Tween 80, n = 10 mice per group

4.4.3 Picrotoxin-induced convulsion

The extract did not protect mice against picrotoxin-induced convulsion

TABLE 4.4: Effect of ethanol extract of *Moringa oleifera* leaves on picrotoxin-induced seizure.

Treatment	Dose mg/kg	24hrs survival	%
Tween 80	10ml/kg	0/10	-
EMOL 2	500	0/10	-
EMOL 3	1000	0/10	-
EMOL 4	2000	0/10	-
Phenobarbitone	40	10/10	100

Results are expressed ns percentage protection of ethanol extract of *Moringa oleifera* leaves in picrotoxin-induced convulsion

Test of significance was carried out using Chi square.

EMOL = ethanol extract of *Moringa oleifera* leaves; Control mice received Tween 80, n = 10 mice per group

4.5 EFFECT OF THE ETHANOL LEAF EXTRACT OF *Moringa oleifera* ON LPS INDUCED COGNITIVE DEFICIT IN MICE

4.5.1 Learning and memory measured by Y maze in mice

The administration of ethanol extract of *Moringa oleifera* leaves (100 - 400 mg/Kg, p.o.) showed a significant increase in % alternation [F (4, 29) = 12.60; P < 0.0001] in LPS induced cognitive deficit. Cognitive deficit was reduced on treatment with LPS (100 ng/mL; 49.13± 1.23) alone. The highest % alternation was at 400 mg/Kg (64.25 ± 4.47) (Figure 4.9).

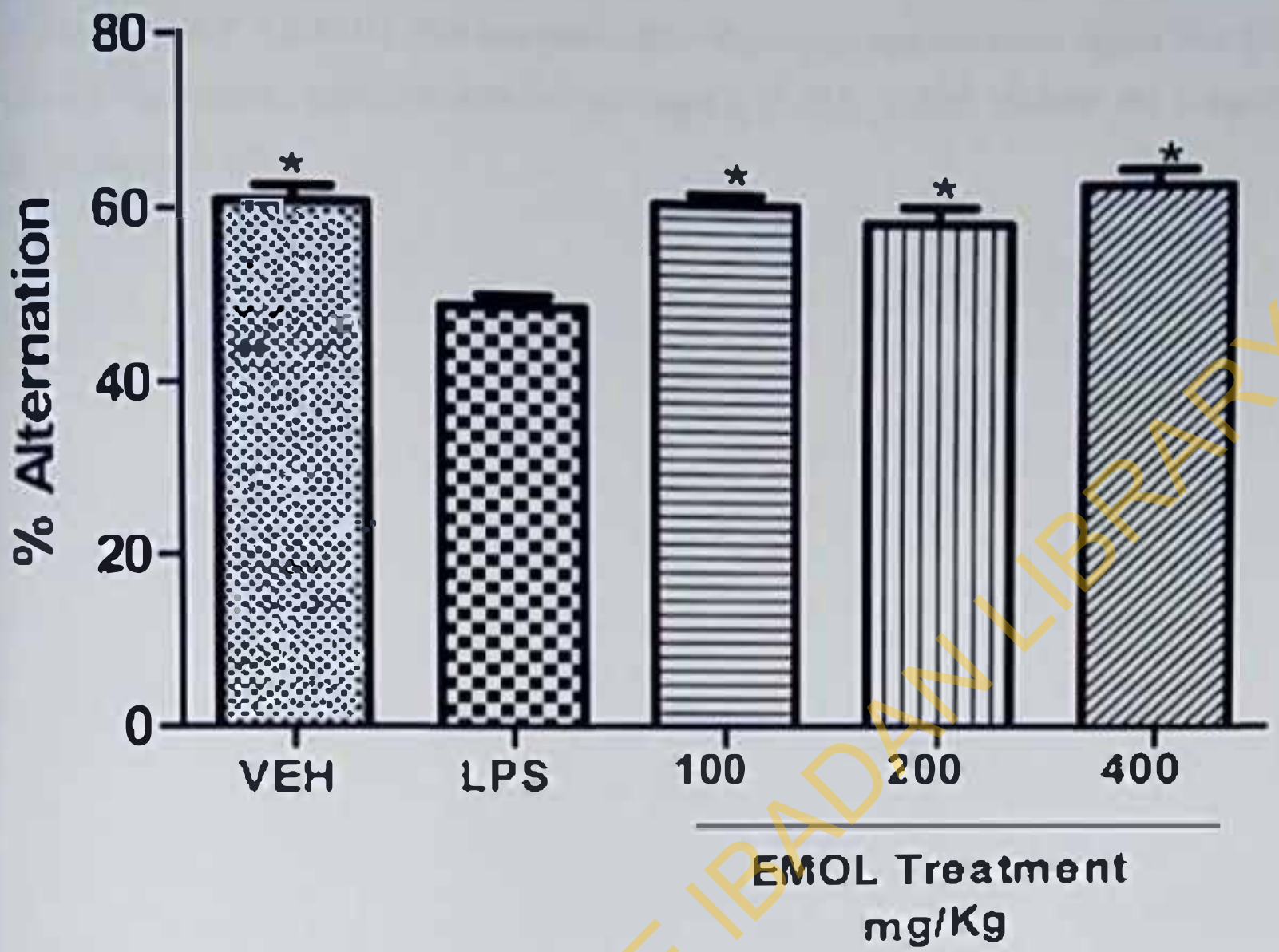


Figure 4.9: Effects of crude extract of *Moringa oleifera* on spatial memory in mice.

Mice were pretreated (p.o.) with either extract or saline for 7 days followed by daily i.p. LPS (250 µg/Kg) or saline (10 ml/Kg) for another 7 days. The Y-maze test was performed 24hr after last administration. Values were presented as mean ± S.E.M (n=6).

One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from LPS only

EMOL: Ethanol extract of *Moringa oleifera* leaves

4.5.2 Cognitive memory measured by novelty object recognition test in mice

The administration of ethanol extract of *Moringa oleifera* leaves (100 - 400 mg/Kg, p.o) showed an increase time spent exploring the novel object introduced after the training session [F (4, 29) = 1.398; P = 0.2635]. The increase in time spent exploring the novel object was not statistically significant. Administration of 400 mg/Kg (3.682± 3.385) showed the longest duration (Figure 4.10).

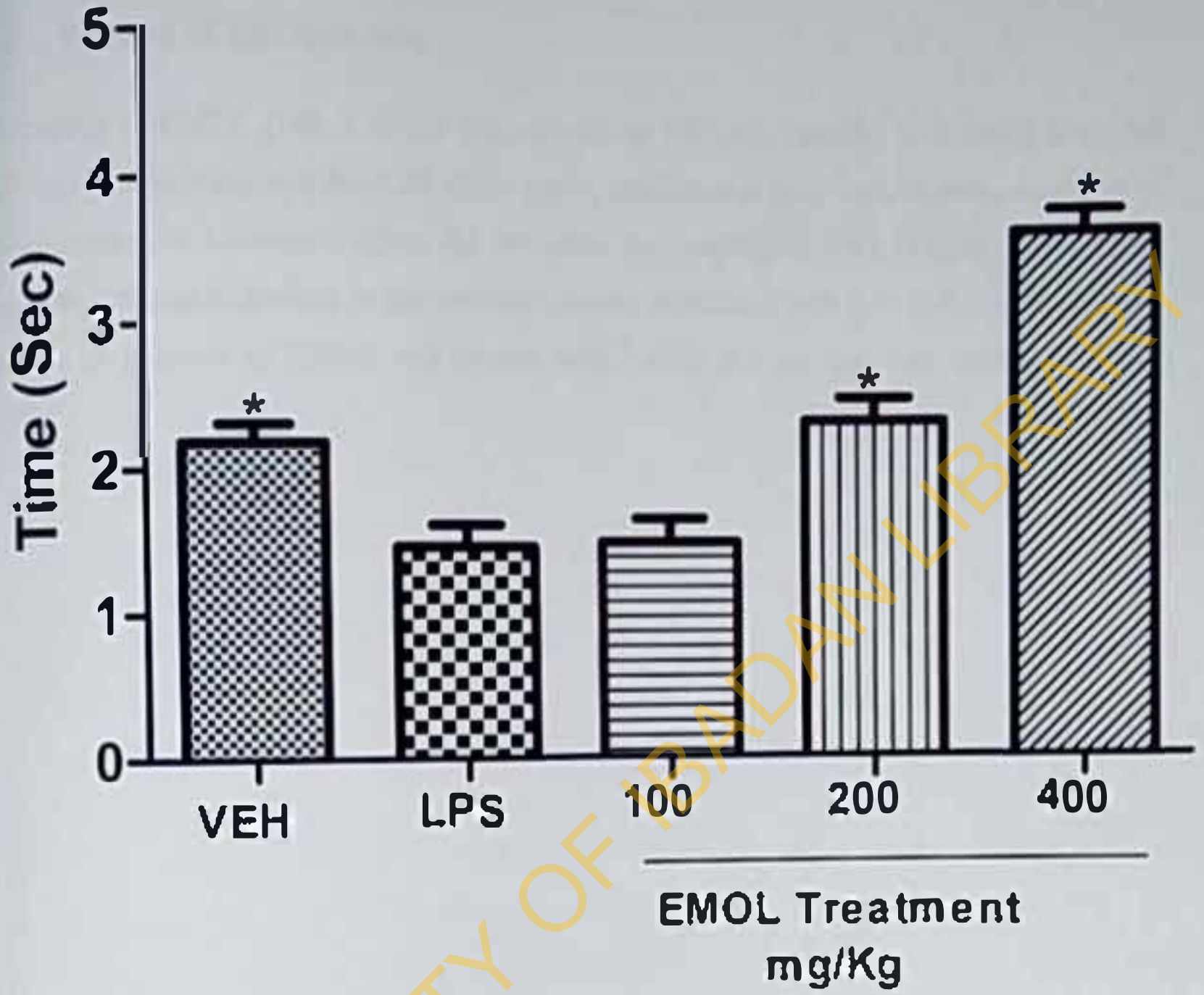


Figure 4.10: Effect of crude extract of *Moringa oleifera* on LPS-induced cognitive memory in mice.

Mice were pretreated (p.o.) with either extract or saline for 7 days followed by daily i.p. LPS (250 µg/Kg) or saline (10 ml/Kg) for another 7 days. The novel object recognition test was performed 24hr after last administration. Values were presented as mean ± S.E.M (n=6). One way ANOVA followed by Dunnet's multiple comparison test.

* $P < 0.05$, indicate significant difference from LPS only.

EMOL: Ethanol extract of *Moringa oleifera* leaves

4.6 ANTINEUROINFLAMMATORY EFFECT OF THE ETHANOL EXTRACT OF *Moringa oleifera* ON LPS INDUCED NEUROINFLAMMATION IN MICROGLIA CELLS

4.6.1 Viability of microglia cells

The toxicity of EMOL (100, 150 and 200 µg/mL) on BV2 cell viability was tested using the MTT assay. Result showed that LPS alone and in combination with various concentrations of ethanol extract of *Moringa oleifera* did not affect the viability of BV2 (Figure 4.11). There was no significant difference in the viability of cells stimulated with LPS (100 ng/mL) in the presence or absence of EMOL and control cells which did not received neither LPS nor EMOL.

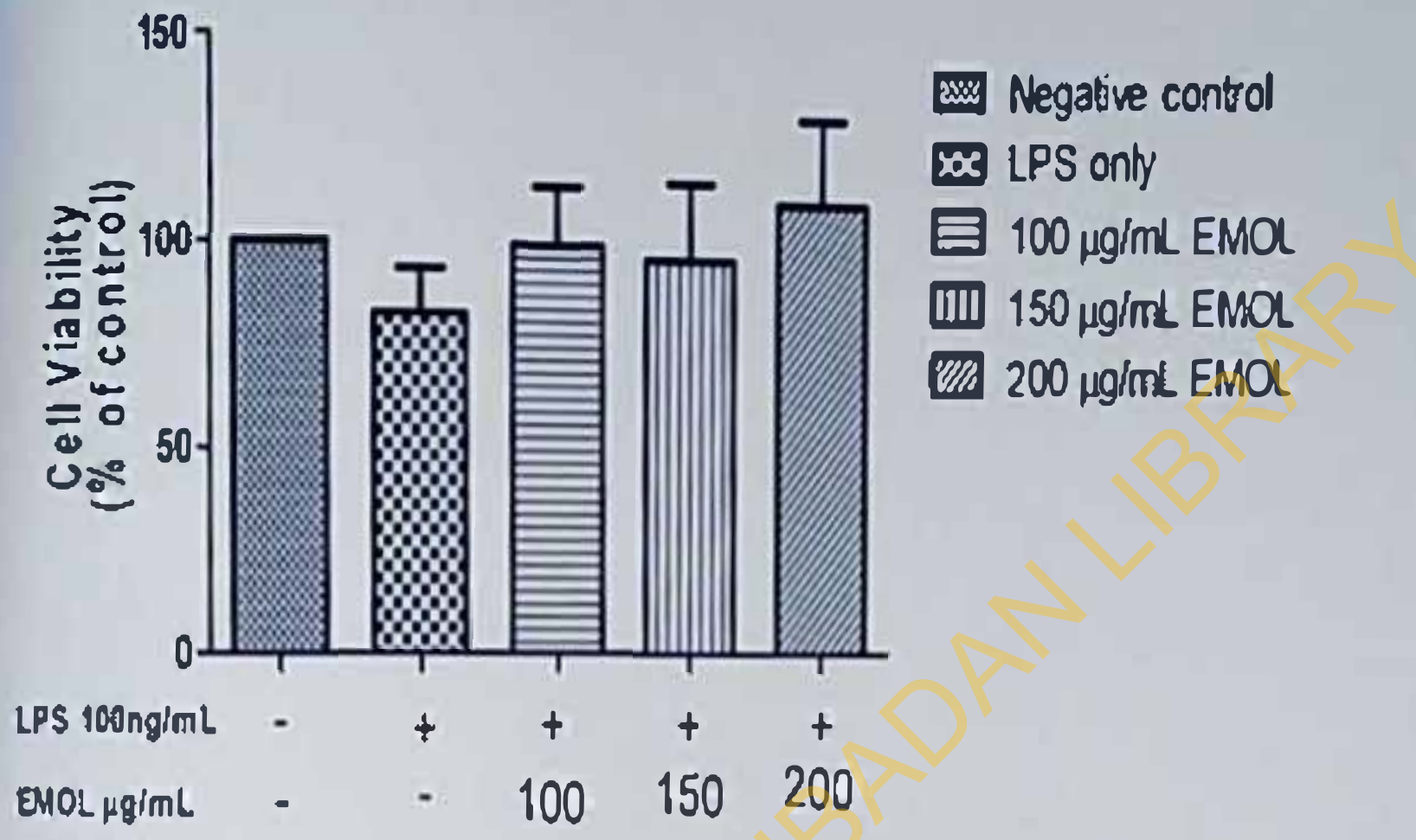


Figure 4.11 Viability of microglia cells.

Cells were stimulated with LPS (100 ng/ml) in the presence or absence of EMOL for 24 hours. At the end of the incubation period MTT assay was carried out. All values were expressed as mean ± SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test.

4.6.2 Inhibition of NO production in LPS stimulated BV-2 cells

LPS alone induced a marked production of NO from BV-2 cells when compared to the unstimulated control. EMOL (150 and 200 µg/mL) significantly [F (4, 14) = 15.09; p = 0.0003] inhibited the level of NO production from LPS stimulated cells (Figure 4.12).

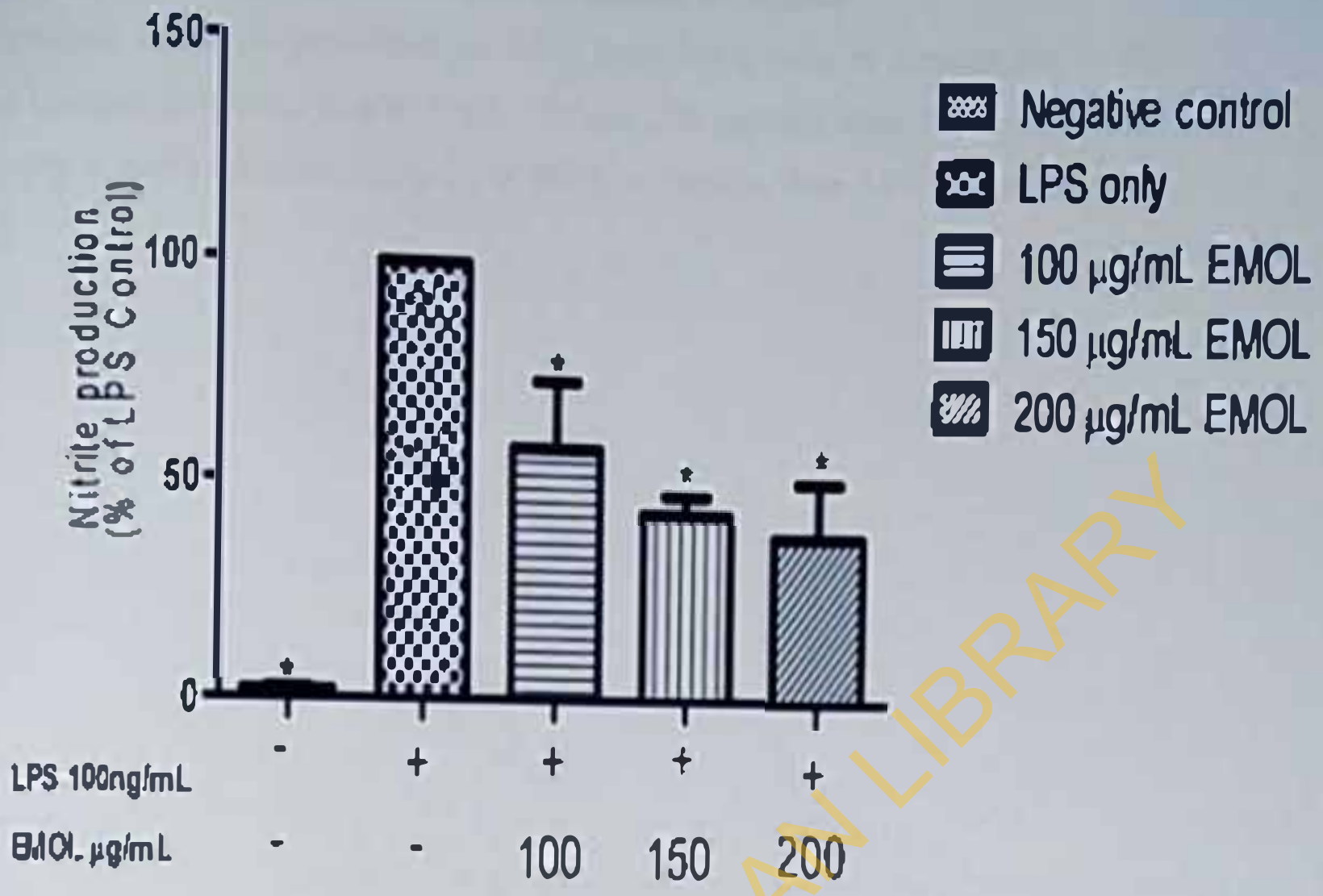


Figure 4.12: Inhibition of NO production in LPS stimulated BV-2 cells

All values were expressed as mean ±SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test. * = significant at $p < 0.05$ when compared with LPS only.

4.6.3 Inhibition of PGE_2 production in LPS stimulated BV-2 cells

LPS alone induced a marked production of PGE_2 from BV-2 cells in comparison to the unstimulated control. However, EMOL (100, 150 and 200 µg/mL) dose dependently and significantly ($^*p < 0.05$) inhibited the level of PGE_2 production from LPS stimulated cells (Figure 4.13).

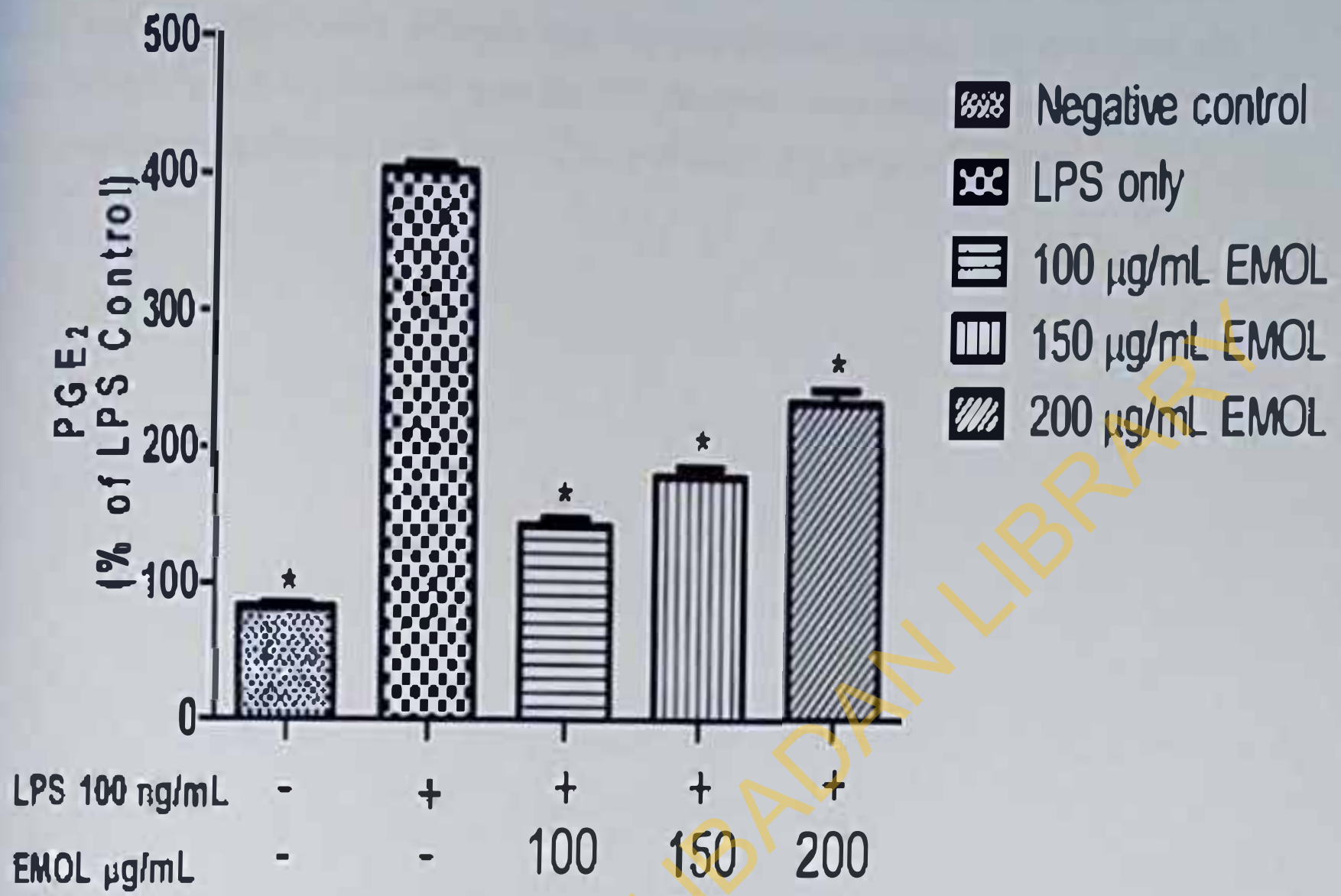


Figure 4.13: Inhibition of PGE_2 production in LPS-stimulated BV-2microglia.

All values were expressed as mean ±SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test. * = significant at $p < 0.05$ when compared with LPS only.

4.6.4 Production of TNF-α and IL-6 in LPS stimulated BV-2 cells

LPS alone and in presence of various concentration of extract induced production of cytokines (TNF-α, IL-6) from BV-2 cells in comparison to the unstimulated control. The values were all significantly different from the unstimulated control, but they were not significantly ($^*p < 0.05$) different from the LPS stimulated cells except EMOL 100 µg/mL which statistical significance [$F(4, 14) = 37.75$; $p<0.0001$] (Figures 4.14 and 4.15).

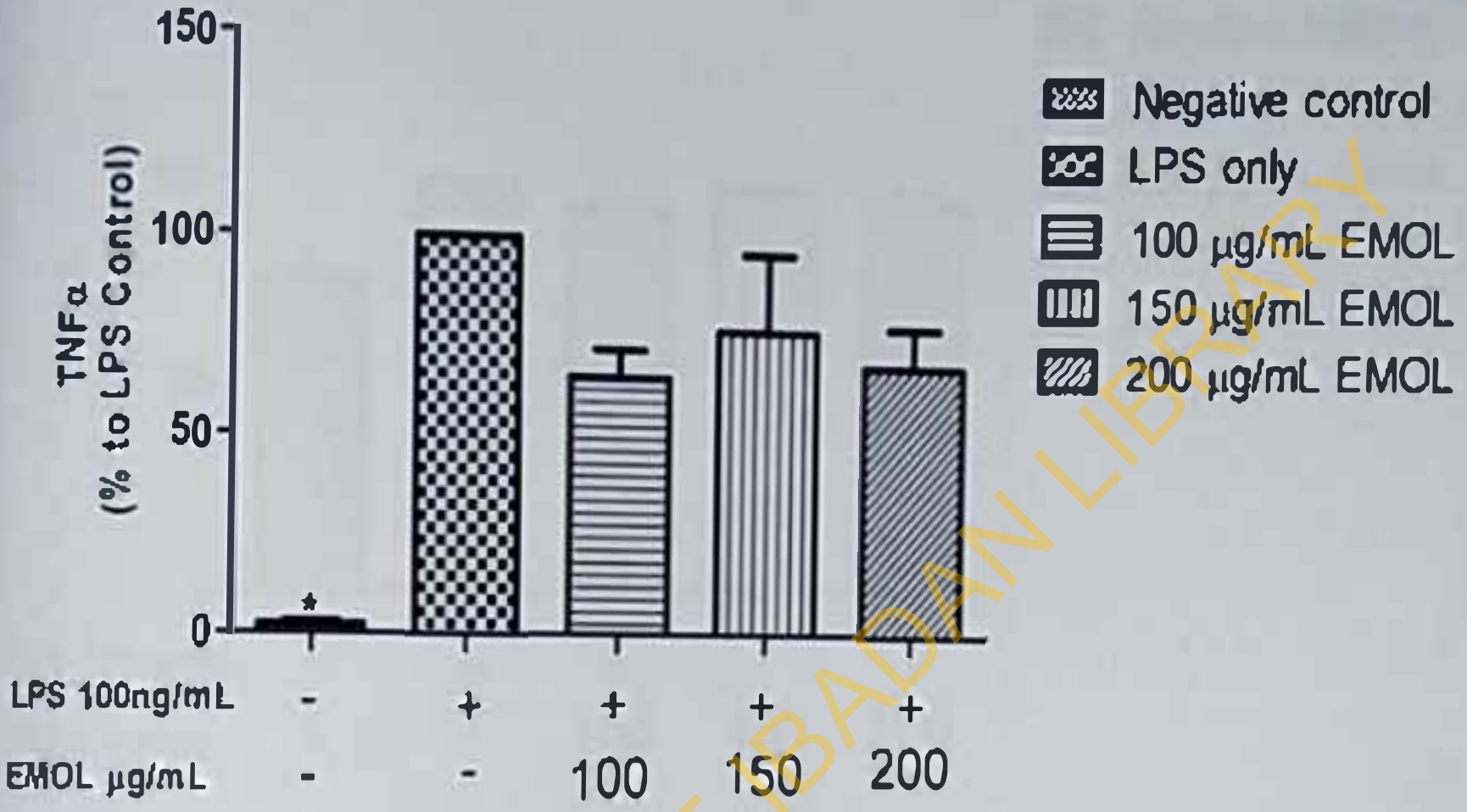


Figure 4.14: Production of TNF-α in LPS-stimulated BV-2microglia.

All values are expressed as mean ±SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test. * = significant at $p < 0.05$ when compared with LPS only.

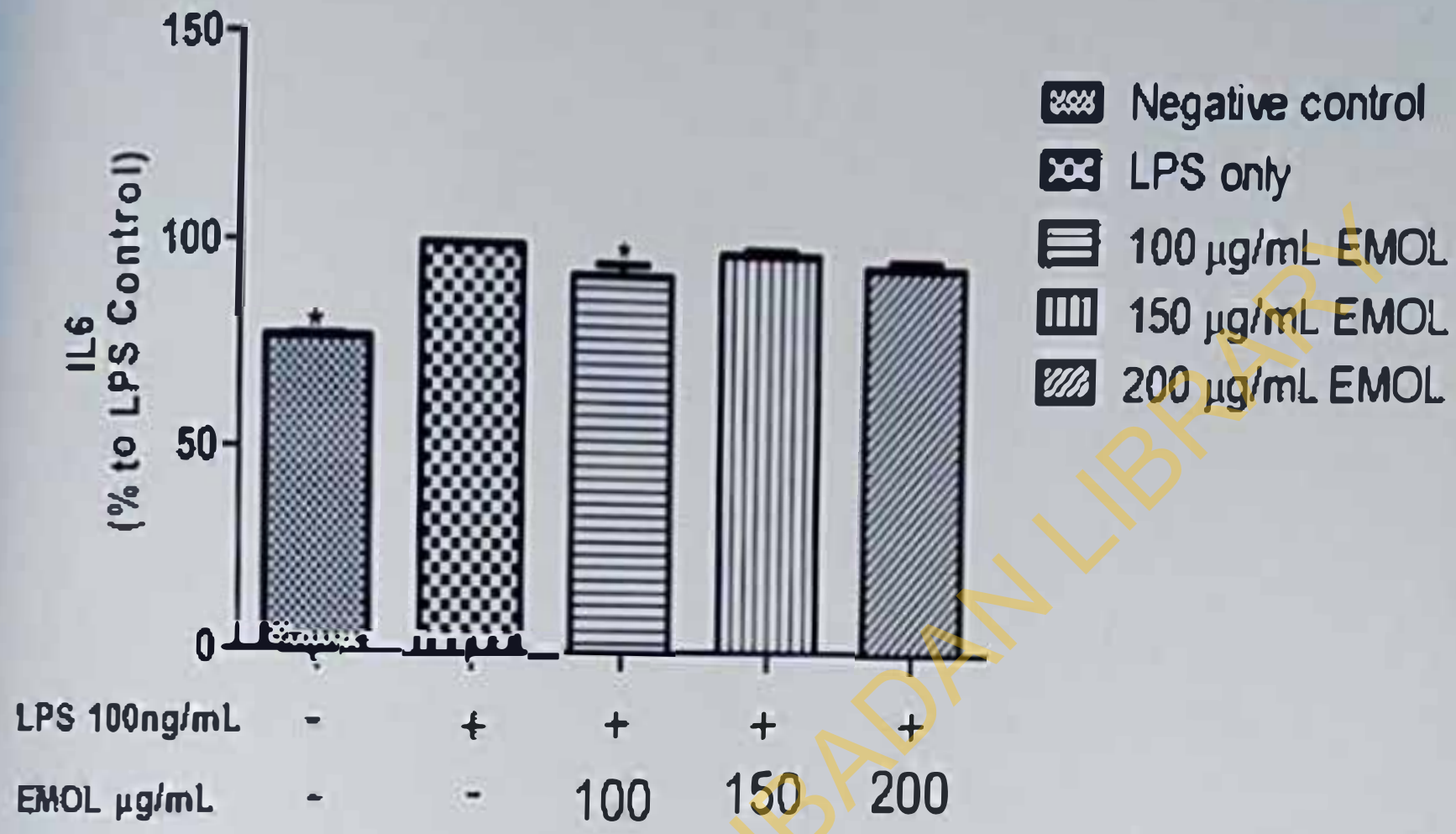


Figure 4.15: Production of IL-6 in LPS stimulated BV-2 microglia

All values were expressed as mean ± SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test. * = significant at $p < 0.05$ when compared with LPS only.

4.6.5 Reactive oxygen species (ROS) production in LPS stimulated BV-2 cells

LPS alone induced a marked production of ROS from BV-2 cells in comparison to the unstimulated control. EMOL significantly reduced the level of ROS production from LPS stimulated cells (Figure 4.16). ROS production was lowest at 150 µg/mL (49.45 ±1.15).

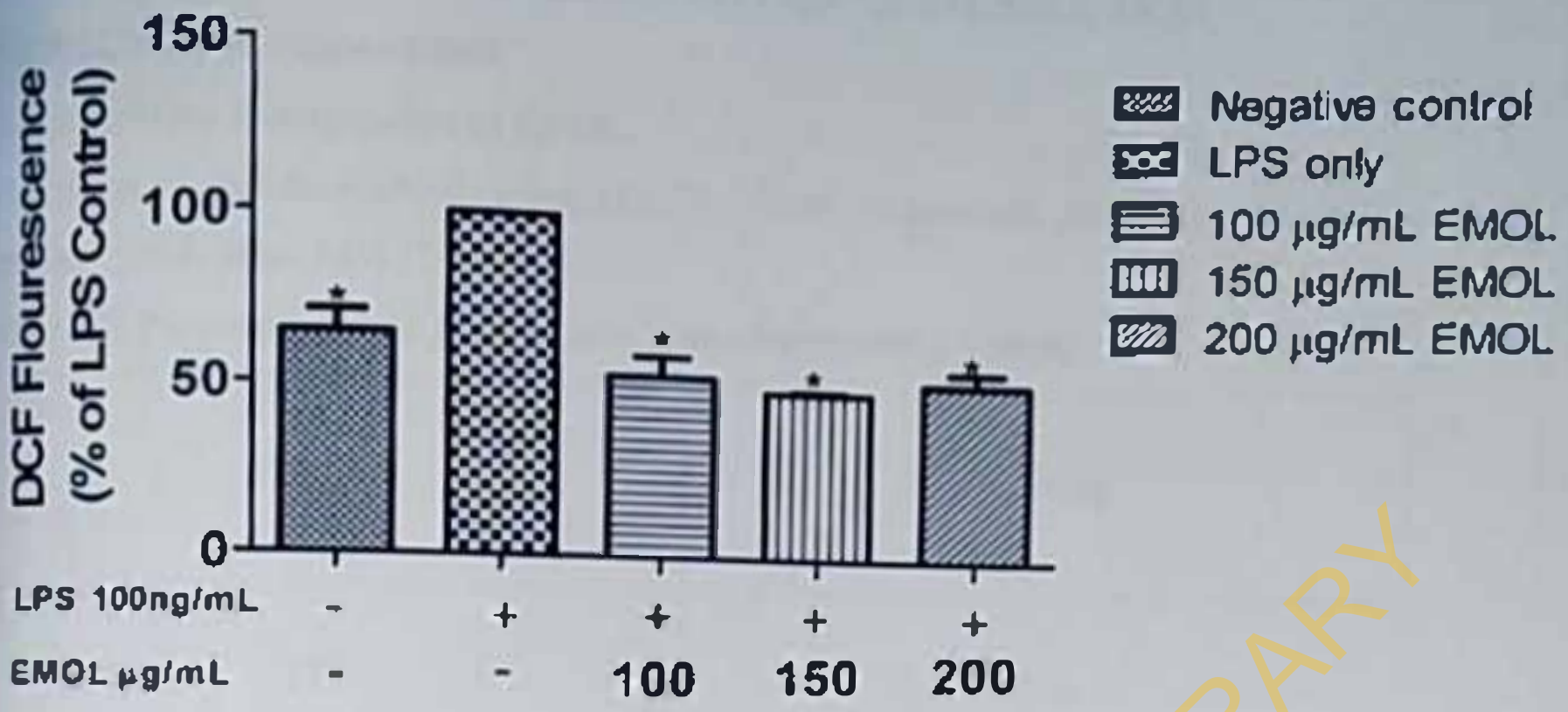


Figure 4.16: Reactive oxygen (ROS) production in LPS activated microglia.

Cells were stimulated with LPS (100 ng/mL) in the presence or absence of crude extract (100-200 µg/mL) for 24 h. At the end of the incubation period, total reactive oxygen and nitrite species production were measured in the cell. All values wer expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only.

DCF: 2', 7' dichlorofluorescein diacetate

4.7 BIOACTIVITY GUIDED FRACTIONATION OF ETHANOL LEAF EXTRACT OF *Moringa oleifera*

4.7.1 Reverse Fractionation of EMOL

Solid phase extraction of EMOL using ACE 10 C18-HL column with 20, 50, 80, and 100% methanol yields about 64% (Table 4.5)

Table 4.5: Percentage Yield from reverse phase fractionation EMOL

Fraction	% Yield
F20	74.00
F50	17.53
F80	00.05
F100	00.03

F20 = 20%; F50 = 50%; F80 = 80%; F100 = 100%

4.7.2 Bioactivity of fractions of EMOL

MTT and Griess assay monitoring of activity of fractions showed that F50 moderately reduced nitrite production and less toxic than F80 and F100 that showed better activity in nitrite assay but is acutely toxic. The following are results obtained for bioactivity of fractions.

4.7.2.1 Viability of BV-2 microglia cells.

The effect of the fractions F20, F50, F80 and F100 at 12.5, 25 and 50 µg/mL on BV2 cell viability was tested using the MTT assay. Result showed that F20 and F50 did not affect the viability of BV-2 cells while F80 and F100 affected the viability of the BV-2 (Figure 4.17).

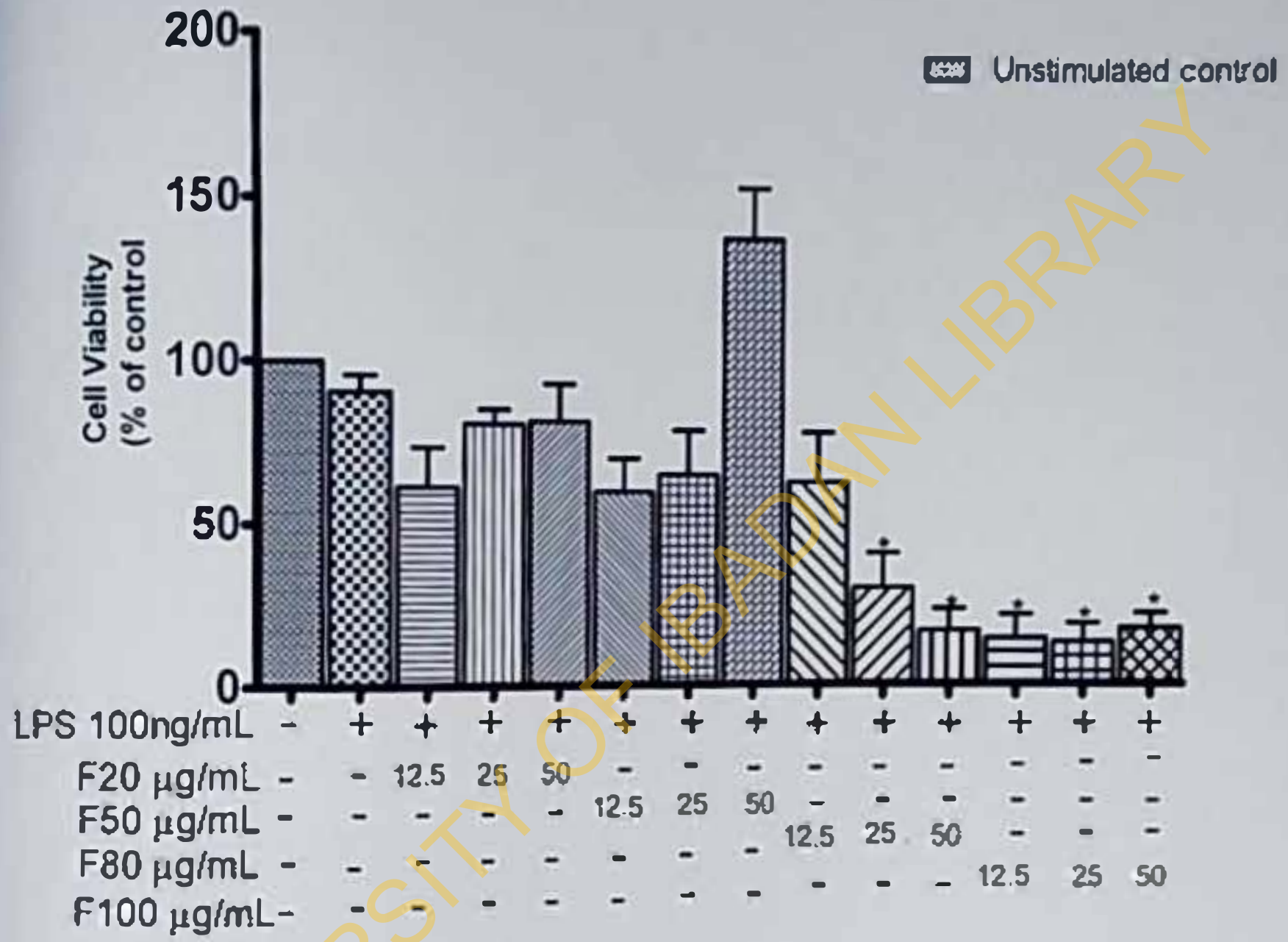


Figure 4.17: The effect of fractions of ethanol extract of *Moringa oleifera* on the viability of BV2 stimulated with LPS.

All values were expressed as mean ± SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only.

4.7.2.2 NO production in LPS stimulated BV-2 cells

LPS alone induced a marked production of NO from BV-2 cells in comparison with unstimulated control. F50 at 25 µg/mL significantly ($^*p < 0.05$) inhibited the level of NO production from LPS stimulated cells (Figure 4.18). F80 and F100 at 12.5, 25 and 50 µg/mL significantly reduced nitrite production (Figure 4.18).

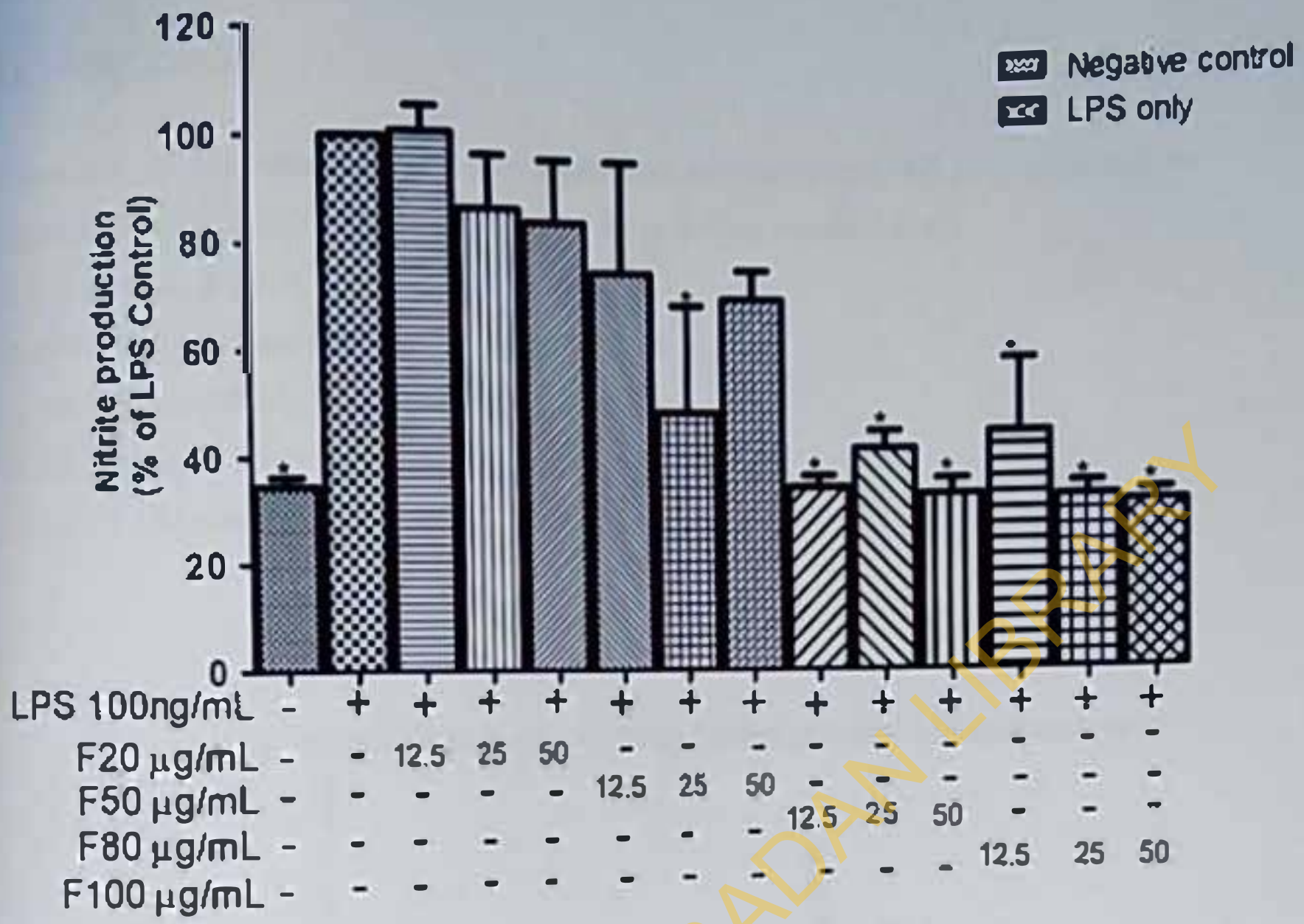


Figure 4.18: The effect of fraction of ethanol extract of *Moringa oleifera* leaves on nitrite production in BV2 stimulated with LPS.

All values were expressed as mean ± SEM for three independent experiments. Data were analysed using one way ANOVA for multiple comparison and post hoc Student Newman-Keuls test. * = significant at $p < 0.05$ when compared with LPS only.

4.7.3 HPLC-DAD

Fingerprint of F50 obtained from semi-preparative reversed-phase HPLC is presented on Figure 4.19. Five compounds were isolated at the following retention times.

12.429 minutes (F50-1),

15.859 -16.185 minutes (F50-2)

17.736 minutes (F50-3)

18.392 minutes (F50-4)

20.863-21.154 minutes (F50-5)

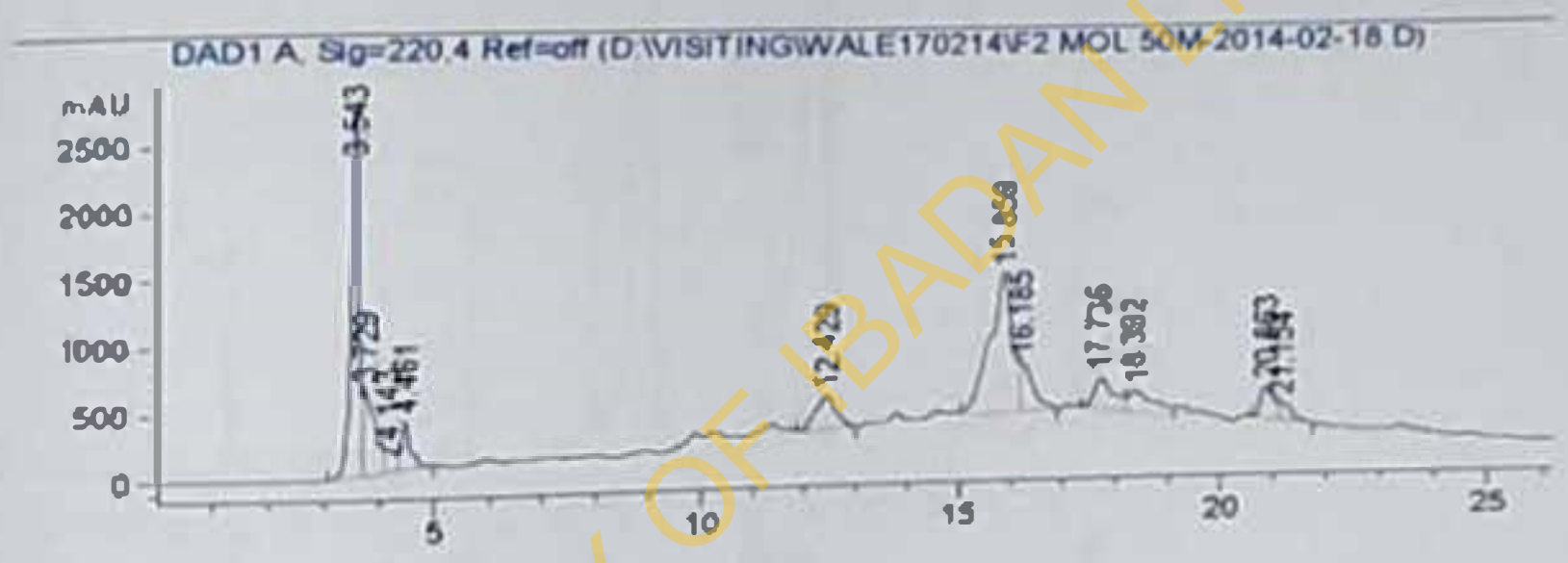


Figure 4.19: Fingerprint of fraction F50 obtained from semi-preparative reversed-phase HPLC

4.7.4 Isolation and Spectra Data of (F50-4)

The proton NMR spectrum, spectra data, UV absorption spectrum and structure of F50-4 (kaempferol-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside) are presented in Figure 4.20, 4.21, 4.22 and table 4.5.

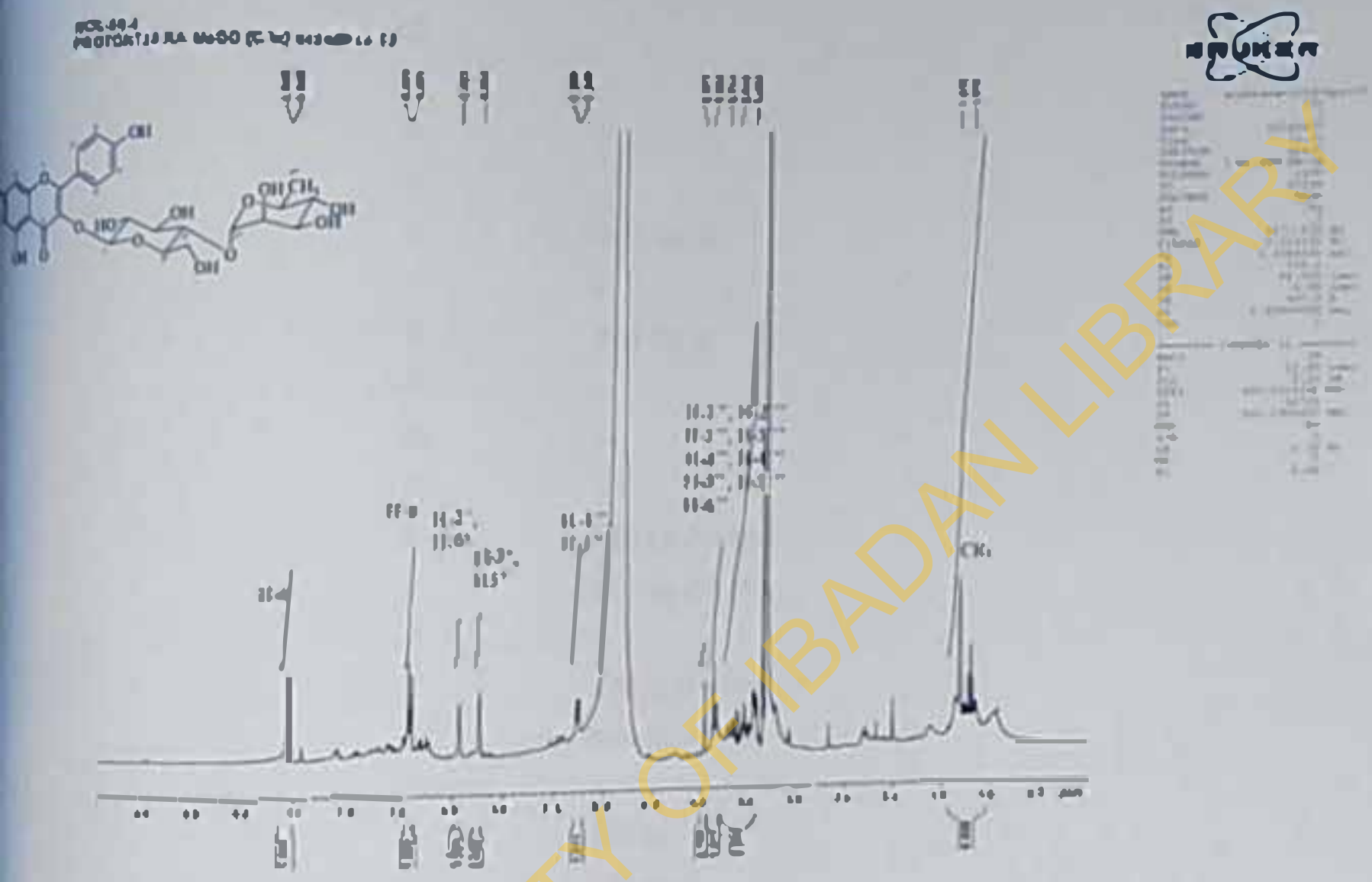


Figure 4.20: ^{1}H NMR spectrum of kaempferol-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside (F50-4)

Table 4.6: ^{1}H NMR (300 MHz, MeOD) data for compound FS0-4

Position	^{1}H NMR δ_H (ppm)
1	-
2	-
3	-
4	-
5	-
6	6.21 (br. s)
7	-
8	6.43 (br. s)
9	-
10	-
1'	-
2'	8.05 (d, J=9 Hz)
3'	6.98 (d, J=9 Hz)
4'	-
5'	6.98 (d, J=9 Hz)
6'	8.05 (d, J=9 Hz)
Glucose	
1''	5.24 (s)
2''	3.59 (m)
3''	3.25 (m)
4''	3.59 (m)
5''	3.41 (m)
6''	3.59 (m)
Rhamnose	
1'''	5.22 (s)
2'''	3.99 (m)
3'''	3.72 (m)
4'''	3.41 (m)
5'''	3.99 (m)
6'''	1.25 (d, J=6 Hz)

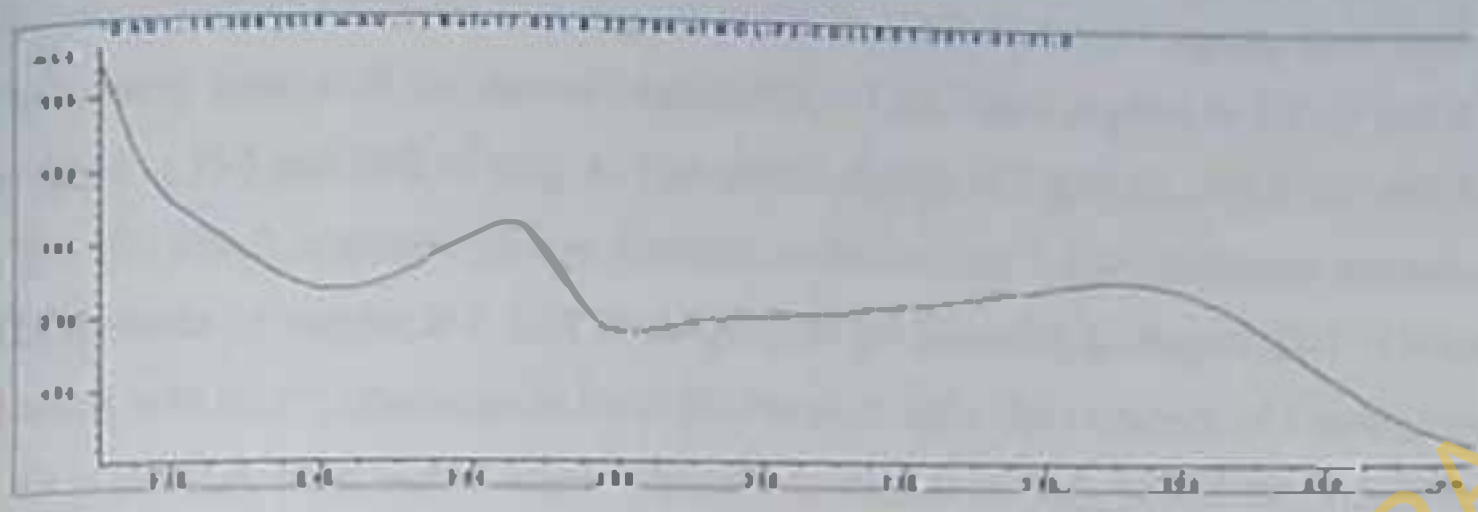


Figure 4.21: UV spectrum of kaempferol-3-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside (F50-4)

Figure 4.22: Kaempferol-3-*O*-β-D-glucopyranosyl-(1→6)-α-L-rhamnopyranoside

4.7.5 Analysis of F50–4 Spectra Data

The UV spectrum (Figure 4.21) obtained from the HPLC/DAD chromatogram showed λ maxima at 350 nm (band I) and 264 nm (band II) characteristic of the kaempferol type. The ^{1}H NMR spectrum of compound (Kaempferol-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside) showed four signals in the aromatic hydrogen region, consistent with the replacement pattern of the flavonol kaempferol. Two broad singlets at δ 6.23 and 6.43 were assigned to H-6 and H-8 of ring A. The proton signals at δ 8.05 (d, *J*=9.0 Hz) and δ 6.98 (d, *J*=9.0 Hz) which appeared as two doublets confirmed the 1,4–disubstituted aromatic ring B. The presence of singlet at δ 5.24 is assigned to the anomeric hydrogen (H-1'') *trans*-diaxial position with H-2'', characterize the β-D-glucoside unit. The existence of a methyl signal at δ 1.25 in the high-field region was attributable to rhamnose. This was further confirmed by the broad singlet at δ 5.22 which assigned to the anomeric dicquatorial hydrogen characteristic for α-linked rhamnose (H-1'''). Signals ranging from δ 3.20 to 5.21 are in association to oxymethine protons together with the signals of anomeric protons (H-1'' and H-1''') confirmed the presence of glucose and rhamnose in the molecule. The assignments of all protons in this molecule are summarized in Table 4.5.

This information together with associated literature data for flavonoid with the same aglycone allowed the identification of F50-4 as kaempferol-3-*O*-β-D-glucopyranosyl-(1→4)-α-L-rhamnopyranoside. This compound has been previously isolated from the leaves of *Oxandra sessiliflora* R.E. Fries (Souza *et al.*, 2014).

4.8 ANTINEUROINFLAMMATORY EFFECT OF ISOLATED COMPOUNDS ON MICROGLIA IN LPS INDUCED NEUROINFLAMMATION

4.8.1 Kaempferol, quercetin and rutin did not affect the viability of microglia cells.

The toxicity of compounds from *Moringa oleifera* on BV2 cell viability was tested using the MTT assay. Result showed that kaempferol, quercetin and rutin at 12.5, 12.5 and 25 µM respectively in combination with LPS did not affect the viability of BV2 (Figure 4.23). There was no significant difference in cell viability of microglia pretreated with or without compounds and LPS.

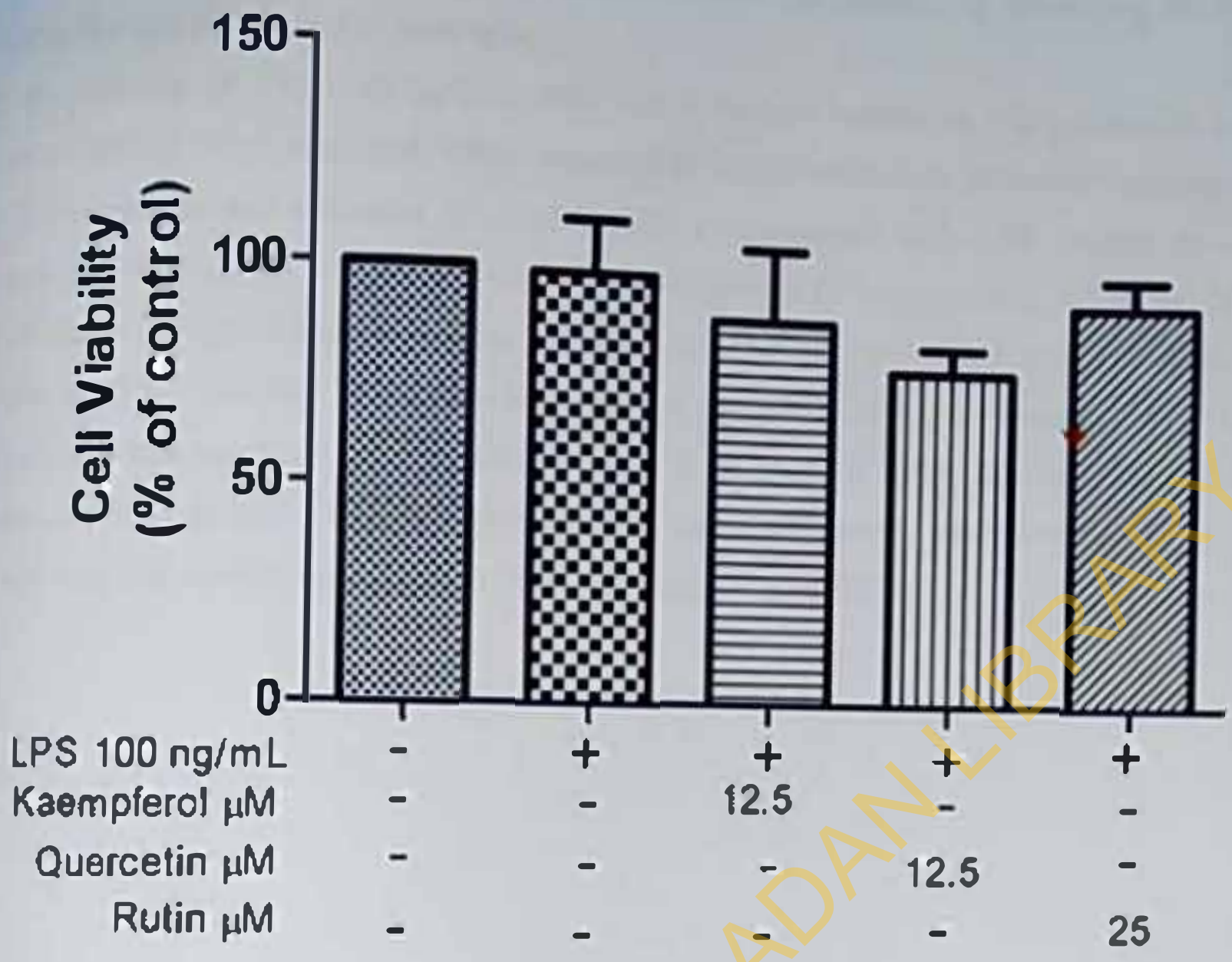


Figure 4.23: Pretreatment with or without Kaempferol, quercetin and rutin on viability of microglia stimulated with LPS.

Cells were stimulated with LPS (100 ng/mL) in the presence or absence of kaempferol, quercetin and rutin for 24 h. At the end of the incubation period. MTT assay was carried out on cells. All values were expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

4.8.2 Kaempferol and quercetin suppresses nitrite production by inhibiting INOS expression in LPS-activated microglia

In the presence of LPS (100 ng/mL), there was a marked increase in NO production in supernatants of BV-2 microglia, when compared to unstimulated cells. However, treatment with kaempferol and quercetin (12.5 μM) prior to stimulation with LPS resulted in a significant reduction in NO production, in comparison with LPS control (Fig. 4.24). NO is synthesized during neuroinflammation through the enzymatic activity of inducible nitric oxide synthase (iNOS). In determining whether the effect of the compounds on NO production was mediated through inhibition of the activities of iNOS, its expression was measured. Interestingly, kaempferol and quercetin caused reduction in expression of COX-2, while nitin had no reduction effect on iNOS expression (Figure 4.25).

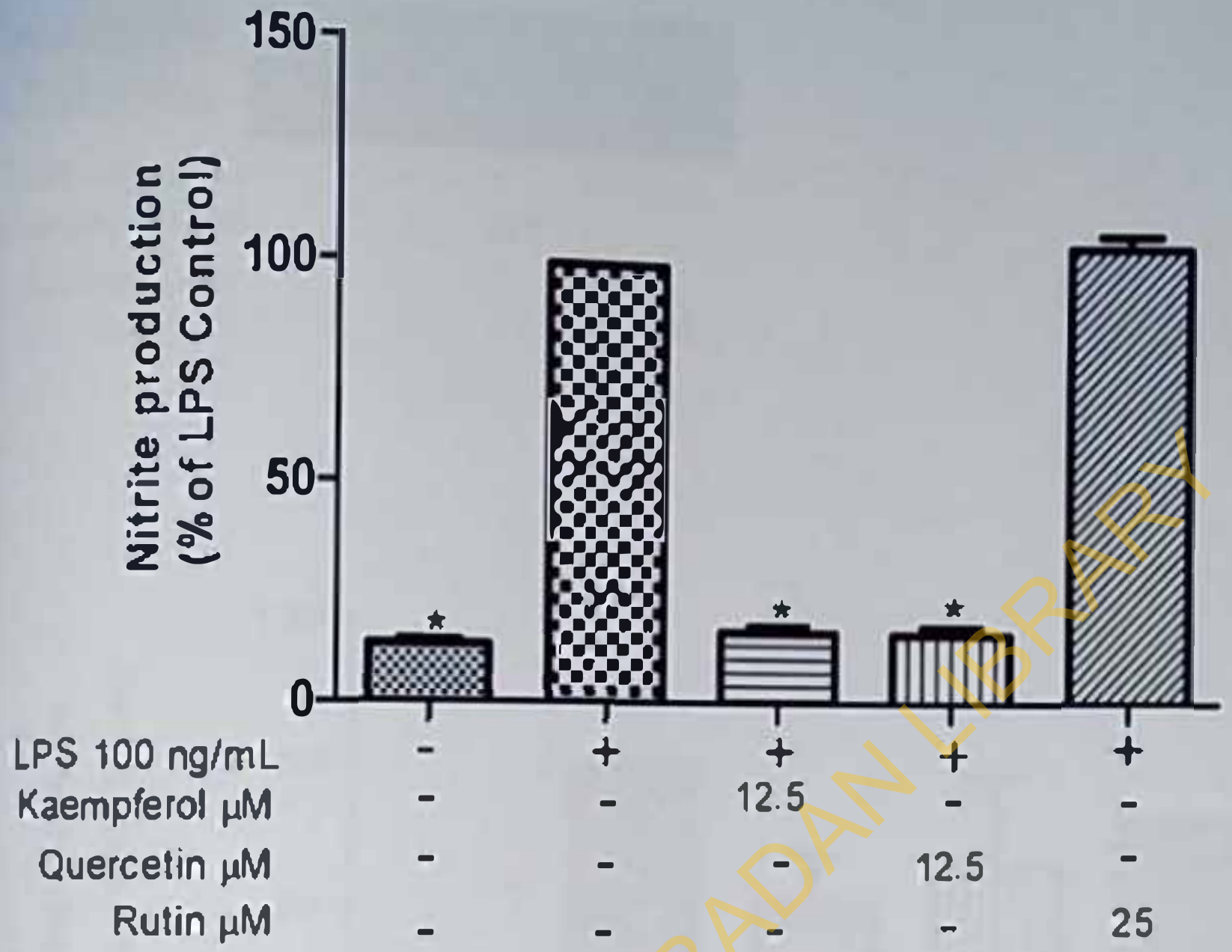


Figure 4.24: Kaempferol and quercetin inhibited nitrite release in LPS-activated microglia.

Microglia were incubated in a medium containing 12.5 µM of both kaempferol and quercetin and 25 µM rutin for 30 min and then activated by100 ng/mL LPS for 24 h. Kaempferol and quercetin significantly diminished nitrite release in microglia. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only.

(a)

LPS (100ng/mL)	-	+	+	+	+
Kaempferol (μM)	-	-	12.5	-	-
Quercetin (μM)	-	-	-	12.5	-
Rutin (μM)	-	-	-	-	25

(b)

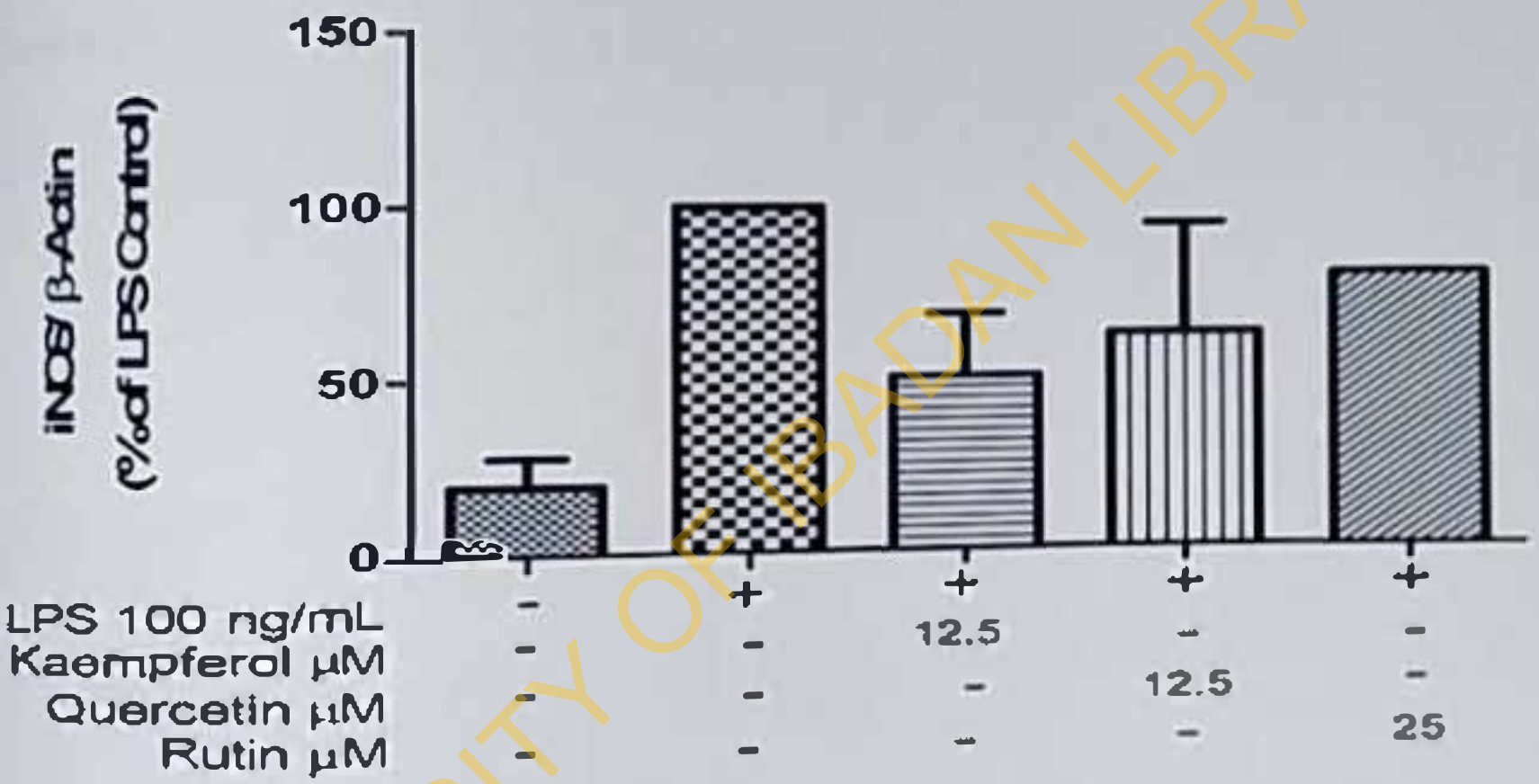


Figure 4.25: Kaempferol and quercetin inhibited iNOS protein expression in LPS-activated microglia

Microglia were incubated in a medium containing 12.5 μM of both kaempferol and quercetin and 25 μM rutin for 30 min and then activated by100 ng/mL LPS for 24h. Kaempferol and quercetin inhibited iNOS protein expression in LPS-activated microglia. Protein expression was determined using western blot with specific anti-iNOS antibody. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post hoc Student Newman-Keuls test.

4.8.3 Kaempferol and quercetin suppresses PGE_2 production by inhibiting COX-2 expressions in LPS-activated microglia

In the presence of LPS (100 ng/mL), there was a marked increase in PGE_2 production in supernatants of BV-2 microglia, when compared to unstimulated cells. However, treatment with kaempferol and quercetin (12.5μM) prior to stimulation with LPS resulted in significant reduction in PGE_2 production when compared with LPS control (Figure 4.26). PGE_2 is synthesized during neuroinflammation through the enzymatic activity of COX-2. It is known that mPGES-1 is coupled to COX-2 in the biosynthesis of PGE_2. In determining whether the effect of the compounds on PGE_2 was mediated through inhibition of the activities of COX-2, the expression of these enzymes was measured. Interestingly, kaempferol and quercetin caused reduction in expression of COX-2, while rutin had no reduction effect on COX-2 (Figure 4.27).

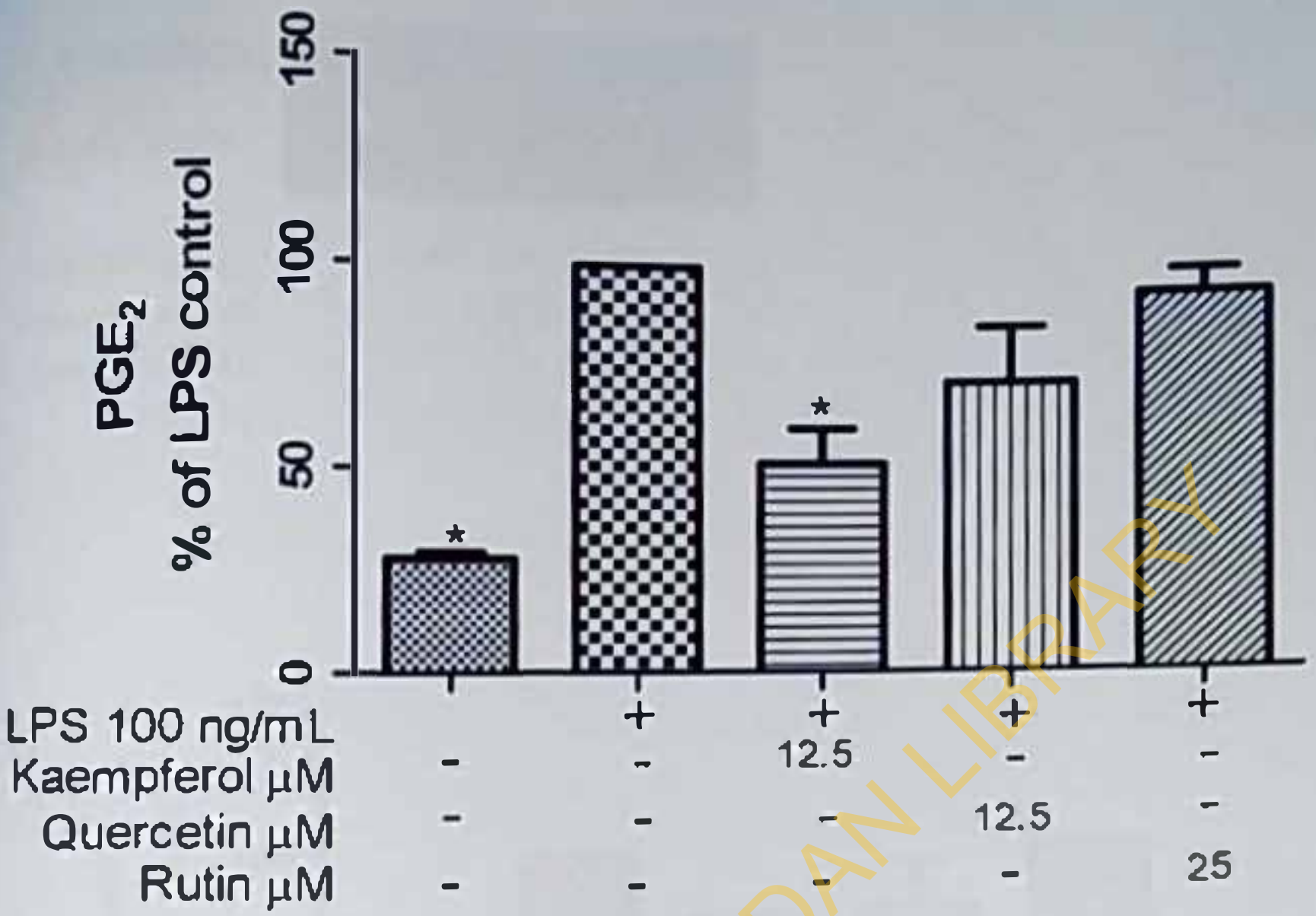


Figure 4.26: Kaempferol and quercetin inhibited PGE_2 release in LPS-activated microglia.

Microglia were incubated in a medium containing 12.5 µM of both kaempferol and quercetin and 25 µM rutin for 30 min and then activated by100 ng/mL LPS for 24 h. Kaempferol significantly diminished PGE_2 release in microglia. .

All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test. * = significant at $p < 0.05$ when compared with LPS only.

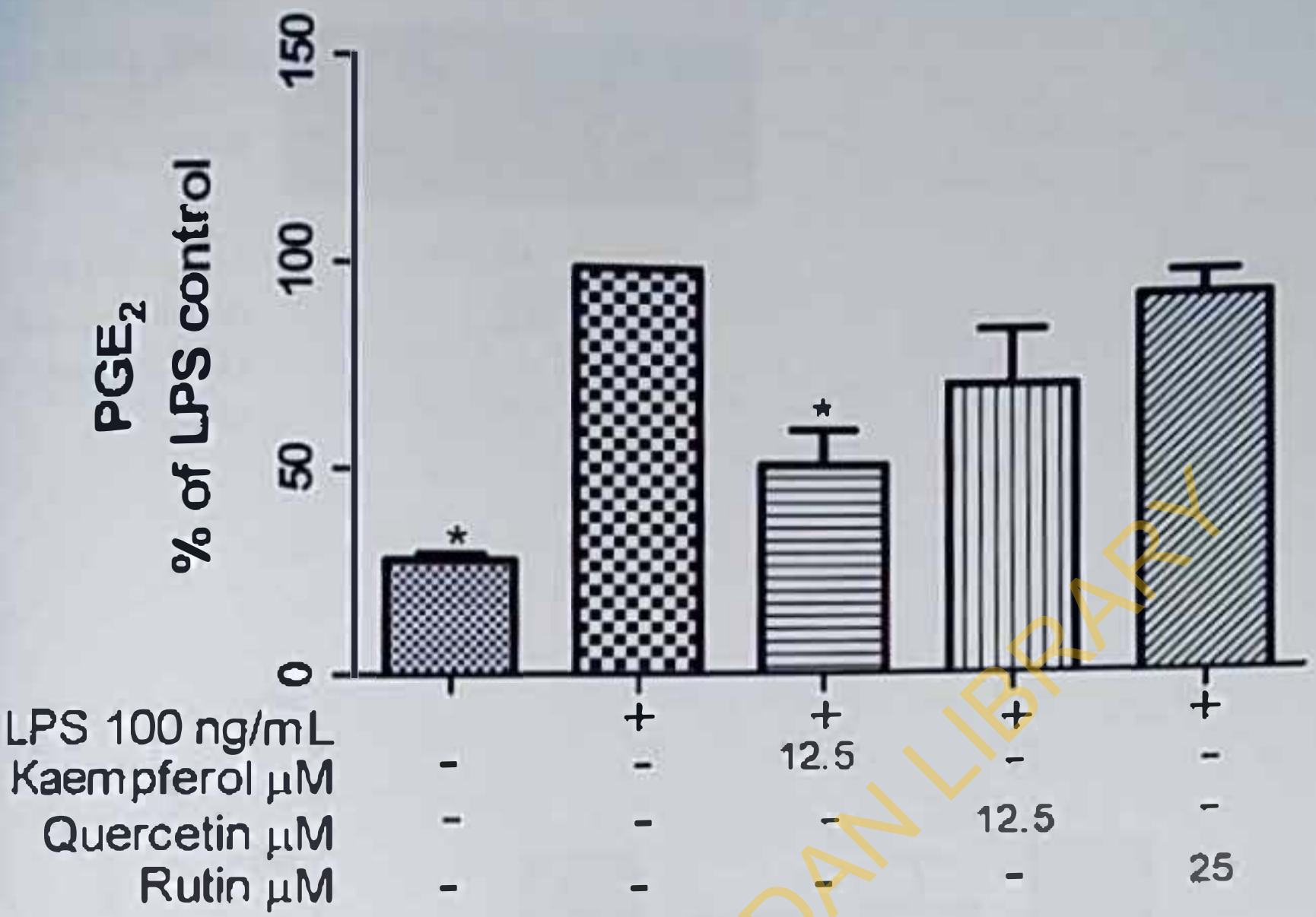


Figure 4.26: Kaempferol and quercetin inhibited PGE_2 release in LPS-activated microglia.

Microglia were incubated in a medium containing 12.5 µM of both kaempferol and quercetin and 25 µM rutin for 30 min and then activated by100 ng/mL LPS for 24 h. Kaempferol significantly diminished PGE_2 release in microglia. .

All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test * = significant at $p < 0.05$ when compared with LPS only.

(a)

LPS (100ng/mL)	-	+	+	+	+
Kaempferol (μM)	-	-	12.5	-	-
Quercetin (μM)	-	-	-	12.5	-
Rutin (μM)	-	-	-	-	25

(b)

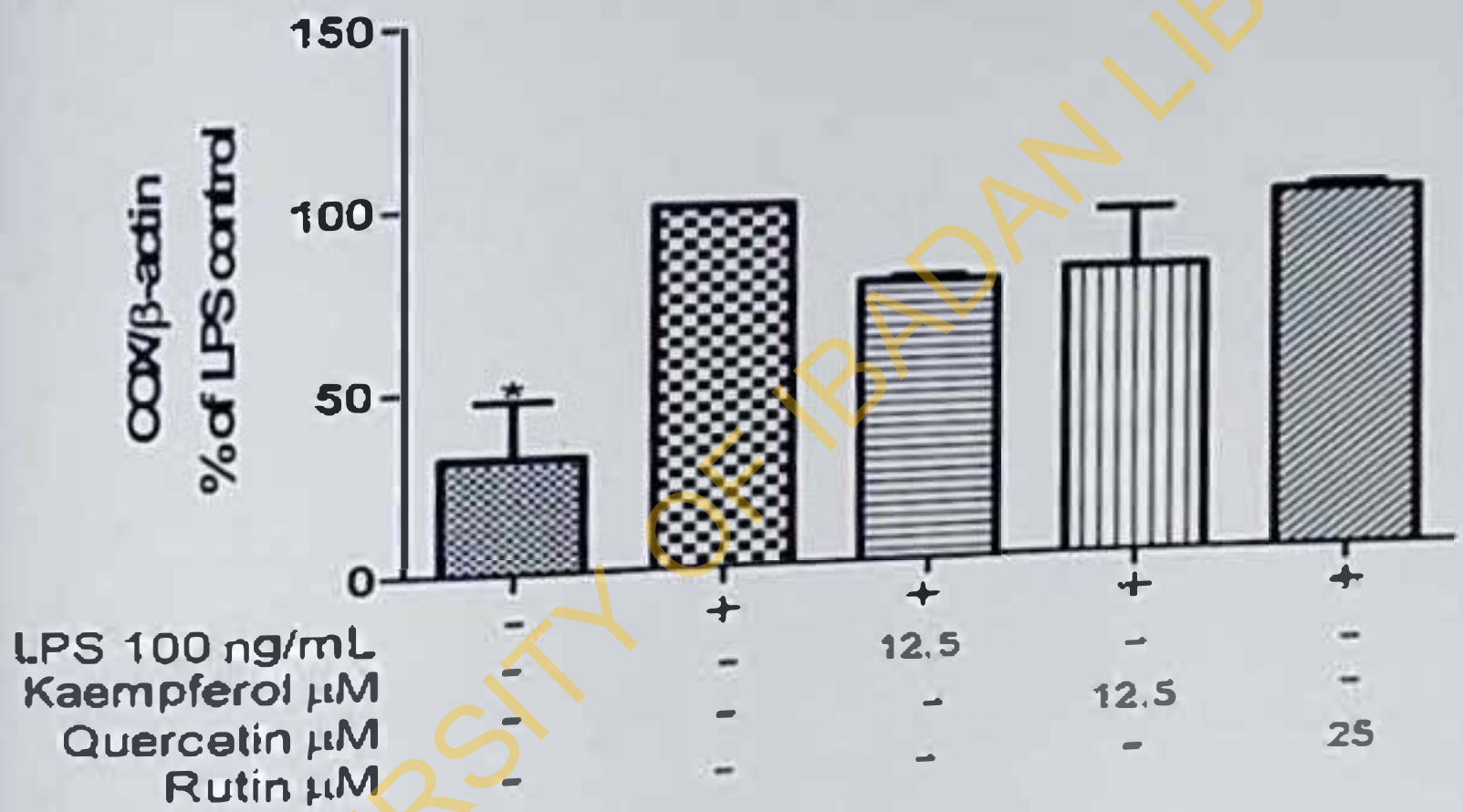


Figure 4.27: Kaempferol and quercetin inhibited COX-2 protein expressions in LPS-activated microglia.

Microglia were incubated in a medium containing 12.5 μM of both kaempferol and quercetin and 25 μM rutin for 30 min and then activated by 100 ng/mL LPS for 24 h. Kaempferol and quercetin inhibited COX-2 protein expression in LPS-activated microglia. Protein expression was determined using western blot with specific anti-COX-2 antibodies. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only.

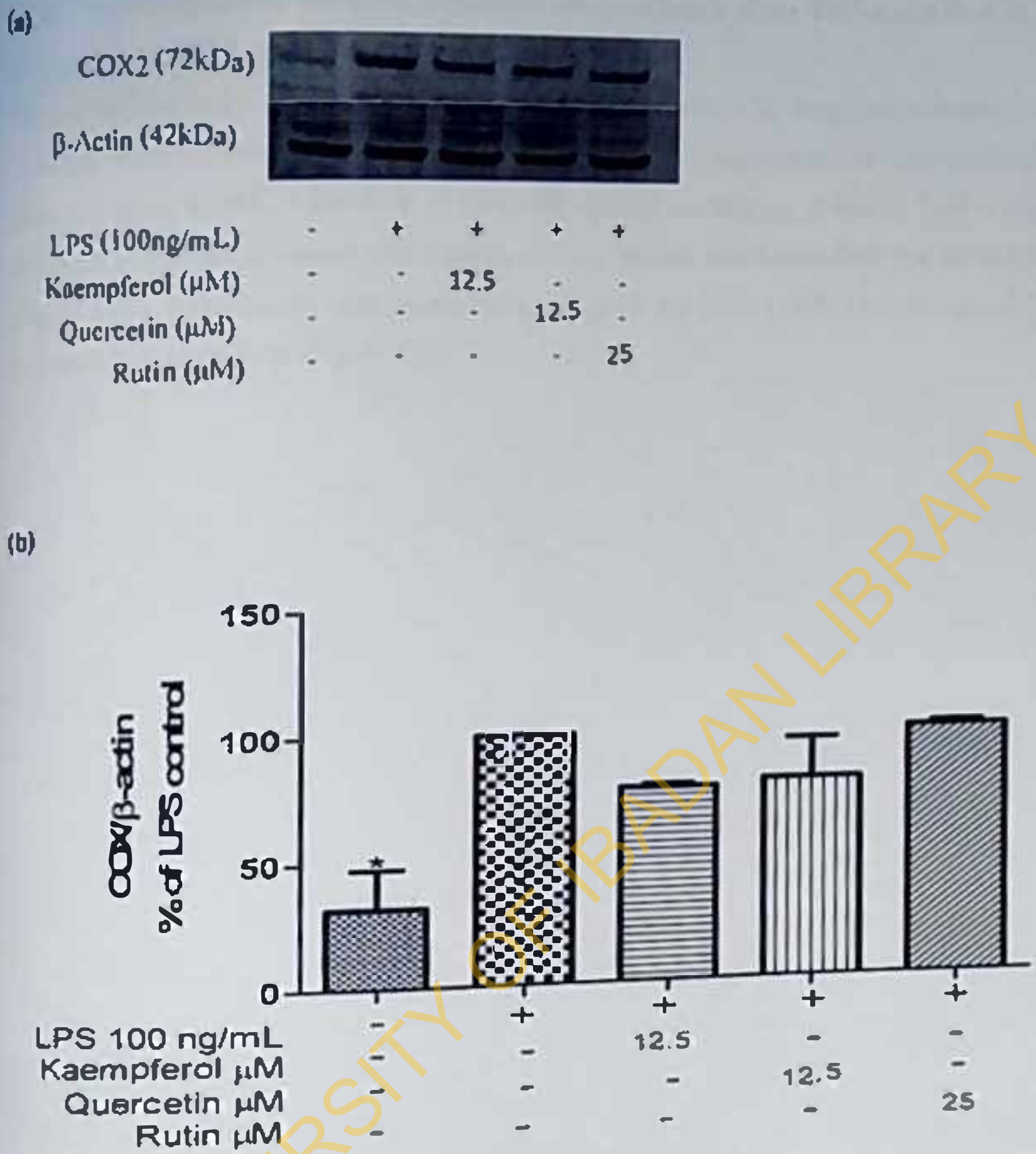


Figure 4.27: Kaempferol and quercetin inhibited COX-2 protein expressions in LPS-activated microglia.

Microglia were incubated in a medium containing 12.5 μM of both kaempferol and quercetin and 25 μM rutin for 30 min and then activated by100 ng/mL LPS for 24 h. Kaempferol and quercetin inhibited COX-2 protein expression in LPS-activated microglia. Protein expression was determined using western blot with specific anti-COX-2 antibodies. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparisons with post-hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only.

4.8.4 Kaempferol and quercetin suppresses the production of the TNF-α and IL-6 in LPS-activated BV-2 microglia

The pro-inflammatory cytokines (TNF-α and IL-6) are known to be important mediators of microglia inflammation. Their production was measured in supernatants of LPS-activated microglia using ELISA. After 24 h of LPS (100 ng/mL) stimulation, levels of TNF-α was significantly reduced in culture supernatants of BV-2 treated with kaempferol and quercetin (Figure 4.28). Pretreatment with kaempferol, quercetin and rutin (12.5, 12.5 and 25 μM) increased IL-6 production (Figure 4.29).

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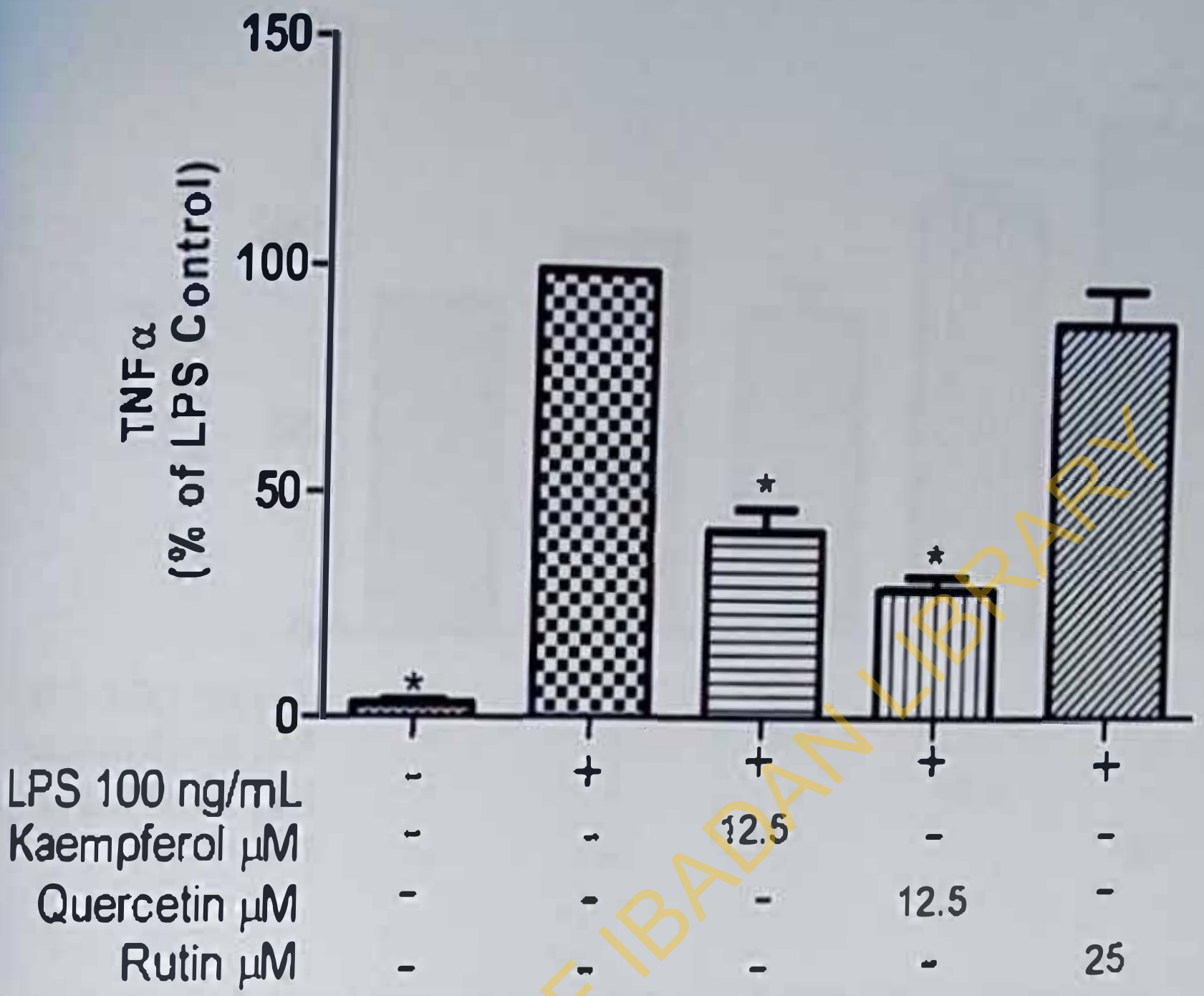

Figure 4.28: Kaempferol and quercetin reduced TNFα production in LPS-activated microglia. Cells were stimulated with LPS (100 ng/mL) in the presence or absence of 12.5 µM kaempferol or quercetin and 25 µM rutin for 24 h. At the end of the incubation period, supernatants were collected for ELISA measurements. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only

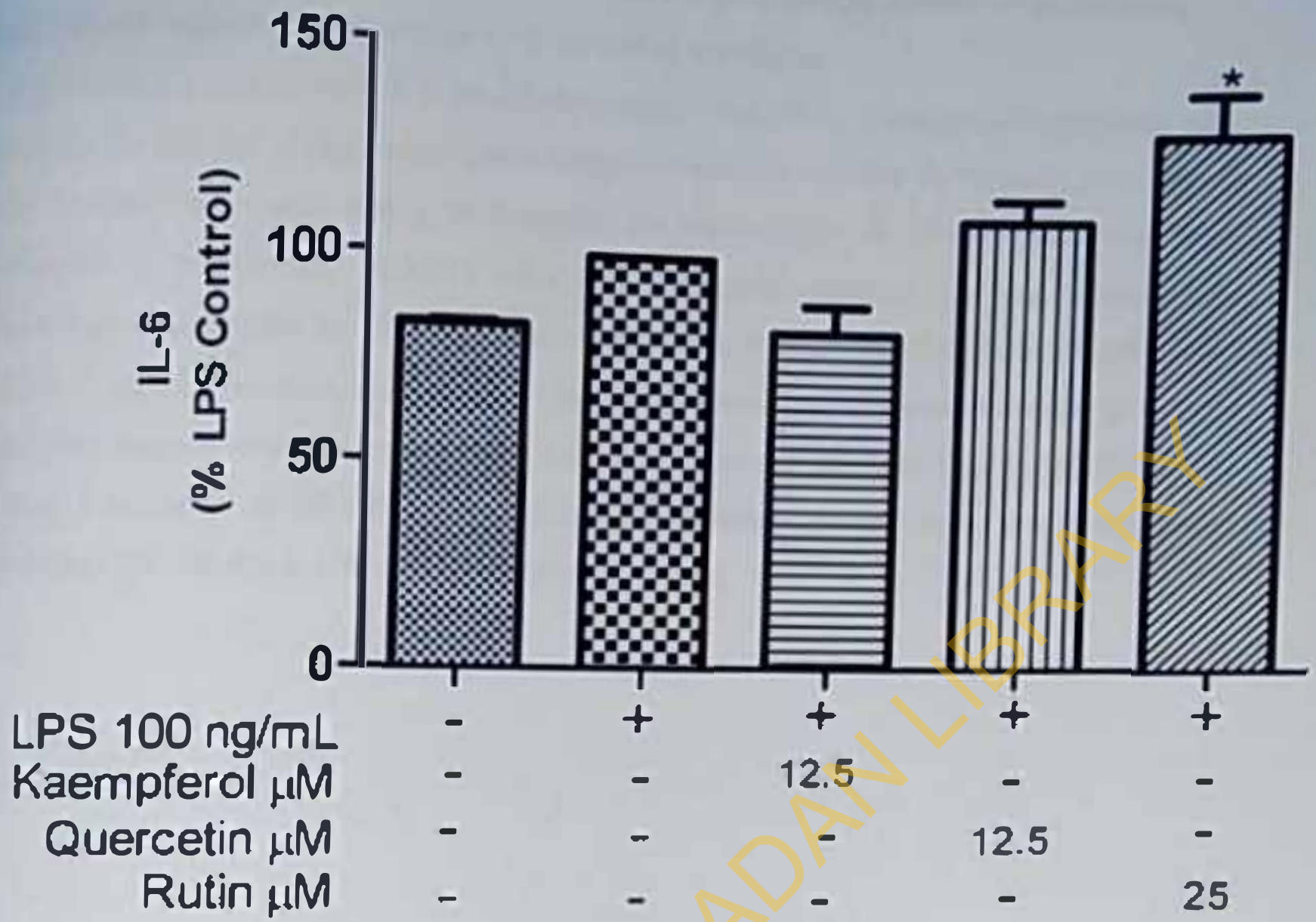

Figure 4.29: Kaempferol and quercetin did not reduce IL-6 production in LPS-activated microglia.

Cells were stimulated with LPS (100 ng/mL) in the presence or absence of 12.5 µM kaempferol or quercetin and 25 µM rutin for 24h. At the end of the incubation period, supernatants were collected for ELISA measurements. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only.

4.8.5 Kaempferol, quercetin and rutin modulate neuroinflammation by interfering with NF-κB signalling pathway in LPS-activated microglia

Considering the role of NF-κB in neuroinflammation, the effect of kaempferol, quercetin and rutin on the activity of this transcription factor was elucidated using the reporter gene assay. The compounds showed ability to modulate the transcription of NF-κB gene. This was measured by transfecting HEK293 cells with a plasmid construct carrying a luciferase reporter gene controlled by NF-κB. It was observed that stimulation of transfected cells with TNF-α (1 ng/mL) resulted in activation of the NF-κB-driven luciferase expression (Fig. 4.30). This phenomenon was affected by kaempferol, quercetin and rutin resulting in significant ($p < 0.001$) inhibition of NF-κB driven luciferase expression, demonstrating that compounds suppresses NF-κB-dependent gene expression in general.

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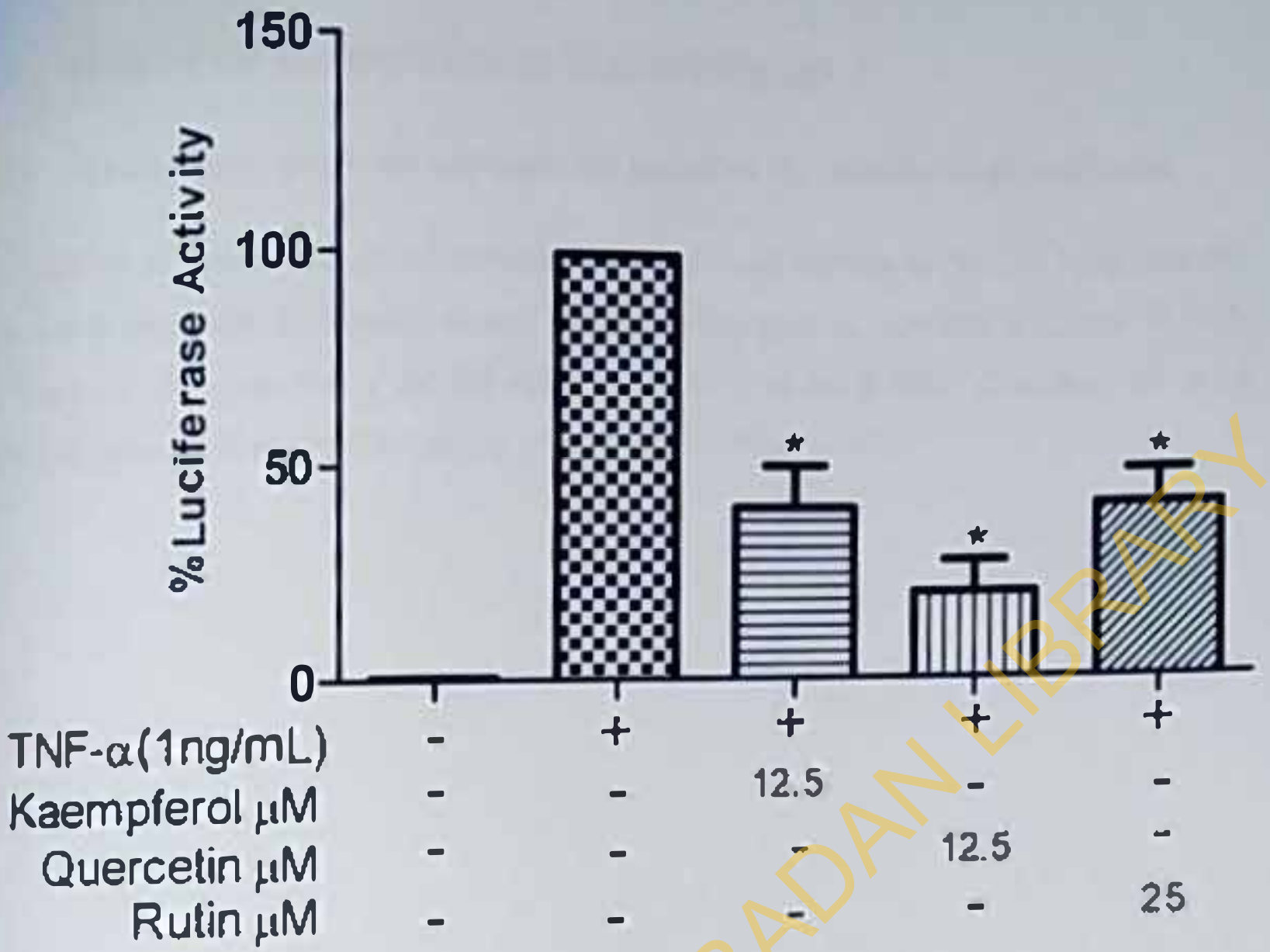


Figure 4.30: Kaempferol, quercetin and rutin inhibited NF-κB-mediated gene expression.

HEK293 cells were transfected with a plasmid construct carrying a luciferase reporter gene controlled by NF-κB, and stimulated with TNF-α (1 ng/mL) in the presence or absence of 12.5 μM kaempferol or quercetin and 25 μM rutin. NF-κB mediated gene expression was measured with ONE-Glo luciferase assay kit and luminescence measured. All values were expressed as mean ± SEM for three independent experiments performed in triplicates. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test. * = significant at $p < 0.001$ when compared with TNF control.

4.9 EFFECT OF COMPOUNDS ON MACROPHAGES

4.9.1 Kaempferol, quercetin and rutin did not affect the viability of macrophages.

The toxicity of compounds from kaempferol, quercetin and rutin on RAW 264.7 cell viability was tested using the ATP assay. Result shows that kaempferol, quercetin and rutin at 12.5, 12.5 and 25 μM respectively did not affect the viability of RAW 264.7 (Figure 4.31). Also LPS 100 ng/mL did not affect viability of RAW 264.7 (Figure 4.31).

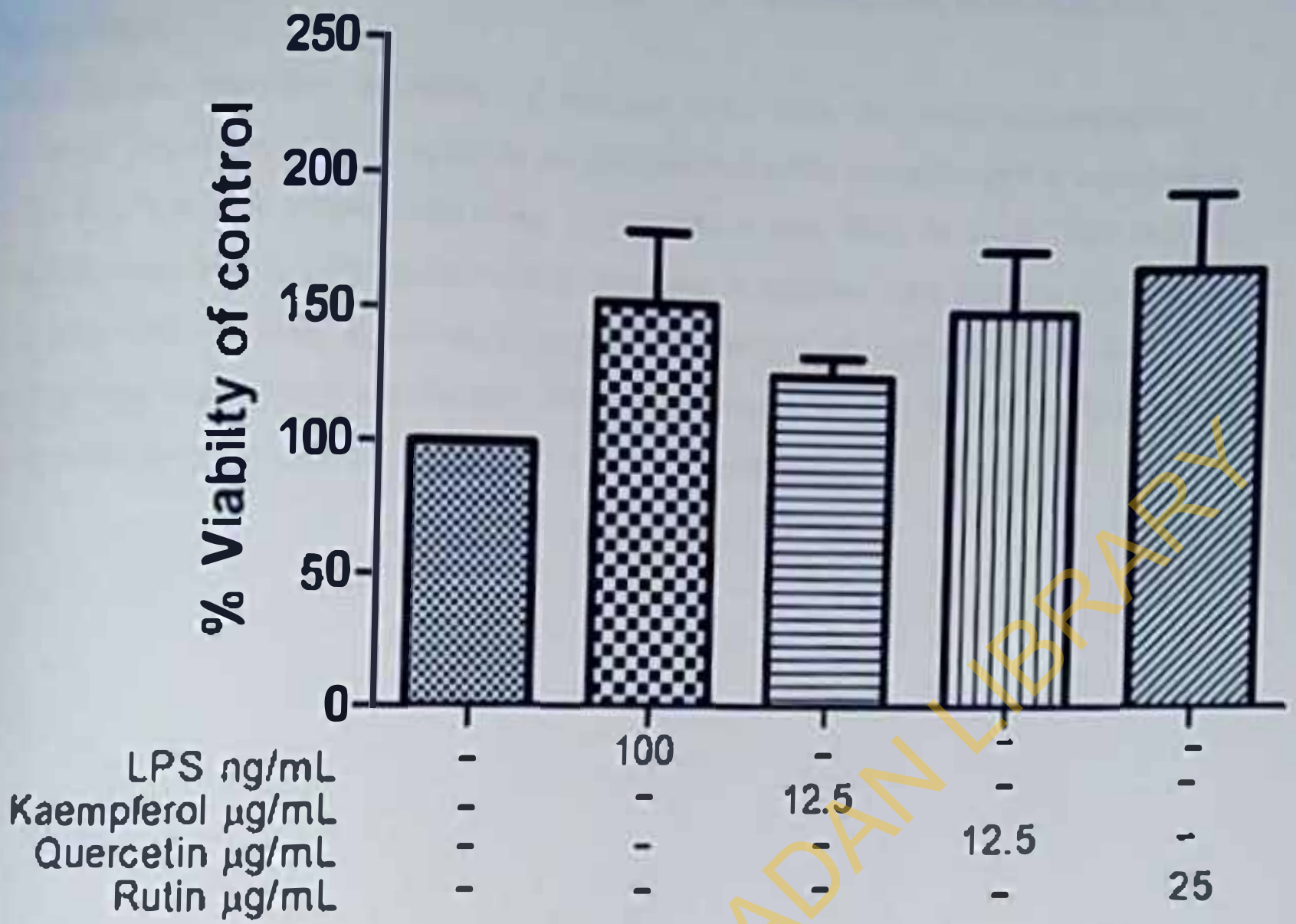


Figure 4.31: Pre-treatment with kaempferol, quercetin and rutin did not affect the viability of microglia.

Cells were stimulated with either LPS (100 ng/mL), kaempferol (12.5 µM), quercetin (12.5 µM) or rutin (25µM) for 24h. At the end of the incubation period, ATP assay was carried out on cells. All values were expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

4.9.2 Kaempferol, quercetin and rutin suppresses the production of cytokines in macrophages

Cytokines are important mediators of immune cells. They are important regulators of immunity. The effect of the compounds on cytokine production was measured in supernatants of RAW 274.6 cells treated with either compounds or LPS after 24 hours. The level of cytokine production in LPS treated macrophages was as expected very high for IFN-γ, IL-6, IL-8 and TNF-α. Rutin significantly inhibited production of IL-6, IL-8 and TNF-α in macrophages. Kaempferol significantly inhibited production of IL-6 and IL-8. None of the compounds showed significant inhibition of IFN-γ (Figure 4.32)

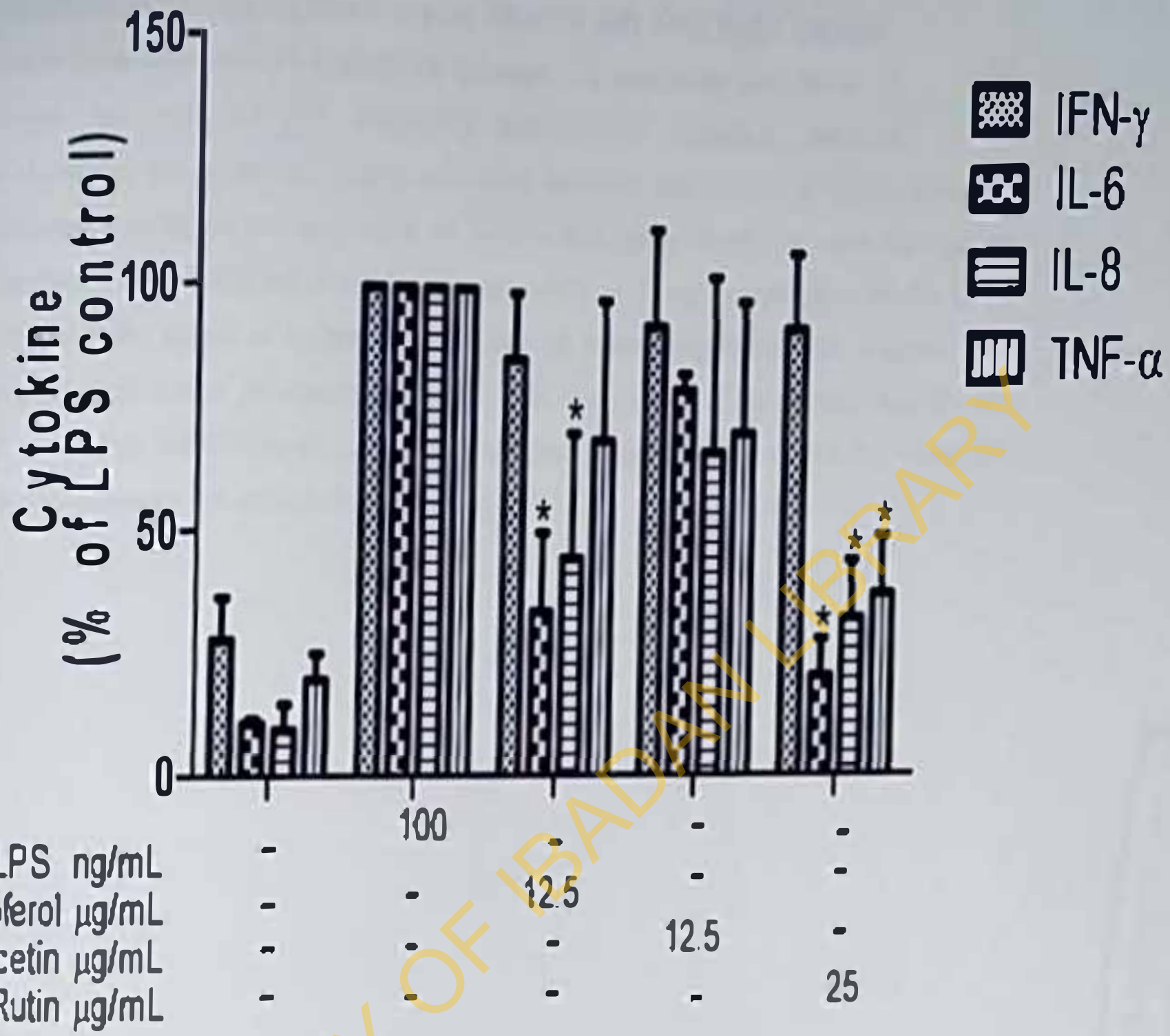


Figure 4.32: Cytokines production in kaempferol, quercetin and rutin treated microglia cells.

Cells were stimulated with either LPS (100 ng/mL), kaempferol (12.5 µM), quercetin (12.5 µM) or rutin (25 µM) for 24 h. At the end of the incubation period, supernatants were collected for ELISA measurements. All values were expressed as mean ± SEM for 3 independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only

4.9.3 Inhibition of phosphorylation of p38, ERK1/2 and JNK MAP kinases contributes to immunodulatory activity of kaempferol, quercetin and rutin

MAP kinases are one of the frequently encountered signaling pathways in the immunomodulation. For example, T cell activation involves the Ras/MAP kinase pathway, with the cascade ending in the activation of ERK which gains ability to pass through the nuclear membrane to activate AP-1 (Fos and Juns) which is a very important regulator of IL-2. Based on this, the effect of isolated compounds on macrophages was investigated. LPS alone increased the level of phosphorylated p38, JNK and ERK. All the compounds showed significant ($p < 0.05$) inhibition of p38. Both phosphorylated JNK and ERK1/2 were also inhibited by the compounds especially rutin (Figure 4.33).

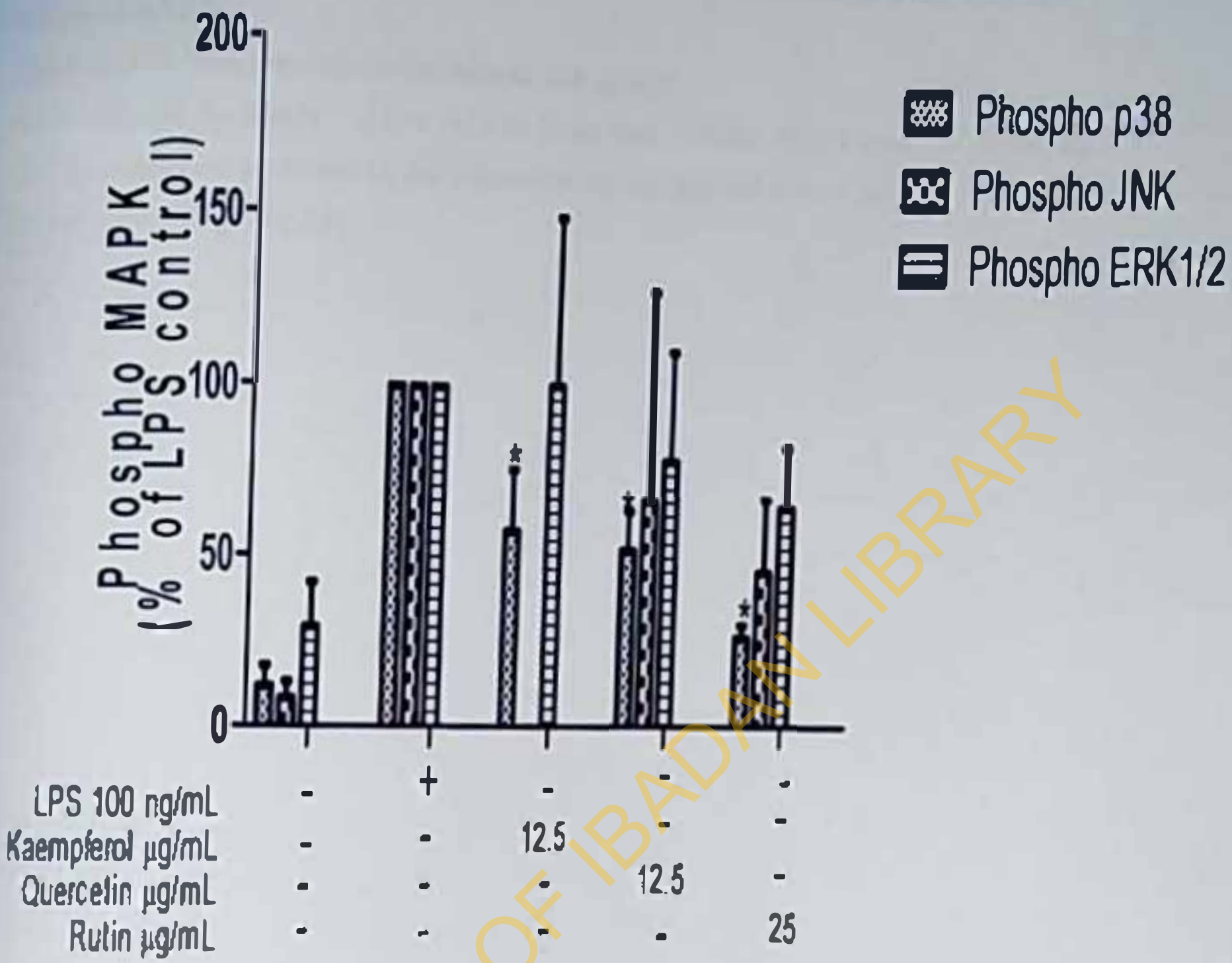


Figure 4.33: Inhibition of MAPK phosphorylation by kaempferol, quercetin and rutin

Cells were stimulated with either LPS (100 ng/mL), kaempferol (12.5 µM), quercetin (12.5 µM) or rutin (25 µM) for 24 h. Data were analysed using one-way ANOVA for multiple comparison with post hoc Student Newman-Keuls test.

* = significant at $p < 0.05$ when compared with LPS only

4.10 IMMUNOMODULATORY EFFECT OF ETHANOL EXTRACT OF *Moringa oleifera* LEAVES

4.10.1 EMOL was toxic on Jurkat cells at 160 µg/mL

EMOL reduced the number of live cells to lower than 100,000 after 6 hours of treatment. After 24 hours post treatment all the concentrations has doubled almost twice except 40, 80 and 160 µg/mL (Figure 4.34)

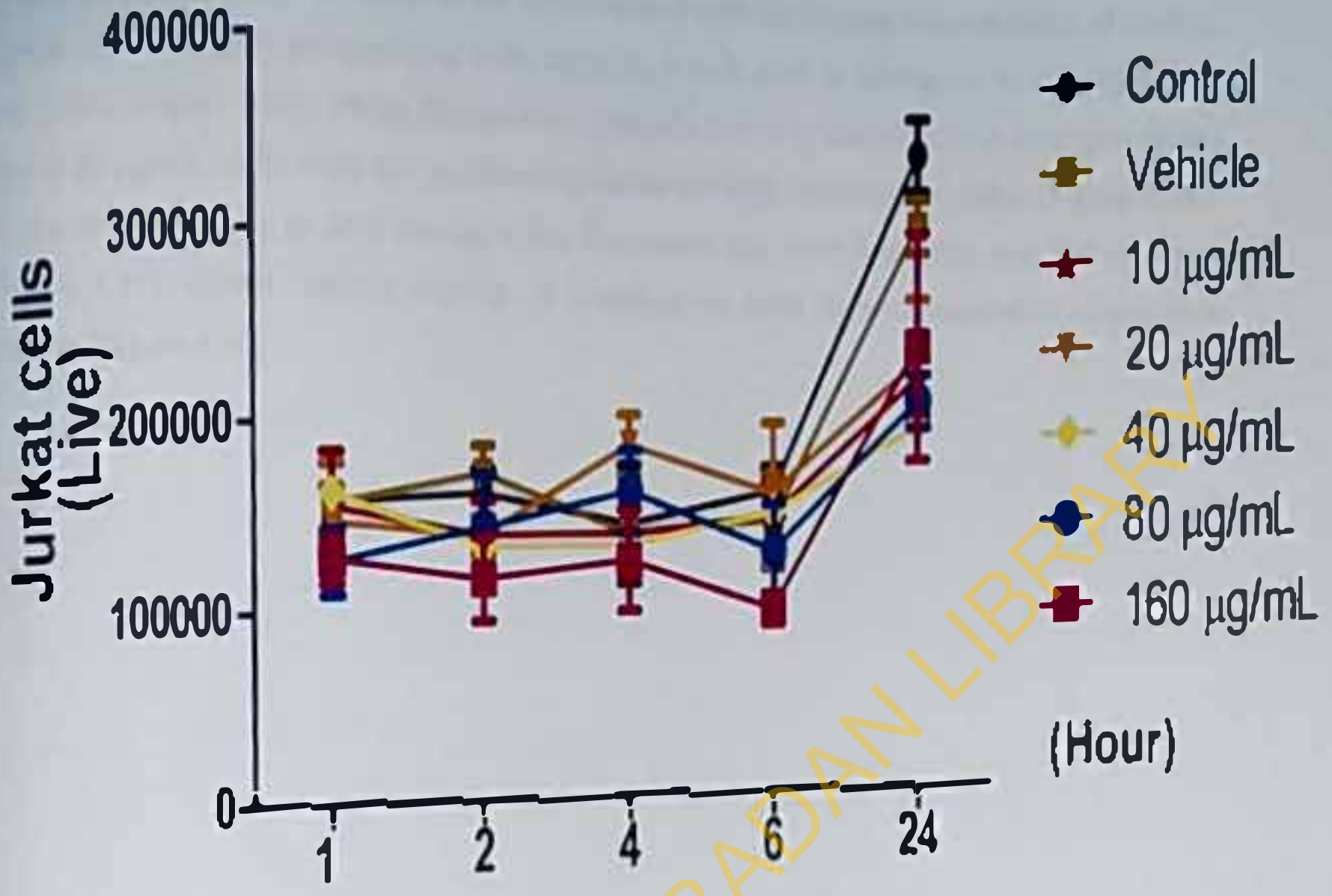


Figure 4.34: Toxicity of crude extract *Moringa oleifera* on Jurkat cells.

Jurkat cells were plated at 1×10^5 cells/well and total number of cells counted using trypan blue cell exclusion assay at time points (1,2,4,6 and 24 hours). Data were expressed as mean ± (SEM) total number of live cells within entire population. Representative of > 4 experiments

4.10.2 EMOL showed a concentration dependent inhibition of proliferation

The number of rapidly dividing Jurkat cells reduced with increasing concentration of EMOL. Jurkat cell are highly proliferating cells showing a bulk shift in histogram to the right from day 1 to 4 (Figure 4.35). Mean fluorescence intensity (MFI) of Jurkat cells in live gate shows that at 80 µg/mL, cells were not proliferating hence the high fluorescence value (Figure 4.36). A plot of fold change in MFI for day 4 and 7 revealed that more inhibition was before day 4 (Figure 4.37). EMOL inhibit number of proliferating cells in a concentration dependent manner (Figure 4.38)

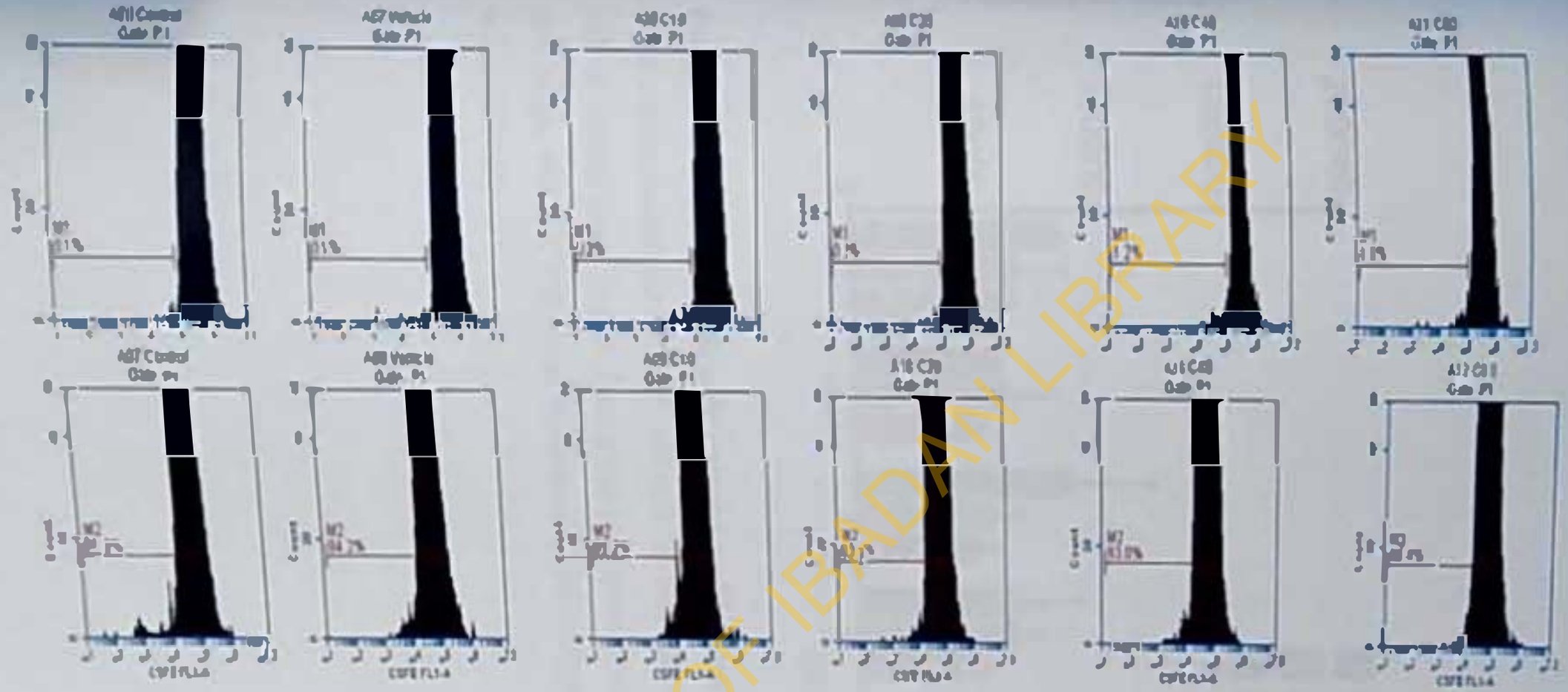


Figure 4.35: Crude *Moringa oleifera* extract reduced the proliferation of Jurkat cells

Jurkat cells sub-cultured at 5×10^5 cells/mL 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points. Each row is a representative histogram plot of CFSE for the various concentrations at days 1 and 4.

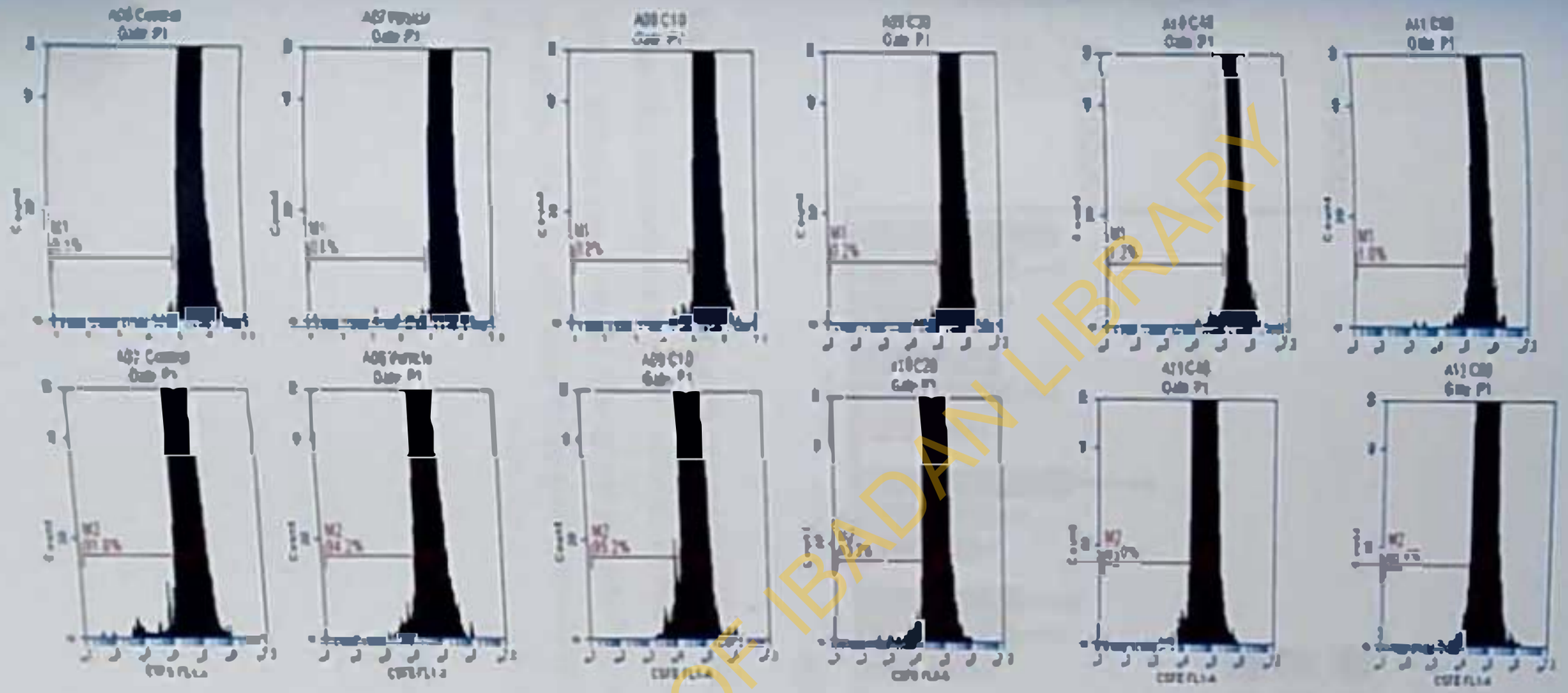


Figure 4.35: Crude *Moringa oleifera* extract reduced the proliferation of Jurkat cells

Jurkat cells sub-cultured at 5×10^{5} cells/mL 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points. Each row is a representative histogram plot of CFSE for the various concentrations at days 1 and 4.

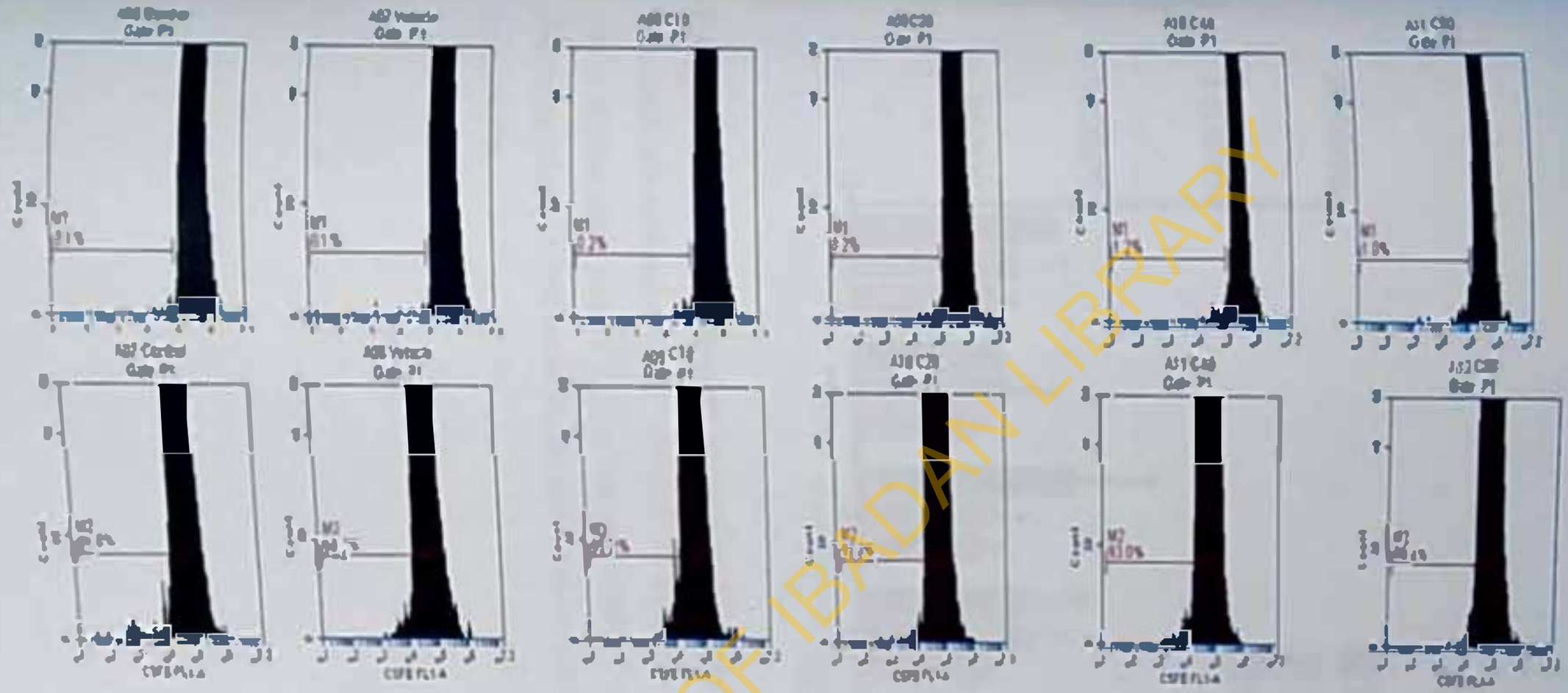


Figure 4.35: Crude *Moringa oleifera* extract reduced the proliferation of Jurkat cells

Jurkat cells sub-cultured at 5× 10^3 cells/ml. 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points. Each row is a representative histogram plot of CFSE for the various concentrations at days 1 and 4.

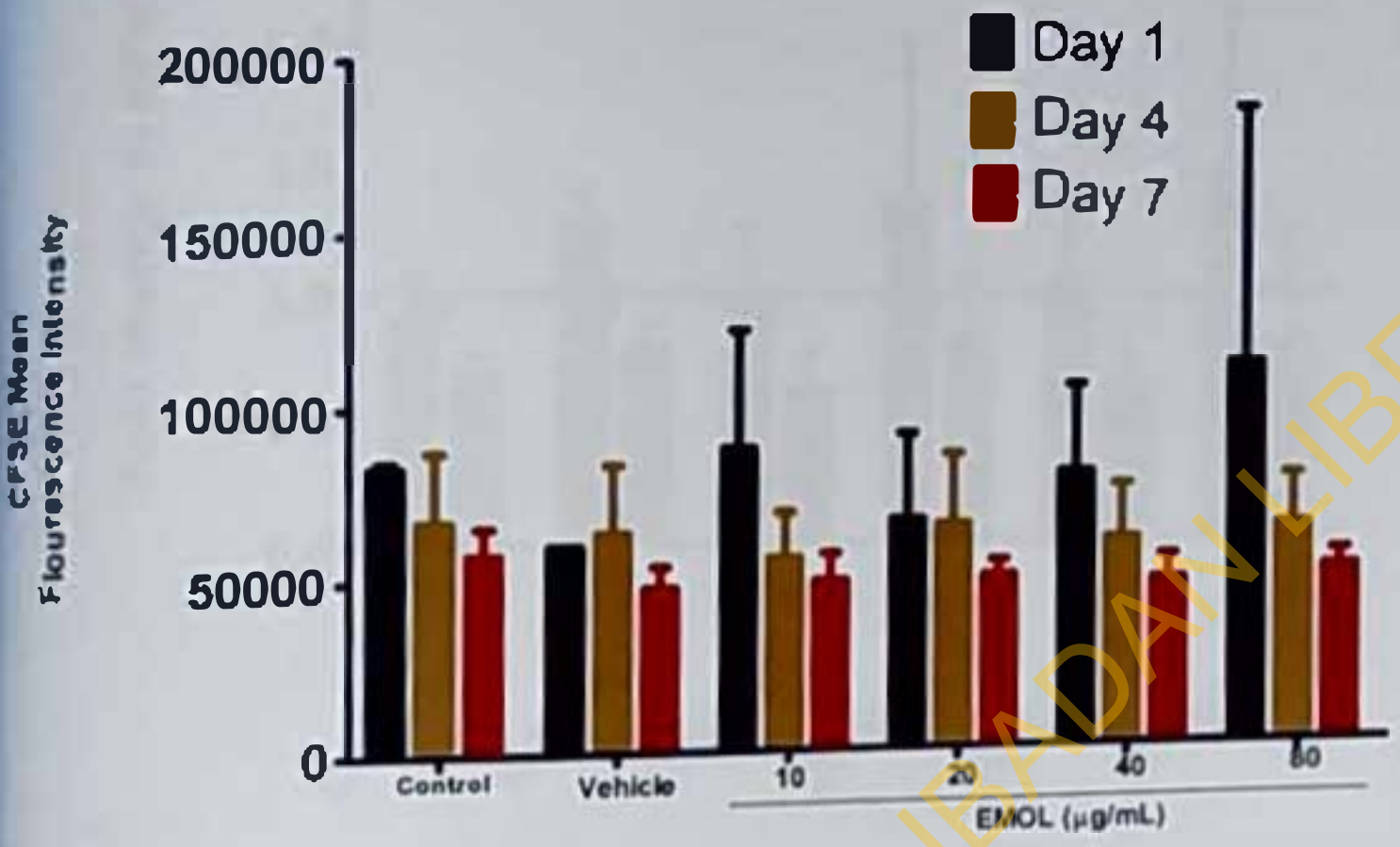


Figure 1.36: EMOL significantly reduced proliferation of Jurkat cells at 80 µg/ml. Jurkat cells sub-cultured at 5×10^5 cells/mL 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points. Plot of CFSE mean florescence intensity (MFI) in presence or absence of extract at time points

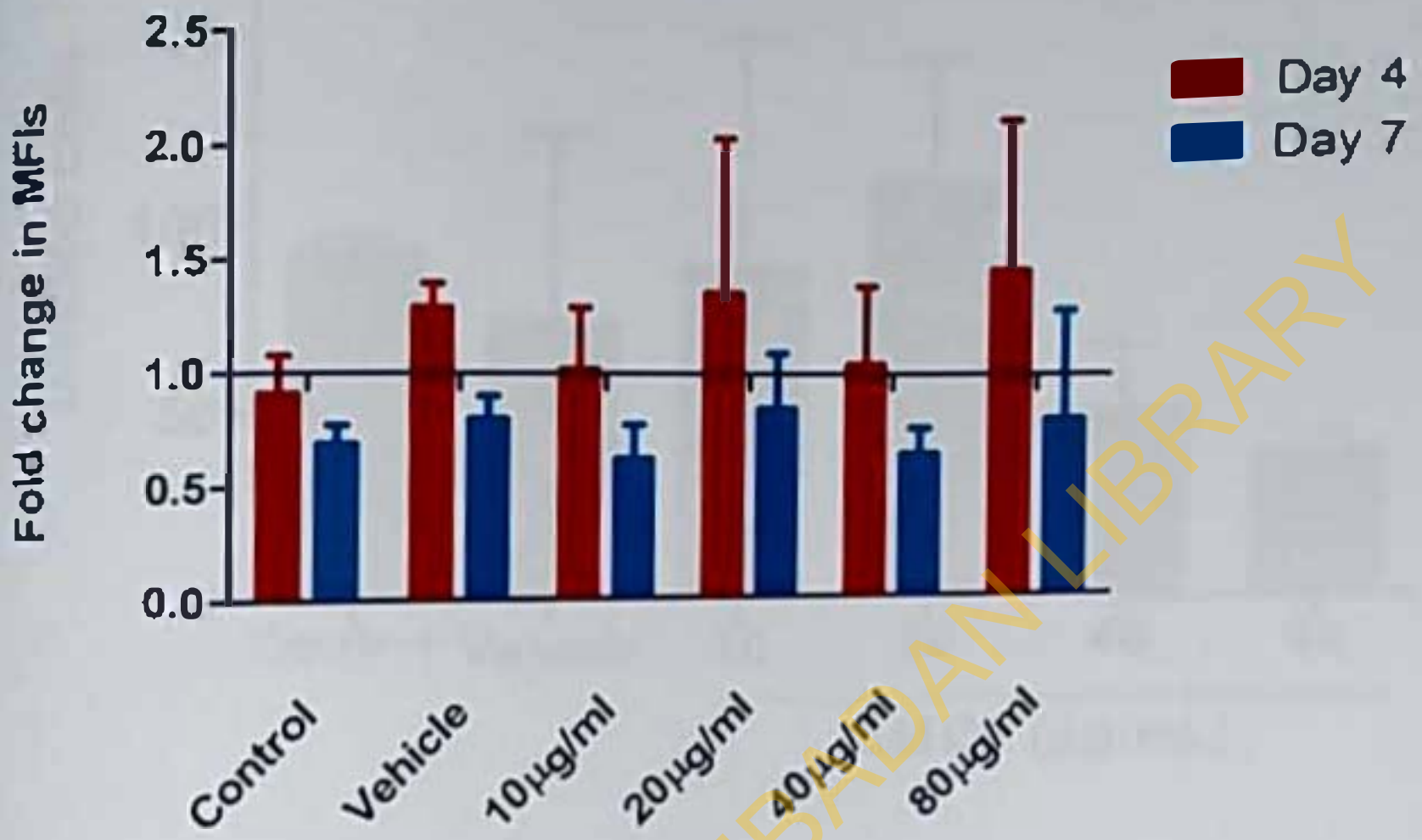


Figure 4.37: Fold change in MFIs at day 4 greater than 1 for EMOL treated Jurkat cells.

Jurkat cells sub-cultured at 5×10^5 cells/ml, 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points. EMOL reduced the proliferation of Jurkat cells.

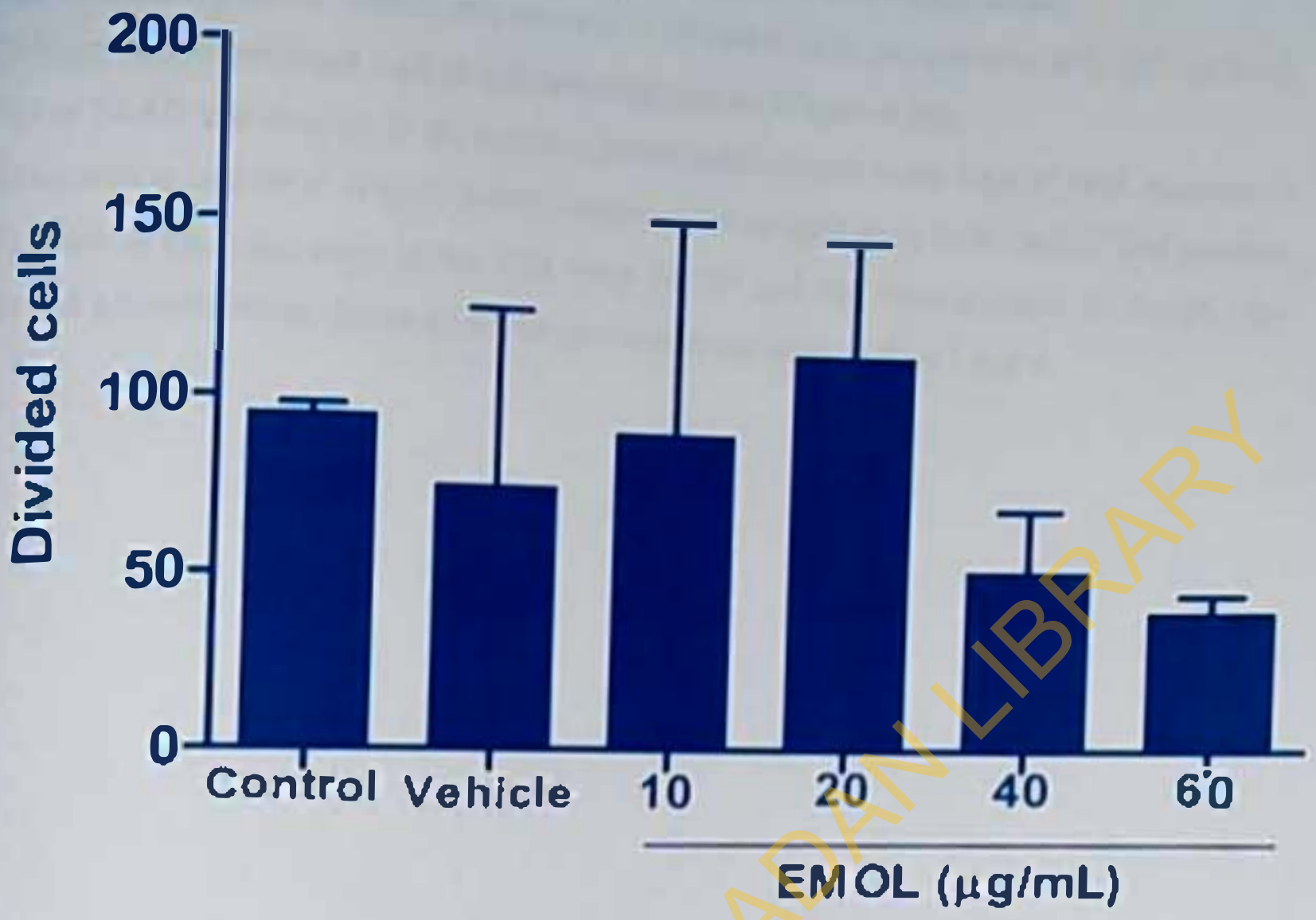


Figure 4.38: EMOL dose dependently reduced the proliferation of Jurkat cells.

Jurkat cells sub-cultured at 5×10^5 cells/mL 24 hours before staining with CFSE and treated with or without crude extract. Gating was performed on rapidly dividing cells and counted at time points.

4.10.3 Apoptosis and necrosis measured by Annexin V and 7AAD stains

Representative plots of 7AAD and annexin V of Jurkat cells sub-cultured at 5×10^5 cells/mL 24 hours before treatment with or without crude extract (Figure 4.39).

Plot of 7AAD and annexin V showed that Jurkat cells become more $7AAD^+$ with increase in concentration of EMOL (Figure 4.40). Untreated Jurkat cells were both $7AAD^-$ and annexin V$^-$, while at 80µg/mL most of the cells were $7AAD^+$ and few were annexin V$^-$. Result also showed a concentration driven effect for necrotic Jurkat cells on days 1 and 4.

Day 1
7AAD vs Annexin V

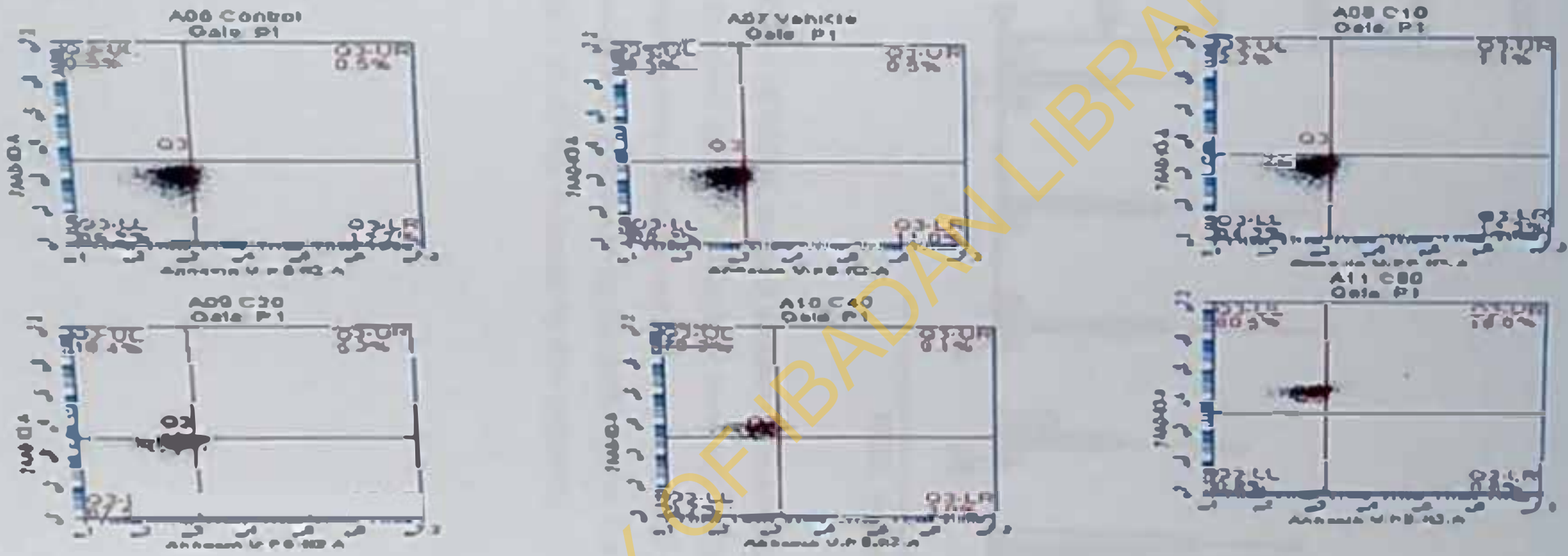


Figure 4.39: EMOL dose dependently increased necrotic death in Jurkat cells

Jurkat cells sub-cultured at 5×10^5 cells/mL 24 hours before treatment with or without crude extract. To directly measure apoptosis and necrosis, Jurkat cells were labelled with annexin V and 7AAD at various time points. Gating was performed on live cells and the total number of viable cells (mean ± SEM) was measured by flow cytometry. Representative plots of 7AAD and annexin V.

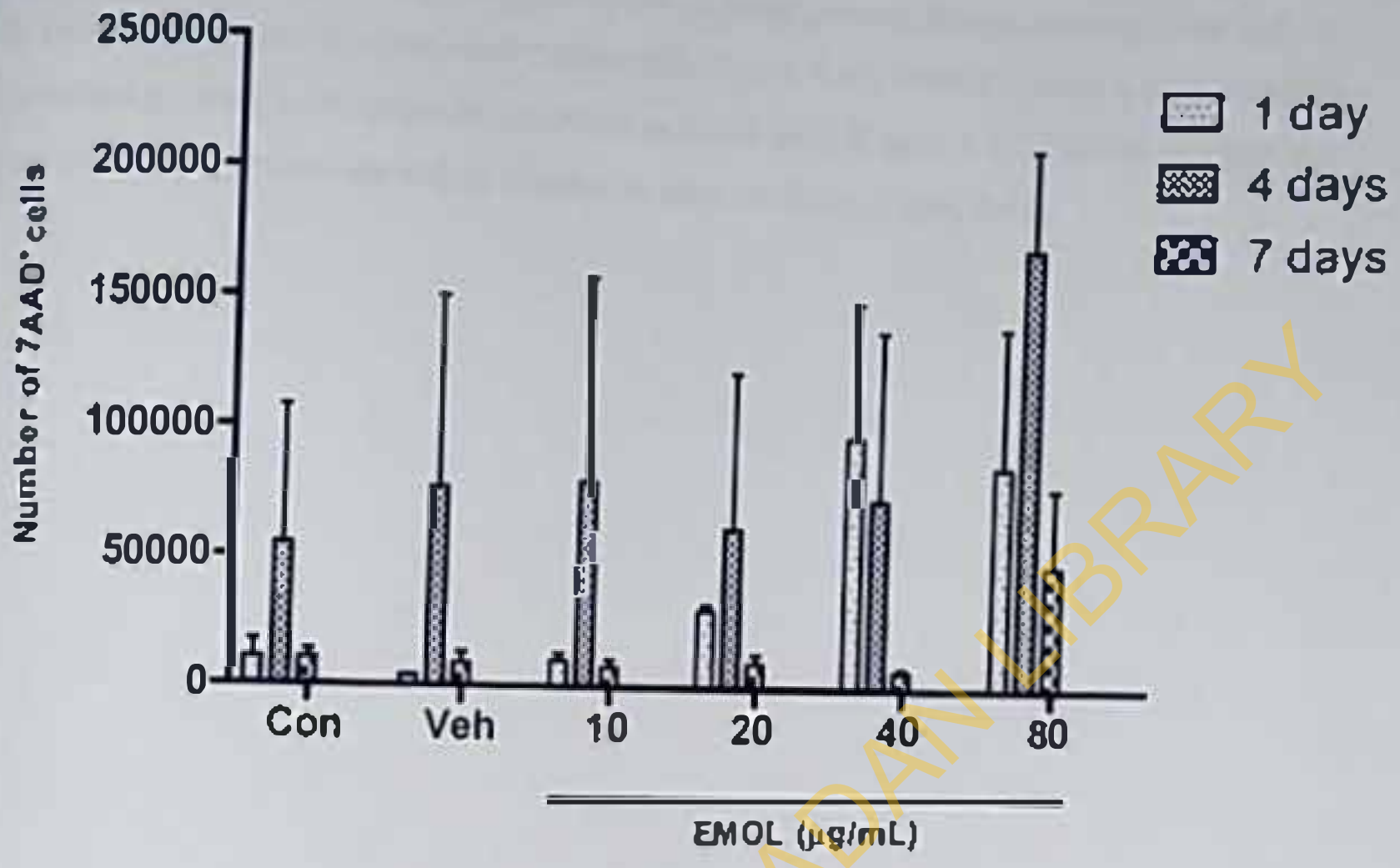


Figure 4.40: Effect of EMOL on total number of 7AAD$^+$ cells.

Jurkat cells sub-cultured at 5×10^5 cells/mL 24 hours before treatment with or without crude extract. To directly measure apoptosis and necrosis, Jurkat cells were labelled with annexin V and 7AAD at various time points. Gating was performed on live cells and the total number of viable cells (mean ± SEM) was measured by flow cytometry.

Con: control

Veh: vehicle

4.10.4 EMOL inhibited apoptosis of Jurkat cell on apoptosis and necrosis in CD3, ICAM and CD28 activated Jurkat cells

CD3 and either CD54 or CD28 co-stimulation of Jurkat prevent anergy making more cells to die by apoptosis than in unactivated Jurkat cells (Figure 4.41). EMOL caused a concentration dependent inhibition of apoptotic cell death in Jurkat cells (Figure 4.42). EMOL as expected showed a concentration dependent increase in necrotic death (Figure 4.43).

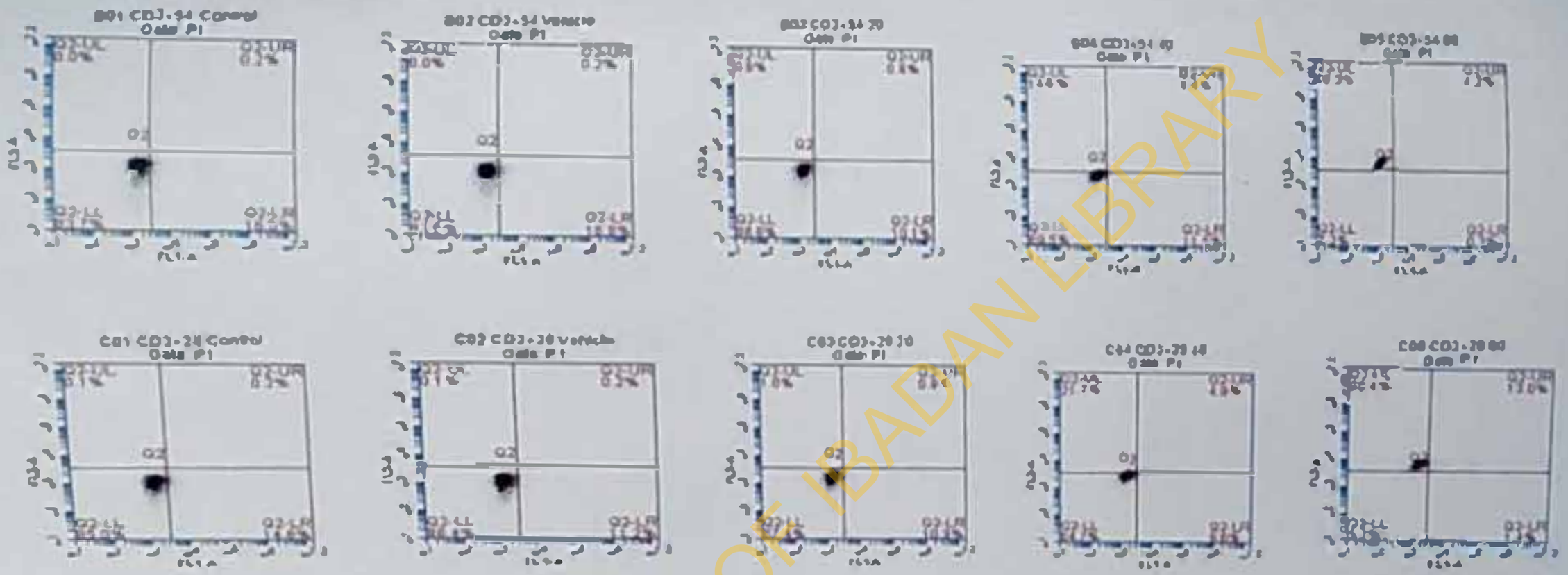
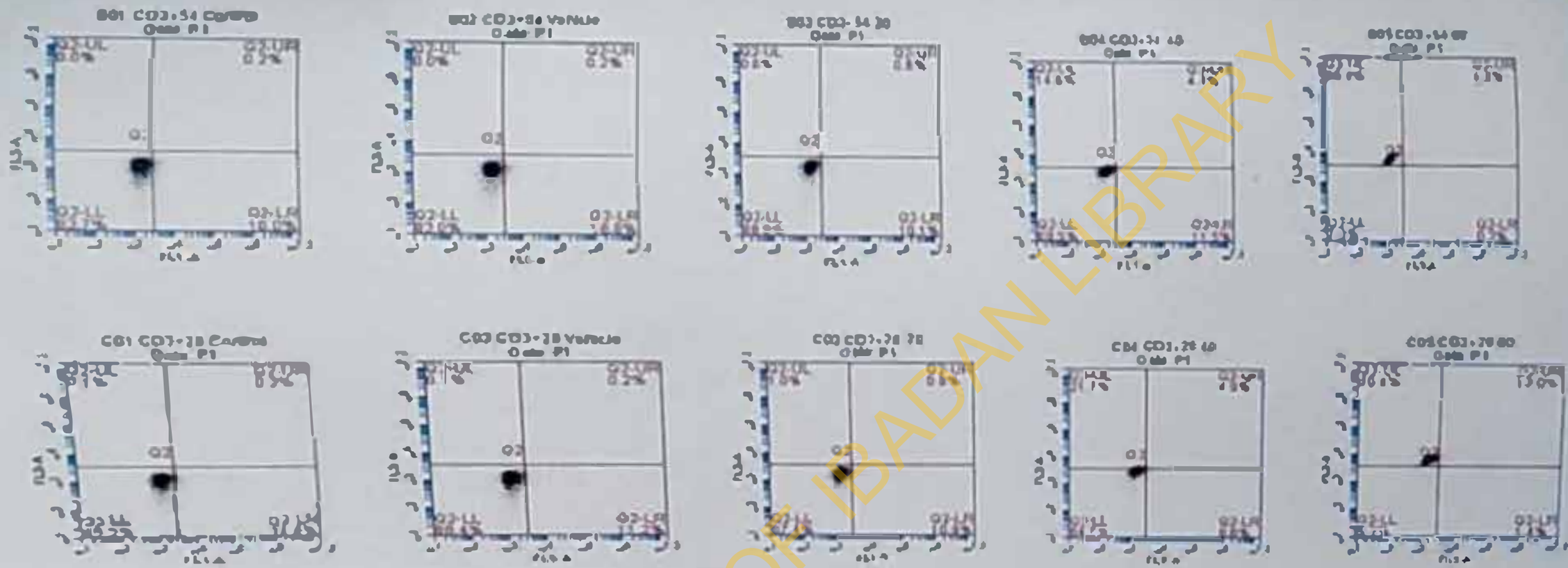


Figure 4.41: Sustained and efficient activation of Jurkat cells following co-stimulation through ICAM-1 and CD28.

Jurkat cells were stimulated with anti-CD3 plus anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean. ± SEM) was measured by flow cytometry. Data (mean ± SEM) were presented as the number of cells relative to the entire population. Representative of three experiments. Representative plots of 7AAD and annexin V.



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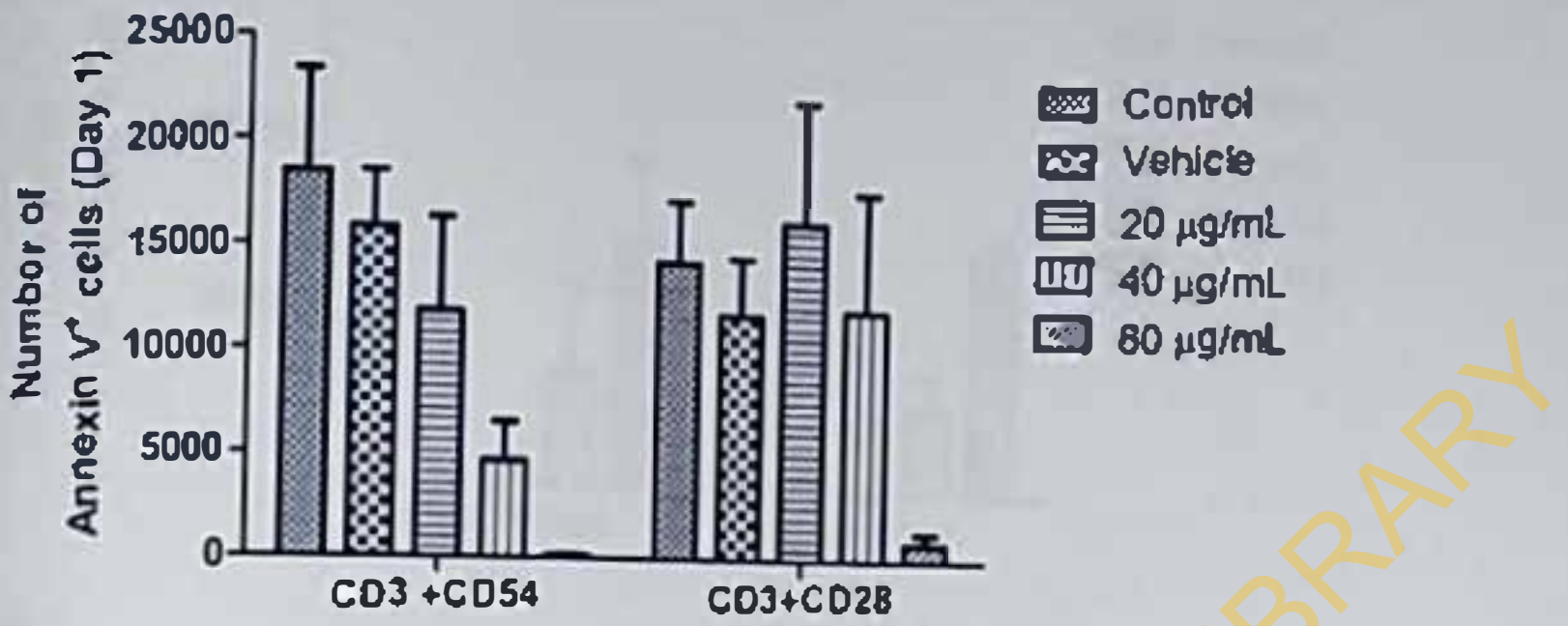
Day 1



Day 4

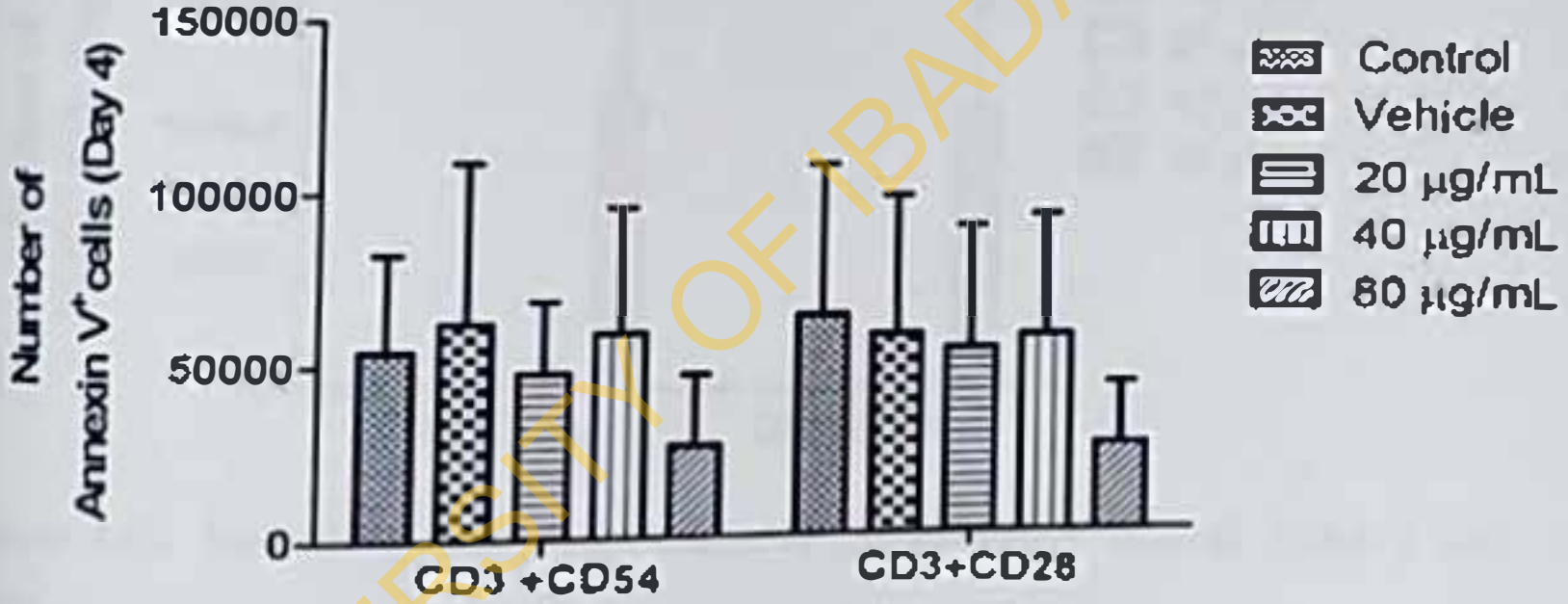
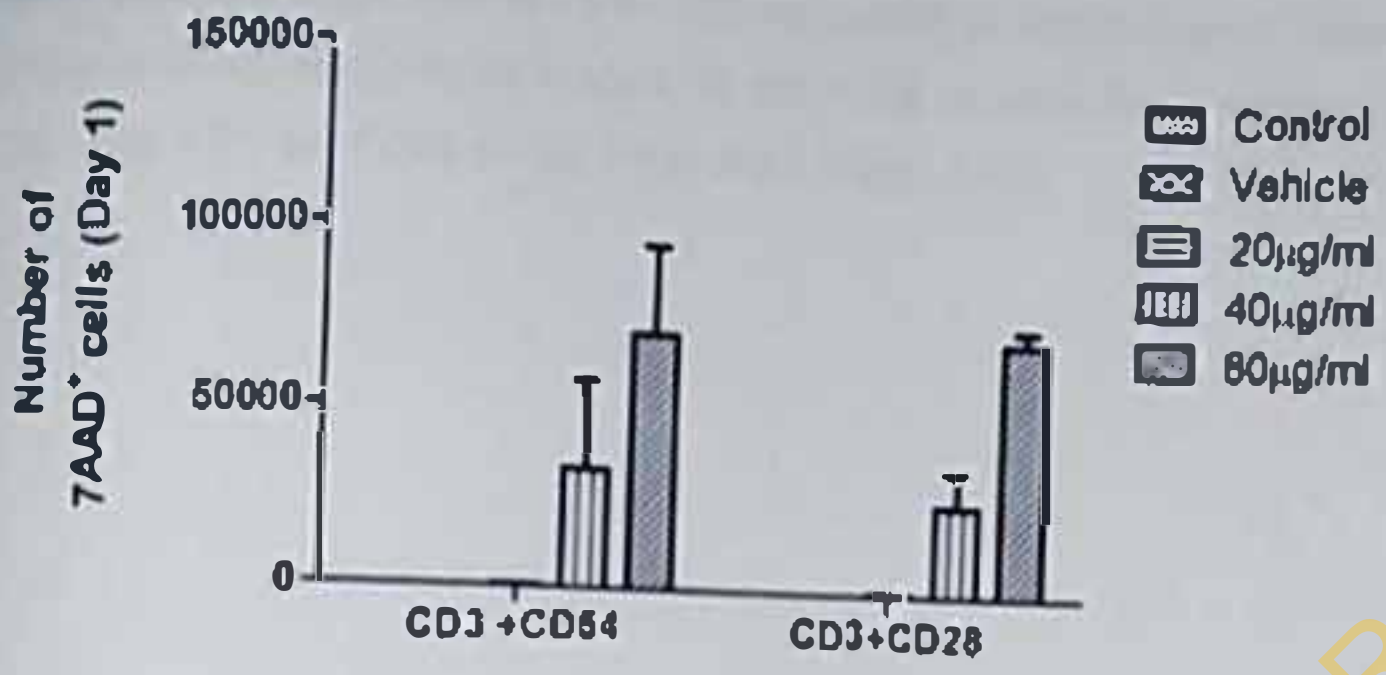


Figure 4.42 : Apoptic (Annexin V$^+$) cells following co-stimulation through ICAM-1 and CD28.

Jurkat cells were stimulated with anti-CD3 plus anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean, ± SEM) was measured by flow cytometry. Data (mean ± SEM) were presented as the number of cells relative to the entire population. Representative of three experiments. Number of annexin V$^+$ cells on day 1 and 4.

Day 1



Day 4

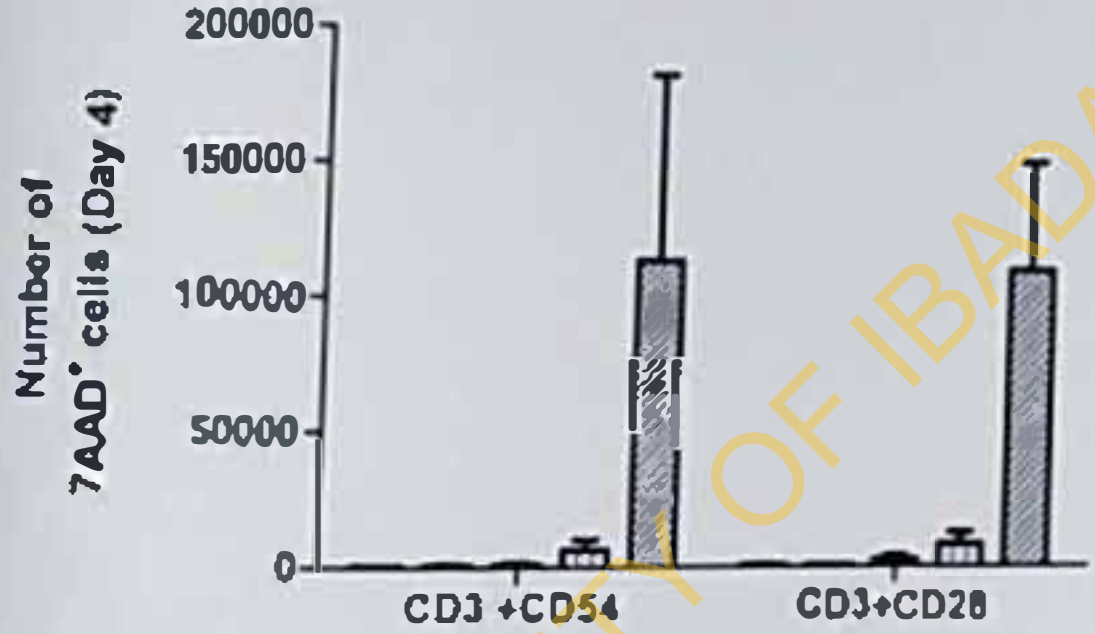


Figure 4.43: Necrotic (7AAD$^+$) cells following co-stimulation through ICAM-1 and CD28.

Jurkat cells were stimulated with anti-CD3 plus anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean, ± SEM) was measured by flow cytometry. Data (mean ± SEM) were presented as the number of cells relative to the entire population. Representative of three experiments. Number of 7AAD$^+$ cell on day 1 and 4.

4.10.5 EMOL on expression of CCR7 in Jurkat cells activated by CD3 and either ICAM or CD28

EMOL caused a slight shift in CCR7 expression MFI of EMOL treated Jurkat cells when compared to untreated (Figure 4.44). CD3 and CD28 activated Jurkat express slightly more CCR7 than CD3 and CD28 on day 1 than day 4 (Figure 4.45).

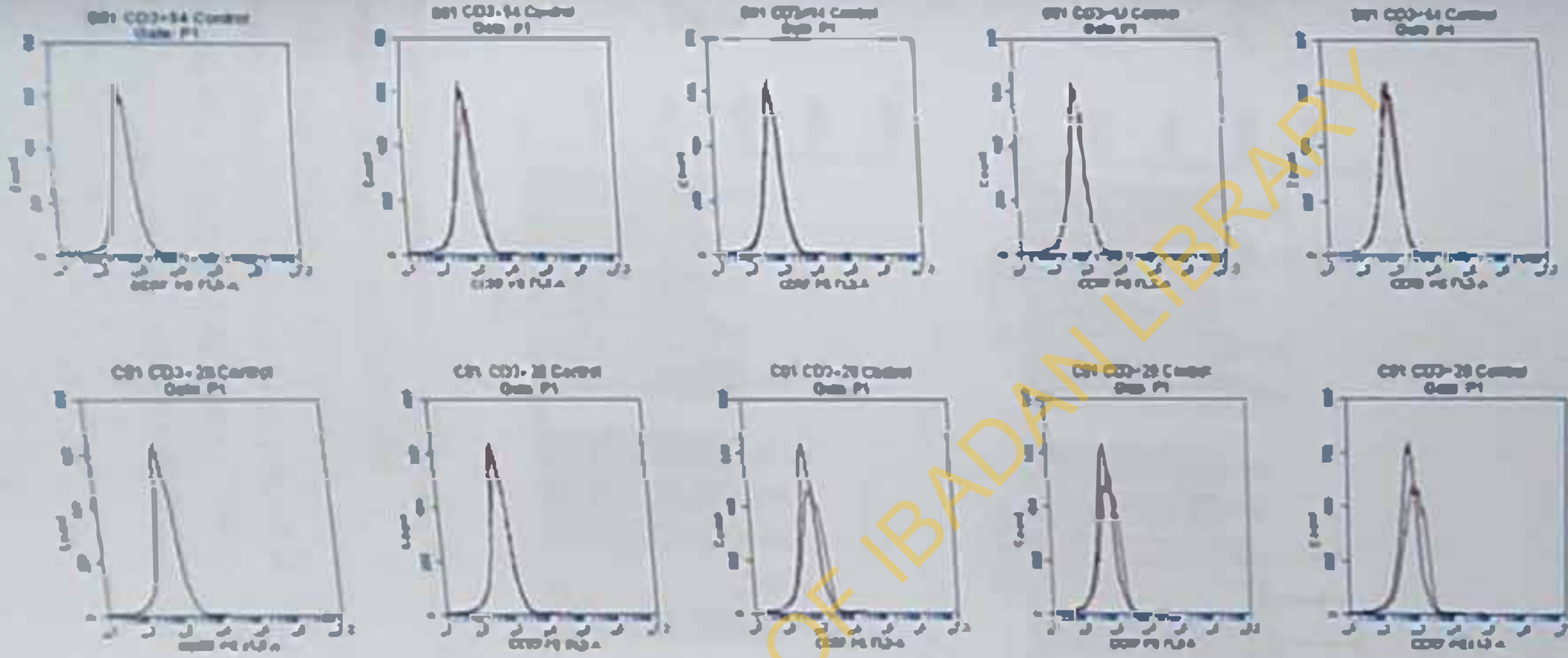
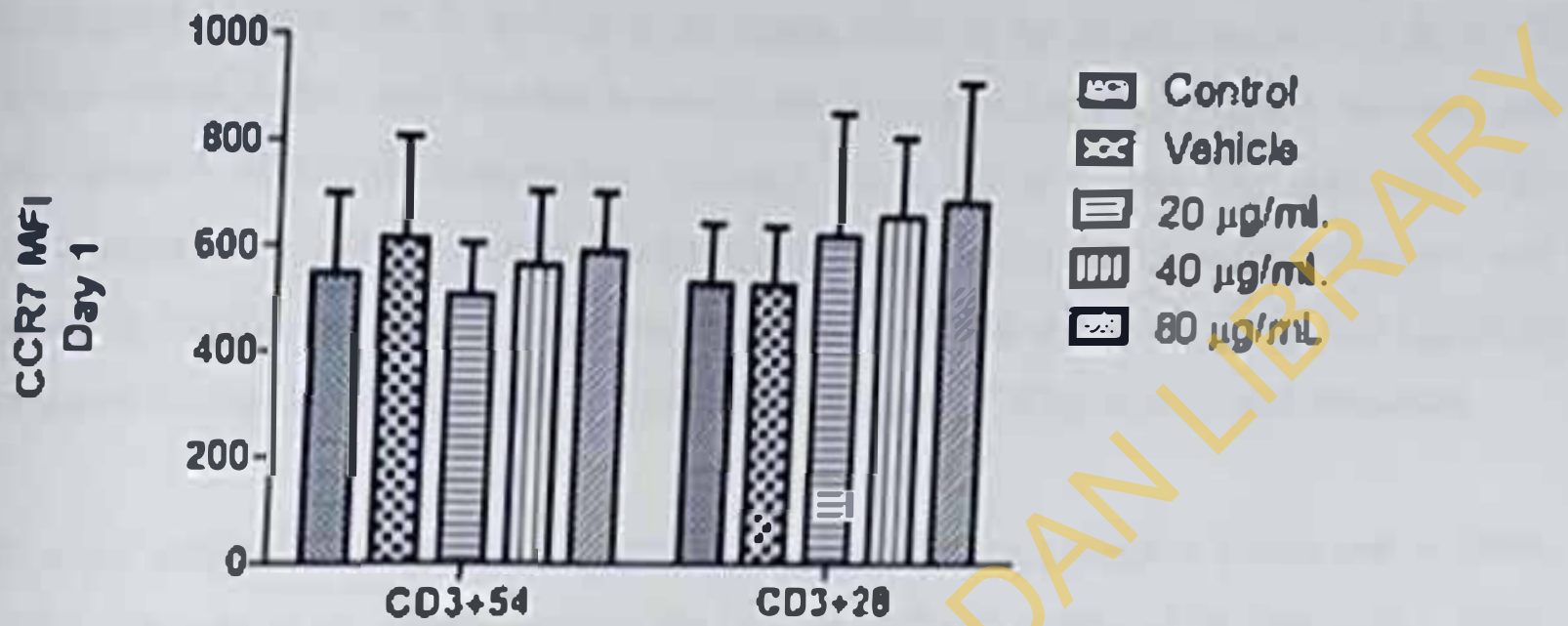


Figure 4.44: Representative histogram plot of CCR7 following co-stimulation through ICAM-1 and CD28.

EMOL relatively increased expression of CCR7 in Jurkat cells following co-stimulation through ICAM-1 and CD28. Jurkat cells were stimulated with anti-CD3 plus anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean ± SEM) was measured by flow cytometry. Data (mean ± SEM) were presented as the number of cells relative to the entire population. Representative of three experiments



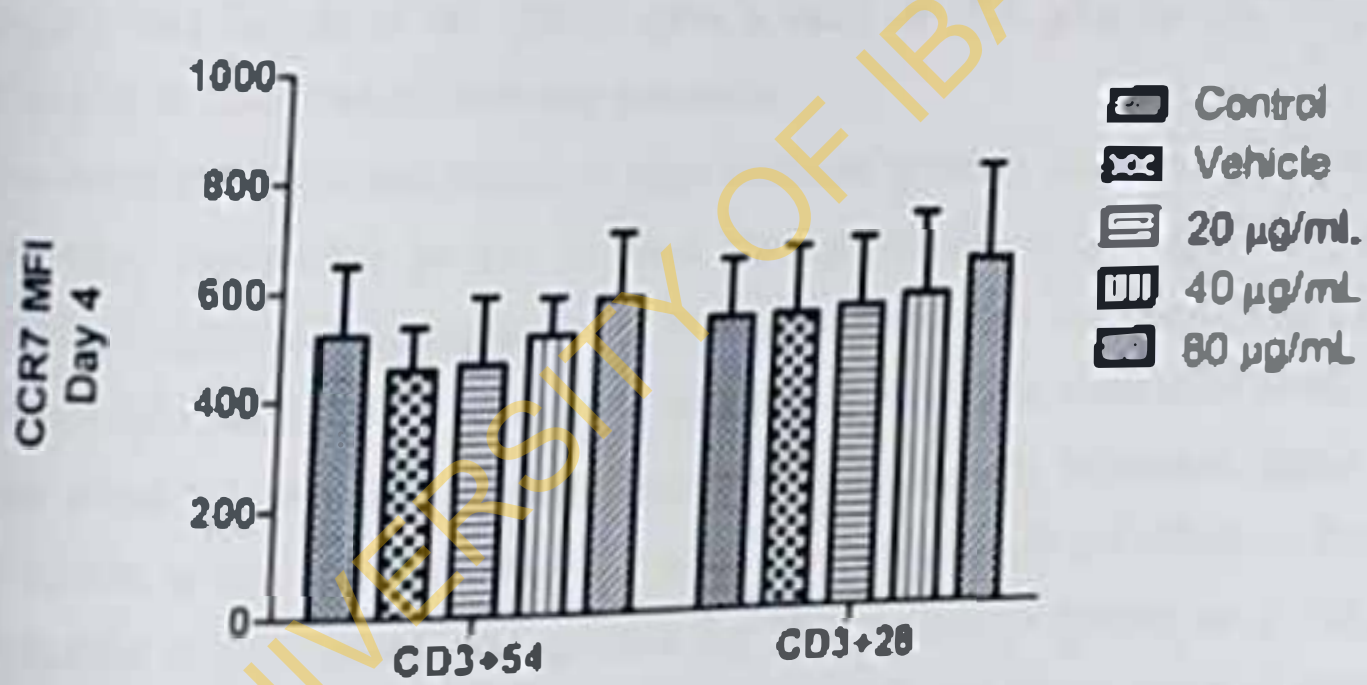


Figure 4.45: EMOL relatively increased expression of CCR7 in Jurkat cells following co-stimulation through ICAM-1 and CD28

Jurkat cells were stimulated with anti-CD3 plus anti-CD54 or anti-CD28. Cells were harvested on the days indicated and the total number of viable cells (mean ± SEM) was measured by flow cytometry. Data (mean ± SEM) were presented as the number of cells relative to the entire population. Representative of three experiments. MFI of CCR7 expression on day 1 and 4.

CHAPTER FIVE

DISCUSSION

5.1 Discussions

The antineuroinflammatory aspects of ethanol extract of *Moringa oleifera* leaves (EMOL) have been investigated in this study. Firstly, the central effects of the extract were investigated to ascertain its activity in the brain, followed by its neuroprotective ability in chronic inflammatory and neurotoxic conditions as exemplified by convulsive epilepsy and LPS induced cognitive impairment. Secondly, in a bid to isolate the principles with neuroprotective property, lipopolysaccharide induced model of neuroinflammation was studied by monitoring various inflammatory mediators, cytokines, chemokines and signaling molecules involved in both upstream and down regulation of inflammation and immunity.

The study established that LD_{50} of ethanol extract of *Moringa oleifera* leaves was > 5000 mg/Kg. Adedapo *et al.*, (2009) reported the LD_{50} of methanol extract of the leaf to be > 2000 mg/Kg, and Kasolo *et al.*, (2010) gave a value of 17.8 g/Kg as oral LD_{50}. EMOL can therefore be classified as relatively non-toxic.

The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolites present in plant, through additive or synergistic action of several chemical compounds acting at single or multiple target sites associated with a physiological process (Briskin, 2000). According to Kaufman *et al.*, (1999), some plant products may exert their action by resembling endogenous metabolites, ligands, hormones, signal transduction molecules, or neurotransmitters and thus have beneficial medicinal effectson human's due to similarities in their potential target sites (e.g. CNS, endocrine system, etc.). These secondary metabolites, individually or in combination, would account for the observed pharmacological effects of this plant. Preliminary phytochemical analysis reveals that ethanol extract of *Moringa oleifera* leaves contains glycosides, tannins, anthraquinones, saponins, but surprisingly no alkaloids. Alkaloids were present in the ethanol extract of leaves from Kenya (Kasolo *et al* 2011). The absence of alkaloids could be as a result of the location, season and time of collection. Tsai *et al.* (2011) reported that winter samples of *Moringa oleifera* had higher ash (except the stalk part), calcium and phenolic compounds (except the leaf part) and stronger antioxidative activity than summer samples.

In-vivo methods using intact animals are considered to be the best method for investigating the action of drugs on the CNS (Sarker *et al.*, 2007). The most important step in evaluating drug action on the CNS is to observe the behavior of the test animals.

The extract (250 – 2000 mg/Kg) produced significant ($P<0.01$) dose-dependent reduction in novelty induced behaviors. Reduction effects at 1000 and 2000 mg/Kg is comparable to diazepam (3 mg/Kg). This observation suggests that EMOL depresses the CNS. The mechanism might be by potentiating the inhibitory pathways (GABA, glycine) or inhibition of the excitatory pathwnys (noradrenaline, acetylcholine). Neuropeptides, dopamine, GABA, endorphins and acetylcholine which are implicated in rearing, grooming and locomotion in mice and small animals might be involved in the EMOL activity. The circuit containing the ventral tegmental area, nucleus accumbens and ventral pallidium is required for expression of locomotor activity elicited by amphetamine like psychostimulants and it was hypothesized as necessary for novelty induced motor activity (Hook and Kalivas, 1995).

The open field test is also used to measure anxiety. Anxiety behavior is triggered by separation of animal from its social group and agoraphobia (large arena induced) (Ambavade *et al.*, 2006). In the open field the animal is expected to show thigmotaxic behavior identified by preference for periphery and reduced ambulation. The reduced movement in the open field shows that EMOL is anxiolytic. The doses of extract used produced a non-significant decrease in the time spent in the open arm. Diazepam (1mg/Kg) produced an increase in time spent in open arm. Anxiety is represented by avoidance of open arm of animals placed in EPM. Doses of the extract increased the index of open arm avoidance. Diazepam, buspirone and other anxiolytics increase time spent in open arm (Rang and Dale, 2006). This finding suggests that the extract is anxiogenic. The mechanism of known anxiogenic agents is via serotonin (5-hydroxytryptamine, 5HT) and GABA (γ-aminobutyric acid) pathways. 5-HT_{1AC} agonists (TFMPP, mCPP), benzodiazepine receptor inverse agonists (FG 7142) and $GABA_A$ receptor antagonists (picrotoxin) and PTZ are anxiogenic. The open arm – closed arm approach for screening for anxiolytic effect has worked well in identifying the anxiolytic potential of benzodinzepine/$GABA_A$ receptor related agents while it is not reliable in detecting anti-anxiety effects through unrelated mechanisms, e.g. 5-HT_{1A} partial agonists like buspirone (Rodgers *et al.*, 1997). Four out of the receptor of 5HT, the major transmitter involved in anxiogenic/anxiolytic have been implicated in anxiety in various animal models (Lucki 1996).

Similarly, the extract significantly diminished the exploratory behavior in mice as demonstrated by the reduction of the number of head-dip. The test is a measure of

exploratory behaviour (Crawley, 1985) and it reveals sedative activity of agents (File and Pellow, 1985; Amos *et al.*, 2001). It has been established that anxiolytics increase the number of head-dips (Takeda *et al.*, 1998). The effect of the extract is therefore suggestive of anxiogenic rather than anxiolytic potentials.

Further evidence of the central depressant activity of the extract is provided by the extract's ability to potentiate pentobarbitone-induced hypnosis, an effect that may be attributed to an action on the central mechanisms involved in the regulation of sleep (Chindo *et al.*, 2003) or an inhibition of pentobarbital metabolism (Kaul and Kulkarni, 1978). It is generally accepted that the sedative effects of drugs can be evaluated by determination of pentobarbital sleeping time in laboratory animals (Lu, 1998). Fujimori, (1965) proposed that prolongation of barbital hypnosis is a good index of central nervous system depressant activity. The extract produced a significant ($P< 0.01$) decrease in sleep latency and prolonged sleep duration induced by pentobarbitone (40 mg/Kg). This is also suggestive of central depressive activity. The extract at 2000 mg/Kg protected 80% of mice from PTZ induced convulsions. There was no protection in strychnine and picrotoxin induced convulsion. Strychnine act via blockade of inhibitory glycine pathway in spinal cord and picrotoxin blocks inhibitory GABA pathway via $GABA_A$ receptor. This suggests that the anticonvulsant action of the EEMOL is mediated by the chloride channel of GABA/benzodiazepine receptor complex and not by the chloride channel of glycine receptors.

Spontaneous alternation behaviour is regarded as a measure of short-term memory in rodents (Hritcu *et al.*, 2007; Heo *et al.*, 2009). A mouse must remember at least the most recently visited arm in order to alternate the arm choice (Lee *et al.*, 2010). Some studies have used arm entries as a measure of locomotion (Ma *et al.*, 2007). Spatial memory as measured by the Y-maze tests is dependent on hippocampal learning and memory function and is related to the NMDA receptor/Ca^{2+} influx signaling pathway (Conrad *et al.*, 2003). The extract did not reduce spontaneous alternation, but diazepam 3 mg/Kg showed a slight decrease in short term memory. In Alzheimer's disease neuronal transmission is seriously compromised, and the cholinergic neurons have been lost. It is associated with memory loss. *Moringa oleifera* restored level of dopamine, noradrenaline, 5HT in rat model of Alzheimer's disease induced with colchicine (Ganguly and Guha, 2007). The result showed that *Moringa oleifera* had a protective role in LPS induced memory deficit. Several studies have shown a close connection between amyloidogenesis and neuro-inflammation induced by systemic injection of LPS, and that neuro-inflammation enhances Aβ generation which impairs memory function. Lee *et al.*, (2010) showed that systemic injections of LPS-induced memory

impairment by $A\beta_{1-42}$ generation in both the cortex and hippocampus resulting in expression of genes involved in inflammation and in amyloidogenesis. Some other studies have demonstrated the influence of LPS on Aβ deposition in AD (Gasparini *et al.*, 2004) and that anti-inflammatory agents such as Ibuprofen prevent Aβ deposition (Yan *et al.*, 2003) by decreasing cytokine production in human neuronal cells, astrocytes (Blasko *et al.*, 2001) and Tg2576 AD mice (Lim *et al.*, 2012). Compared with some other inducer of amyloidogenesis and neuroinflammation, LPS also caused higher co-expression of inflammatory proteins COX-2 and iNOS, and amyloidogenic proteins BACE and C99 in mice brains than with IFN-γ or TNF-α alone or in combination with LPS. Hauss-Wegrzyniak and Wenk (2002) showed that LPS induced extracellular deposition of beta amyloid fibrils into the hippocampus. Going by the protective role of *Moringa oleifera* in systemic LPS induced cognitive deficit, EMOL could be a potential source of compounds with anti-neuroinflammatory effect targeting amyloidogenesis.

Neuroinflammation is a phenomenon that is aimed at protecting the central nervous system (CNS) against infectious insults and injury. It has been closely linked to the pathogenesis of AD and evidence has demonstrated sustained inflammatory responses involving microglia and astrocytes in animal models of neurodegeneration. Although, most cases of neuroinflammation constitutes a beneficial process that ceases once the threat has been eliminated and homoeostasis has been restored (Spencer *et al.*, 2012), sustained neuroinflammatory processes has been suggested to be the factor that drives and contribute to the cascade of events that result in progressive neuronal damage observed in many neurodegenerative disorders like Alzheimer's and Parkinson's disease (Hirsch *et al.*, 2005; McGeer and McGeer, 2003; Wilms *et al.*, 2007; Spencer *et al.*, 2012).

Microglial cells are the primary immune cells in the CNS and their actions are similar to those of peripheral macrophages (Kreutzberg, 1996). Consequently, their primary functions are to promote host defence by destroying invading pathogens, removing deleterious debris, promoting tissue repair and facilitating tissue homoeostasis, partly through their influence on surrounding astrocytes and neurons (Glass *et al.*, 2010). However, sustained, uncontrolled activation of microglia can lead to an excess production of various neuroinflammatory factors which promote neuronal injury. These factors include PGE_2, nitric oxide, pro- inflammatory cytokines, reactive oxygen species (ROS), and glutamate (Strait and Kincaid-Colton, 1995; Gibbons and Dragunow, 2006; Wang *et al.*, 2006; Kim *et al.*, 2007). Consequently,

intervention in a microglia activation process has become a promising therapy used for the treatment of many neurodegenerative conditions (Kim *et al.*, 2007).

Natural phytochemicals have been widely proposed as treatment options for neuroinflammatory disorders especially AD. In this respect, naturally-occurring phytochemicals like curcumin, resveratrol and green tea catechins have been suggested to prevent AD because of their anti-amyloidogenic, anti-oxidative, and anti-inflammatory properties (Kim and Bae, 2010). *Moringa oleifera* has been shown to be neuroprotective (Bakre *et al.*, 2013) and also reported to exhibit anti-inflammatory activity (Singh *et al.*, 2012). A study by Adedapo *et al.* (2015) reports that methanol extract of *Moringa oleifera* (200 mg/Kg) inhibited carrageenan induced rat paw oedema.

One important finding of this study is that extract of *Moringa oleifera* reversed cognitive deficit induced by LPS. These observations seem to justify the use of the plant in treating inflammatory disorders. A major challenge in understanding the therapeutic potentials of plant extracts usually relates to lack of information on the pharmacological profile of its bioactive substances. Consequently, this study investigated the crude extract of *Moringa oleifera* and some compounds isolated from its ethanol extract by activity guided reverse phase fractionation in LPS stimulated microglia (BV-2), unstimulated microphages (RAW 264.7) and T-cell line (Jurkat cells).

Microglial cells are known to release proinflammatory cytokines such as IL-1, IFN γ, IL-6, and TNF-α, when activated (Johnston *et al.*, 2011). Activated microglia has also been reported to produce potentially neurotoxic substances like nitric oxide, oxygen radicals, and proteolytic enzymes, as well as proinflammatory cytokines (Zindler and Zipp, 2010). The effect of *Moringa oleifera* and some isolated compounds on the production of NO, ROS, TNFα, IL6, and PGE_2 in LPS stimulated microglia cells was investigated. Results indicate that concentrations of milligrams/mL of extract of *Moringa oleifera* significantly suppressed the production of these mediators and cytokines in activated microglia compared to large amount required for effects *in-vivo*. Mouse and rat microglia have been shown to be potent producers of NO and proinflammatory cytokines and mediators upon activation with LPS (Minghetti and Levi, 1998). The study demonstrated that LPS induces an increase in iNOS immuno-reactivity and NO release in microglial cells, which were inhibited by *Moringa oleifera*. LPS and other inflammatory stimuli have been reported to activate the MAPK and

NF-κB signalling pathways in microglia (Liu *et al.*, 2011). In particular, NF-κB is an important upstream regulator of cytokine, COX-2, and iNOS expressions (Pahl, 1999). Studies have also shown that blockade of NF-κB transcriptional activity in the CNS can suppress expression of iNOS, COX-2, and the proinflammatory cytokines, such as IL-1β, IL-6, and TNFα (Moon *et al.*, 2007). It is also widely known that LPS stimulation increases NF-κB activation through IκBα phosphorylation and degradation, leading to nuclear translocation of the p65 subunit. The results also confirmed that kaemferol, quercetin and rutin which are compounds isolated from *Moringa oleifera* showed regulation of transcription of NF-κB in activated microglia.

TNF-α and IL-6 are two of the main pro-inflammatory cytokines produced by activated microglia during CNS inflammation, and their excessive production has been linked to many neurodegenerative disorders, including AD (Jung *et al.*, 2009). In this study, EMOL significantly inhibited the LPS-induced release of TNF-α, but not IL-6 in BV-2cells. On their release from activated microglia, NO and PGE_2 have been implicated as critical mediators in the processes of neuroinflammation (Rock and Peterson, 2006). Furthermore, high levels of NO and PGE_2 produced by the activities of iNOS and COX-2 have been shown to be cytotoxic to neuronal cells (Strauss *et al.*, 2000; Munhoz *et al.*, 2008). The present study showed that EMOL significantly inhibited LPS-induced iNOS and COX-2 protein expressions in BV-2 cells. These results show that the inhibition of NO and PGE_2 production by EMOL is possibly due to the inhibition of iNOS and COX-2 up-regulation during microglia activation by LPS. PGE_2 is one of the most critical mediators of neuroinflammation and neuronal damage in AD. Elevated levels of PGE_2 and over expression of COX-2 have been observed in the brains of AD patients (Hoshino *et al.*, 2011). These authors further suggested that the extent of COX-2 expression correlates with the amount of Aβ and the degree of progression of AD pathogenesis. PGE_2 and COX-2 have also been shown to be major neurotoxic factors in the brain (Jung *et al.*, 2010). Studies by Ganter *et al.*, (1992) demonstrate that neuronal cells are capable of producing inflammatory and acute phase proteins. In this study, it was shown that ethanol extract of *Moringa oleifera* inhibited PGE_2 production as well as COX-2 protein expression in microglia cells stimulated with LPS probably because it contains kaempfetol and quercetin. These observations might explain the mechanisms involved in the earlier observed anti-inflammatory actions of *Moringa oleifera*.

NF-κB has been demonstrated to be one of the important intracellular signal transduction pathways leading to expression of COX-2 and iNOS in LPS-stimulated microglia cells. In its inactive form, NF-κB is bound to cytoplasmic protein IκB. On phosphorylation by IκB kinase (IKK), IκB becomes degraded followed by translocation of NF-κB to the nucleus. Once in the nucleus, NF-κB binds to specific DNA leading to activation of cellular expression of pro-inflammatory genes, including COX-2 and iNOS. This activation has been shown to be stimulated by several factors, such as lipopolysaccharide (LPS), TNF-α, interleukin-6 (IL-6) and interleukin-1β (IL-1β) (Ghosh *et al.*, 2013). The effect kaempferol, quecetin and rutin on role of this transcription factor showed in the anti-inflammatory action of *Moringa oleifera* might be via inhibition NF-κB as measured by NF-κB mediated luciferase transcription. Based on our result, it can be proposed that the antineuroinflammatory effect of *Moringa oleifera* (kaempferol and quercetin) might be throogh suppression of PGE_2 and iNOS production through inhibition of NF-κB signalling in LPS-stimulated microglia cells. This finding also seem to indicate that *Moringa oleifera* might serve as a potential template for the design of novel compounds in neurodegenerative disorders.

ROS are diverse and abundant in biological systems. While excessive ROS production clearly damages DNA, low levels of ROS affect cell signaling particularly at the level of redox modulation. The pathology of neurodegeneration is also associated with oxidative and nitrosative stress mediated by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Halliwell, 1992; Finkel and Holbrook, 2000), thus implicating the use of antioxidants as potentially beneficial strategies. ROS are known to stimulate signaling of numerous cellular pathways. Hydrogen peroxide treatment of cells causes activation of NF-kB/Rel, AP-1, and mitogen-activated kinases (Powis *et al.*, 1997). Oxidants can also simulate receptor tyrosine kinases even in the absence of ligand as well as the downstream effectors in signal transduction pathways including ras, protein kinase C, phospholipase C gamma, mitogen activated kinase, and c-jun-N-terminal kinase (Steal *et al*, 1994; Liou *et al*, 2000). EMOL lowered the H_2O_2 generated in LPS activated microglia cells. Although, the beneficial level of ROS is not certain, it is well accepted that relatively low levels of ROS promote cellular proliferation rather that cause cell degeneration or death (Finkel, 2011). This observation is in conformity with the suggestions that flavonoids and their metabolites may have neuroprotective effects (Spencer, 2010) and might be potential novel therapeutics capable of reducing the risk of degenerative brain diseases, including Alzheimer's disease.

Rutin seems to have a better immunomodulatory activity than kaempferol and quercetin as shown by the stimulation of macrophages and production of cytokines. These cytokines include inflammatory cytokines IL-6, IL-8, TNF-α and IFN-γ. Interferon-gamma (IFN-γ) is regarded as a Th1 cell cytokine which coordinates crosstalk between innate and adaptive immunity and inflammatory responses by stimulating the macrophage to increase its production of a broad range of mediators including autacoids, ROS, arachidonic acid species, and pro-inflammatory cytokines. *Moringa oleifera* derived compounds seems to be unique in their ability to enhance IFN-γ production. IFN-γ is a potent macrophage activator that also helps to activate and shape the adaptive response. It is generated by NK cells and this might explain the usefulness of *Moringa oleifera* in boosting immunity. IL-6 belong to IL-1 family of pro-inflammatory cytokines which are secreted very early in the immune response by dendritic cells and monocytes or macrophages in recognition of viral, parasitic, or bacterial antigens by innate immune receptors. The main function of IL-6 is signaling onset of B-cell differentiation to plasma cells. IL-8, also called CXCL8 is a chemoattractant with a key role of attracting neutrophils to infection site. TNF-α, like IL-6 and IL-8 are proinflammatory cytokines. Differences in intracellular signaling were observed between compounds isolated from *Moringa oleifera*. But result shows that compounds induced phosphorylation of p38, JNK1/2/3 and ERK1/2. Thus, it is clear that compounds might be exerting their biological activities through different signaling pathways, and a better understanding of these signaling events will be important in expanding knowledge of such complementary and alternative medicines.

The trypan blue exclusion assay is used to determine the amount of cells that are viable, but cannot differentiate between necrotic and apoptotic death as the cause of death. The extract killed more than 50% of Jurkat cells at concentration higher than 80 µg/mL; surprisingly the cells were able to recuperate after their doubling time of twelve hours. Though the extract cause cell death, its nutritive potential was brought to bear by promoting doubling at the concentrations higher than number of death. The higher concentration still has a total number of live cells similar to the control and lower concentrations despite the high number of dead cells. *Moringa oleifera* like some other natural products contains several numbers of chemical constituents used as hormones and secondary constituents (Ganatta *et al.*, 2012). Leaf extract of *Moringa oleifera* has been reported to contain a high number of nutrients and has been used in folkloric medicine to boost milk production in lactating mothers. The

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difference in toxicity *in-vivo* and *in-vitro* might be explained by the pharmacokinetics of the extract.

With interest in the mechanism of cell death, further experiment was performed at optimum concentrations. This is defined as concentrations showing death lower than 50%. The experiment showed that the mechanism of cell death might be necrosis. The three major morphologies of cell death include apoptosis, cell death associated with autophagy and necrosis (Krysko *et al.*, 2008). Apoptosis is an active process of self destruction associated with profound structural changes including morphological alteration, increased membrane permeability and nuclear collapse characterised by chromatin condensation and DNA fragmentation (Wyllie *et al.*, 1980). Two standard cytofluorometric methods of apoptosis quantification, each assay detecting distinct cellular alterations of the apoptotic process ie. 7AAD and annexin V. 7AAD evaluates the alteration in plasma membrane integrity and annexin V measures the translocation of phosphatidylserine from the inner to the outer layer of the plasma membrane. 7AAD staining has since been used to replace propidium iodide (PI) stain because of its ability to simultaneously identify cells in the various stages of apoptosis and death. Apart from these, it is also able to stain necrotic cells and it is not radioactive like PI. Staining with 7AAD at 15 µg/100 µl in Annexin V buffer for fifteen minutes in the dark at room temperature indicated cell death by necrosis. $7AAD^+$ cells increased in a dose-dependent manner for day 1. The preponderance of $7AAD^+$/Annexin V^+ cells cannot be overemphasized. The number of $7AAD^+$/Annexin V^+ cells increased in a dose dependent manner. Measuring of these multiple parameters (7AAD and Annexin V) permit precise quantification of apoptosis. The forward and side scatter plot easily detects cell shrinkages (Petit *et al.*, 1995). This is reflected in the decrease in average number of cells in the live gate across concentration. The death might be as a result of progressive loss of membrane permeability and flipping out or translocation of phosphatidylserine (Van Engeland *et al.*, 1996). In the absence of phagocytosis, apoptotic cells proceed to a stage of secondary necrosis, which shares many features with primary necrosis (Krysko *et al.*, 2008). The ability of *Moringa oleifera* to increase $7AAD^+$/Annexin V^+ cells might be an intrinsic pro- apoptotic property which could be beneficial in neoplastic diseases. Antiproliferative activity of *Moringa oleifera* leaves might be related to its intrinsic apoptotic property which has been shown to cause emerging of apoptotic bodies, chromatin condensation, cell shrinkage, DNA fragmentation and induce generation of ROS in carcinoma KB cells.

Antiproliferative effect of the extract was measured using CFSE. Although the assay has its challenge, the protocol was strictly followed to rule out interference of concentration of

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Antiproliferative effect of the extract was measured using CFSE. Although the assay has its challenge, the protocol was strictly followed to rule out interference of concentration of

cells/CFSE and duration of labeling on toxicity by CFSE. The number of cells dividing reduced in group treated with the extract. Though these seem very small compared to the proliferating cells, but this can be attributed to the intrinsic ability of Jurkat cells to proliferation. Cell proliferation is controlled by growth factors which activates transcription factors that binds DNA to either turn on or turn off production of proteins which results in cell division. This pathology is very important in neoplastic diseases and the ability of *Moringa oleifera* to negatively regulate this mechanism might probably portray some kind of futuristic purpose for which it could be pursued.

Moringa oleifera might probably contain some immunomodulatory compounds which interfere with CD3 and CD28 prestimulated Jurkats. T cells requires TCR signaling and a co-receptor stimulation for activation. A third signaling which is also important in determining the outcome of the activation is provided by stimulation of any of the over 200 accessory molecules expressed on T cells. These accessory molecules are receptors for diverse molecules ranging from cytokines to adhesion molecules and chemokines etc. The *Moringa oleifera* leaves might contain compounds which might bind to some of the accessory molecule, resulting in a more regulated programmed cell death. The pre-stimulated cells died more by apoptosis when not treated with the extract. This death might be as a result of anergy, because treated cells show lesser death by apoptosis. Pre-stimulated with CD3 and CD28 turned more cells into early apoptosis than CD3 and CD54. The T cell phenotype, a naive T cell differentiates to depend on the type of stimulus it receives. CD3 and CD 54 form part of central supramolecular activating complex (cSMAC) and peripheral SMAC (pSMAC) respectively. CD54 is an adhesion molecule expressed on APCs for LFA-1 found on T cells. It is being implicated in helping to sustain the signal generated by allowing long term cell interactions. CD28 is a costimulatory receptor. It is expressed by T cell and its ligand is naturally CD80/86 expressed on APC like CD54. But unlike CD54, CD80/86 does not serve as a mere adhesion molecule but as a ligand for a costimulatory receptor. It provides the second signal required for activation. The positive modulation of apoptosis by *Moringa oleifera* in CD3 and CD28 might point to some of the benefits in neoplasmic conditions and immunity.

CCR7 is an important surface marker broadly used to distinguish dedicated memory T cells. Along with CD44 and CD62L which indicate TCR stimulation and tendency for a T cell to take residency in secondary lymphoid organs, CCR7 expression is used to characterize types of memory T cells. The relative increase in the expression of CCR7 by CD3 and CD28 stimulated T cells in comparison with the CD3 and CD54 stimulated cells might be indicative

of *Moringa oleifera* ability to boost immunity. Nominal use of the extract has been reported in TAM as immunity boosting. This might be explained by its ability to increase CCR7 expression making an already tending to memory T cell take residency in the secondary lymphoid organ as a central memeory T cell (T_{CM}). Effector memory T cells (T_{EM}) like T_{CM} expresses CD62L and CD44 but does not express CCR7.

CHAPTER SIX

6.1 SUMMARY AND CONCLUSION

Results obtained in this study suggest that the ethanol extract of *Moringa oleifera* leaf possessed a dose-dependent effect on rearing, grooming and locomotion. This is a CNS depressive effect which is possibly mediated via positive modulation of GABA. Also the finding of this study showed that the extract has sedative, anticonvulsive and anxiogenic activity. The anti-convulsant activity of the extract can be concluded to be via its neuroprotective ability. Our data have shown that ethanol of extract *Moringa oleifera* leaf has an anti-neuroinflammatory property related to inhibition of inflammation associated by NO, PGE and TNF-α production. Also the anti-neuroinflammatory principles are abundant in the polar fraction of the leaves of ethanol extract of *Moringa oleifera* which resulted in isolation of isoquercetin, kaempferol and rutin. Isoquercetin and kaempferol possessed very good antineuroinflammatory effect mediated through NF-κB, while rutin proved to be more potent in protecting against peripheral immunity. On the overall, *Moringa oleifera* possessed a beneficial immunomodulatory activity.

Contributions to Knowledge

- Leaf extract *Moringa oleifera* is scientifically demonstrated to have central effects.
- Leaf extract of *Moringa oleifera* is endowed with anti-Alzheimer's property.
- The leaf extract of *Moringa oleifera* increase expression of CCR7 which provides the basis for its use in immunity boosting

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