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ROLE OF NEROLIDOL, AN ALIPHATIC SESQUITERPENE, IN COLON INFLAMMATION

Vishnu Raj

United Arab Emirates University

College of Medicine and Health Sciences

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This dissertation is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Under the Supervision of Dr. Sandeep Subramanya

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Declaration of Original Work

I, Vishnu Raj, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this dissertation entitled "*Role of Nerolidol, an Aliphatic Sesquiterpene, in Colon Inflammation*", hereby, solemnly declare that this dissertation is my own original research work that has been done and prepared by me under the supervision of Dr. Sandeep Subramanya, in the College of Medicine and Health Sciences at UAEU. This work has not previously formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my dissertation have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this dissertation.

Student's Signature:  Date: 25-04-2021

Advisory Committee

1) Advisor: Dr. Sandeep B. Subramanya

Title: Associate Professor

Department of Physiology

College of Medicine and Health Sciences

2) Co-advisor: Dr. Shreesh K. Ojha

Title: Associate Professor

Department of Pharmacology

College of Medicine and Health Sciences

3) Member: Prof. Ernest A. Adeghate

Title: Professor and Chair

Department of Anatomy

College of Medicine and Health Sciences

4) Member: Prof. Basel Ramadi

Title: Professor and Chair

Department of Medical Microbiology

College of Medicine and Health Sciences

Approval of the Doctorate Dissertation

This Doctorate Dissertation is approved by the following Examining Committee Members:

1) Advisor (Committee Chair): Dr. Sandeep B. Subramanya
   Title: Associate Professor
   Department of Physiology
   College of Medicine and Health Sciences

   Signature _____ Date 25-04-2021

2) Member: Prof. Milos Ljubisavljevic
   Title: Professor and Chair
   Department of Physiology
   College of Medicine and Health Sciences

   Signature _____ Date 27-04-2021

3) Member: Shreesh K. Ojha
   Title: Associate Professor
   Department of Pharmacology
   College of Medicine and Health Sciences

   Signature _____ Date 25-04-2021

4) Member: Pradeep Dudeja
   Title: Professor
   Department of Physiology & Director, Divisional Scholarly Activities Division of Gastroenterology & Hepatology, Dept. of Medicine, UIC Institution: University of Illinois, Chicago, IL, USA

   Signature _____ Date 26-04-2021

This Doctorate Dissertation is accepted by:

Acting Dean of the College of the College of Medicine and Health Sciences: Professor Juma Al Kaabi

Signature  Date 27 June 2021

Dean of the College of Graduate Studies: Professor Ali Al-Marzouqi

Signature  Date 27 June 2021

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Abstract

Inflammatory bowel diseases, which comprise Crohn's disease (CD) and ulcerative colitis (UC), are chronic and progressive immune-mediated inflammatory conditions of the gastrointestinal (GI) tract. Both genetic and environmental factors influence this condition. Conventional therapy to suppress aberrant immune responses seen in IBD with corticosteroid or with biological agents has a high relapse rate that limits their use. Therefore, approximately 40% of IBD patients switch to alternative therapies that include dietary supplements rich in phytochemicals. Nerolidol (NED) is a naturally occurring sesquiterpene alcohol present in various plants and has potent anti-inflammatory properties. Therefore, in the current study, the role of NED in preclinical models of colon inflammation were investigated. Initial experiments were carried out to investigate the anti-inflammatory properties of NED in lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophage cells. NED significantly decreased proinflammatory cytokine such as TNF-α, IL-1β, and IL-6 both at protein and mRNA level in LPS-stimulated RAW macrophages. In addition, NED also significantly decreased expression of proinflammatory mediators such as COX-2 and iNOS. Based on these results NED was further investigated as a putative anti-inflammatory compound in *in vivo* and *in vitro* models of colon inflammation. C57BL/6J male black mice administered with 3% dextran sodium sulfate (DSS) used as *in vivo* model of colon inflammation. DSS-induced colitis groups received either vehicle or NED (50, 100, and 150 mg/kg body weight/per day) by oral gavage. NED significantly decreased the DAI , colon length and preserved microscopic architecture of the colon to near control level. NED significantly decreased tissue MPO concentrations, CXCL-2, CCL2 mRNA expression and proinflammatory cytokines (IL-1β, IL-6, and TNF-α) and mediators (COX-2 and iNOS) level both at the protein and at mRNA level in DSS administered mice. NED promoted Nrf-2 nuclear translocation and increased antioxidant enzyme (SOD and CAT) activity, HO-1 and SOD-3 mRNA levels. *In vitro* model of colon inflammation was established by challenging HT-29 cells with TNF-α (1ng/ml concentration) and treated with NED (25μM and 50μM). NED treatment significantly decreased proinflammatory chemokines (CXCL-1, IL-8, CCL2, and COX-2) mRNA levels.

NED significantly decreased phosphorylation of MAPK (p38, JNK, and ERK$^{1/2}$) and NF-κB in the DSS-induced colitis and in LPS-stimulated RAW macrophages.

Intestinal epithelial barrier dysfunction leading to enhanced intestine permeability is associated with IBD pathogenesis. Therefore, the effect of NED on intestine tight junction integrity using DSS-induced colitis and in LPS challenged Caco-2 monolayers was investigated. NED significantly decreased FITC-dextran permeability in DSS group and decreased transepithelial electrical resistance (TEER) in LPS-treated Caco-2 monolayers. NED significantly increased expression of tight junction proteins such as claudin-1, -3, -7, and occludin in *in vitro* and *in vivo* models. Collectively, results from the present study indicates that NED supplementation attenuates colon inflammation through its potent antioxidant and anti-inflammatory activity and enhances intestine tight junction integrity.

Keywords: Nerolidol, DSS colitis, Nrf-2/Keap-1, MAPK, NF-κB signalling, Caco-2 cells, HT-29 cells, FITC-Dextran permeability, Transepithelial electrical resistance (TEER), Intestine epithelial tight junction.

Title and Abstract (in Arabic)

دور نيروليدول ، سيسكيتيربين أليفاتي ، في التهاب القولون

الملخص

أمراض الأمعاء الالتهابية ، والتي تشمل مرض كرون ومرض التهاب القولون التقرحي ، تعد أمراض التهابية مزمنة ومستفحلة تصيب الجهاز الهضمي. تلعب العوامل الوراثية والبيئية دورا في الإصابة بهذه الأمراض. الكورتيكوستيرويد أو العوامل البيولوجية المستخدمة في العلاج التقليدي لقمع المناعة الشاذة في مرض التهاب الأمعاء تعد ذات انتكاس مرتفع ،مما يؤدي للحد من استخدامها. يلجأ ما يقرب من 40٪ من مرضى داء الأمعاء الالتهابي إلى العلاجات البديلة والتي تشمل المكملات الغذائية الغنية بالمواد الكيميائية النباتية.

نيروليدول عبارة عن كحول سيسكيتيربين طبيعي موجود في نباتات مختلفة ذات خصائص قوية مضادة للالتهاب. في الدراسة الحالية ، قمنا بتحري دور نيروليدول في النماذج قبل السريرية لالتهاب القولون. في تجاربنا المبدئية قمنا بدراسة الخصائص المضادة للالتهابات لـنيروليدول في خلايا البلاعم المحفزة عديدات السكاريد الدهنية (LPS) باستخدام فئران (RAW 264.7) . ثبط نيروليدول عديدات السكاريد الدهنية التي تفعلها مواد تنتج في مواقع الالتهاب مثل TNF-α و IL-1β و IL-6 . وذلك عن طريق تحفيز انتاج حمض الريبونوكلييك الخاص بها. بالإضافة إلى ذلك ، منع نيروليدول الزيادة في التعبير عن الوسائط المؤيدة للالتهابات مثل COX-2 و iNOS مما يشير إلى خصائصه المحتملة كمضاد للالتهابات. وهذا الدور تم دراسته مخبريا في نماذج مخبرية معروفة لمرض التهاب القولون في الفئران والمخبر. تم إنشاء نموذج التهاب القولون باستخدام الفئران السوداء الذكور (C57BL / 6J) C57BL / 6J التي يتم اطعامها 3 ٪ كبريتات الصوديوم ديكستران (DSS) في مياه الشرب لمدة 7 أيام للحث على التهاب القولون بها. خلال التجربة العلمية تلقت مجموعات التهاب القولون مركب نيروليدول بجرعات متفارتة (50 ، 100 ، و 150 ملغم / كغم من وزن الجسم / يوميًا بالتزقيم). تم قياس طول القولون ، مؤشر نشاط المرض (DAI) ، أنسجة القولون ، والعديد من المعلمات البيوكيميائية. تم إنشاء نموذج التهاب في المختبر عن طريق تحفيز خلايا HT-29 (خلية سرطان القولون والمستقيم البشرية) مع TNF-α (تركيز 1 نانوقرام/ملم 1) باستخدام تركيزات مختلفة من نيروليدول (25 ميكرومتر و 50 ميكرومتر) في التجارب المخبرية. ونتج عنها تقليل كبير

في حدة الالتهاب بالأنسجة المقيمة مجهريا وقلل من التقلص في طول القولون. ونتج عن استخدام نيروليدول تغير في التركيز الخلوي لمادة MPO بالأنسجة ، وانخفاض بحمض الريبونوكلييك لمادتي CXCL-2 و CCL2 الذي تفرزة خلايا

العدلات والبلاعم بالإضافة لانخفاض ملحوظ لمواد المؤيدة للالتهاب و منها IL-1β و IL-6 و TNF-α على مستوى انتاج حمض الريبونوكلييك والبروتين الخاص بها.

ارتفاع في انتاج الوسطاء المؤيدين للالتهابات ومنهم COX-2 و iNOS ونترات الأنسجة الناجم عن استخدام مركب DSS تم حجبه عند استخدام نيروليدول.

روّج نيروليدول لجرعة الإزاحة النووية لـ Nrf-2 بالاعتماد على الجرعة. كمت زاد بشكل كبير من نشاط إنزيم مضادات الأكسدة (SOD و CAT) ومستويات HO-1 و SOD-3 mRNA. أدى علاج نيروليدول في خلايا HT-29 التي تواجه TNF-α إلى انخفاض كبير في مستويات المواد المؤيدة للالتهابات (CXCL-1 و IL-8 و CCL2 و COX-2) على مستوى حمض الريبونوكلييك .

كينازات البروتين المنشط بالميتوجين (MAPKs) والعامل النووي كابا ب (NF-κB) هي مسارات إشارات مهمة تشارك في الالتهاب. لذلك ، قمنا بالتحقيق من تأثير نيروليدول على آلية إشارات MAPK و NF-κB. تشير نتائجنا إلى أن فسفرة MAPK (p38 و JNK و $ERK^{1/2}$) و NF-κB قد زادت بشكل كبير في نموذج التهاب القولون الناجم عن استخدام DSS ، وحفز LPS في خلايا البلاعات من نوع RAW. أدى استخدام نيروليدول كعلاج في نموذج الالتهاب هذا إلى تثبيط الفسفرة بشكل كبير لبروتينات إشارات MAPK و NF-κB في كل من نموذج الفئران وفي غلايا البلاعات من نوع RAW المحفزة بـ LPS. تشير هذه النتائج إلى أن عمل نيروليدول المضاد للالتهابات يتم بوساطة تثبيط آلية إشارات MAPK / NF-κB.

يؤدي ضعف الحاجز الظهاري المعوي إلى تعزيز نفاذية الأمعاء في مرض التهاب الأمعاء. لذلك ، قمنا بالتحقيق في تأثير نيروليدول على ترابط الخلايا الخلوي بالأمعاء في التهاب القولون باستخدام نموذج التهاب القولون الناجم عن استخدام مادة DSS (في نموذج الجسم الحي) و LPS الذي يحفز نموذج المختبر Caco-2 monolayers. قلل نيروليدول بشكل كبير من تركيز FITC-dextran في مصل الفئران مقارنة بالمجموعة التي تم استخدام DSS فيها فقط ، مما يشير إلى تقليل نفاذية القولون. أظهرت نتائجنا أيضًا أن LPS تسبب في حدوث انخفاض في

المقاومة الكهربائية بطريق الظهارة (TEER) في Caco-2 monolayers والذي تم منعه بشكل كبير من خلال العلاج باستخدام نيروليدول. لوحظ أن نيروليدول يحمي سلامة الحاجز المعوي من خلال تعزيز التعبير عن بروتينات الوصلة الضيقة مثل كلودين 1 ، 3 ، 7 ، وإكلودين في النماذج المختبرية والحيوية لالتهاب القولون. توضح نتائجنا بشكل جماعي أن مكملات نيروليدول تخفف التهاب القولون من خلال نشاطها الفعال كمضاد للأكسدة ومضاد للالتهابات في كل من نماذج التهاب القولون في الجسم الحي وفي المختبر. عزز نيروليدول أيضًا سلامة تقاطع الأمعاء الضيقة لمنع اختلال وظيفة الحاجز الملحوظ في التهاب القولون.

مفاهيم البحث الرئيسية: كلمات البحث الرئيسية: نيروليدول, التهاب القولون (DSS), الجرعة (Nrf-2), إشارات (NF-κB / MAPK), خلايا Caco-2,خلايا HT-29, الإزاحة النووية
, المقاومة الكهربائية بطريق الظهارة TEER, تقاطع الأمعاء الضيقة, الاكلودين, و الكلودين. نفاذية FITC-dextran.

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Dedication

To my beloved parents and family

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List of Abbreviations

ASA	Aminosalicylic Acid
CAM	Complementary and Alternative Medicine
CAT	Catalase
CD	Crohn's Disease
COX	Cyclooxygenase
CVD	Cardiovascular Diseases
DAI	Disease Activity Index
DSS	Dextran Sodium Sulphate
EGCG	Epigallocatechin Gallate
ERK	Extracellular Signal Related Kinases
FBS	Fetal bovine serum
FD-4	FITC-Dextran
FITC	Fluorescein Isothiocyanate Conjugated
GI	Gastrointestinal
GSH	Glutathione
GPx	Glutathione Peroxidase
H_2O_2	Hydrogen Peroxide
IBD	Inflammatory Bowel Diseases
IEC	Intestinal Epithelial Cells
IFN-γ	Interferon Gamma
IL	Interleukin
iNOS	Inducible Nitric Oxide Synthase
JAMA	Junction Adhesion Molecule

JNK	c-Jun N-terminal Kinases
LPS	Lipopolysaccharides
MAPK	Mitogen Activated Protein Kinases
MDA	Malondialdehyde
MLC	Myosin Light Chain
MLCK	Myosin Light Chain Kinases
MPO	Myeloperoxidases
NED	Nerolidol
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
Nrf-2	Nuclear Factor Erythroid 2-Related Factor 2
PBS	Phosphate Buffered Saline
PRR	Pattern Recognition Receptor
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SAZ	Sulfasalazine
SCID	Severe Combined Immunodeficiency
SOD	Superoxide Dismutase
STAT	Signal Transducers and Activators of Transcription
TEER	Transepithelial Electrical Resistance
Th	T Helper Cells
TJ	Tight Junction
TLR	Toll-Like Receptor
TNBS	Trinitrobenzene Sulfonic Acid

TNF-α	Tumor Necrosis Factor - Alpha
UC	Ulcerative Colitis
ZO	Zonula Occludins

Chapter 1: Introduction

1.1 Overview

Inflammatory bowel disease (IBD) is a broader term, which defines all general inflammatory conditions of gastrointestinal (GI) tract. Based on the location of GI tract inflammation, IBDs can be classified broadly into Crohn's disease (CD) - inflammation can occur anywhere in the GI tract, and ulcerative colitis (UC) - inflammation mostly confined only to the colon and rectum (Kaser, Zeissig, & Blumberg, 2010; Pervin et al., 2016). Recent data signifies that approximately 40% of IBD patients consume/prefer complementary medicines and alternative therapies, with a hope of relieving associated morbidities (Opheim, Bernklev, Fagermoen, Cvancarova, & Moum, 2012; Rosillo et al., 2012). Epidemiological data indicated a steady rise in the incidence/prevalence of IBD in the Middle Eastern region in recent decades (Molodecky et al., 2012). These increased incidences have been attributed to preferred consumption of westernized diet, antibiotics abuse, and the associated development of resistance resulted in gut microbiota alterations (Prideaux, Kamm, De Cruz, Chan, & Ng, 2012). The entire mechanisms involved in IBD pathogenesis are not completely understood. However, an interplay between environmental factors and genetic susceptibility together with a dysregulated immune response are reported to be the major causes behind the onset of this inflammatory condition (Abraham & Cho, 2009). IBD is associated with an impaired intestinal barrier due to compromised epithelial barrier integrity (Gecse et al., 2012). Inflammation is manifested as lesions and associated immune responses along with elevated oxidative stress in the mucosa (Barbosa et al., 2003; Kruidenier, Kuiper, Lamers, & Verspaget, 2003; Monk et al., 2015; Xavier & Podolsky, 2007). Macrophages are activated due to the entry of

luminal microbes into the lamina propria and are associated with enhanced production of proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), interleukin (IL)-1, IL-6, and IL-12 (de Souza & Fiocchi, 2016; Shepherd et al., 2018).

Current pharmacological therapies available for UC or CD treatment offer no complete cure. The treatment goal has been to induce and maintain remission with the primary objective of preventing long-term complications, which in UC is colorectal cancer (Ekbom, Helmick, Zack, & Adami, 1990a, 1990b). There are differences in the clinical features and symptoms of UC and CD. Despite these differences, the current pharmacological options of these conditions overlap.

The first line of therapy for IBD is 5-aminosalicylic acid (5-ASA) and its derivatives for patients with mild to moderate conditions. These compounds act by regulating the inhibition of cyclooxygenase (COX) and lipoxygenase (LOX) pathways. In acute or severe inflammation, steroid therapy (prednisolone or budesonide) is more preferred than 5-ASA drugs. Steroids are given either orally or as intravenous injection, depending on IBD severity. In severe inflammatory conditions, continuous monitoring of the patient's inflammatory response is recorded either by endoscopy of mucosa or by estimating markers such as C-reactive protein. More potent immunomodulatory drugs like azathioprine or 6-mercaptopurine are recommended to treat this condition (Dignass et al., 2012).

If patients are unresponsive to conventional therapies, monoclonal antibodies that can inhibit the proinflammatory cytokine TNF-α is of great significance. Anti-TNF-α drugs like infliximab and adalimumab have already been reported to be potent in inducing and maintaining remission in moderate UC cases. Severe forms of IBD

have shown to respond to these drugs (Jarnerot et al., 2005; Miyagi, 1979; Rutgeerts et al., 2005; Sandborn et al., 2014). Vedolizumab (an approved drug for UC treatment currently) is a humanized monoclonal antibody raised against α4β7 integrin, a glycoprotein expressed on the surface of circulating B- and T-lymphocytes at variable extends. These glycoproteins interact with the intestinal mucosal cell adhesion molecule (Colombel et al., 2017; Mora & Von Andrian, 2006). There are many newer drugs in the clinical trials stage or recently completed trials designed to target the other inflammatory response components. Even after recent advances in IBD pharma therapeutics, statistics indicated a 30-40% lifetime surgery risk involved among UC patients (Hancock, Windsor, & Mortensen, 2006).

The research focused on novel IBD treatment approaches have made substantial progress in recent years. However, reports documented that 25-30% of IBD patients did not respond to biological therapies like anti-TNF-α antibodies (Hazel & O'Connor, 2020). Moreover, these drugs are very expensive; and associated with adverse effects over prolonged use and high relapse rate (Braus & Elliott, 2009; de Souza & Fiocchi, 2016). Hence, there is an unfulfilled task in developing potentially effective drugs or combinations of drugs with the best safety profile for IBD patients. Presently, many compounds are at different phases of clinical trials, including anti-adhesion agents like etrolizumab and abrilumab, JAK inhibitors such as tofacitinib, and anti-trafficking molecules require an extended period to assess their safety profile (Weisshof, El Jurdi, Zmeter, & Rubin, 2018).

Drug development focus is always towards formulating synthetic (therapeutic) agents using high throughput screening (HTS) platforms for drug discovery. However, the achieved outcomes are not up to the level of expectation, as noticeable from the

declined rate of emergence of new drugs (Scannell, Blanckley, Boldon, & Warrington, 2012). Therefore, the research focus is gaining momentum towards identifying active compounds from medicinal plants and its phytochemicals. For centuries, medicinal plants have been explored for many pharmacologically active agents, and still, a large number of currently used medications are plant-derived products or their derivatives (Kinghorn, Pan, Fletcher, & Chai, 2011; Newman & Cragg, 2012).

Recent scientific studies indicate that phytochemicals based interventions are promising, because of its enhanced tolerability and improved therapeutic efficiency among IBD patients (Bertomoro et al., 2010; Oxelmark et al., 2016; Zezos & Nguyen, 2017).

1.2 Importance of dietary phytochemicals in health and diseases

Phytochemicals are non-nutrient bioactive secondary metabolites of plants (Zhang et al., 2015). Review of literature documented that more than 10,000 different phytochemicals have been identified so far from fruits, vegetables, grains, and other plant parts. These phytochemicals comprise tannins, flavones, triterpenoids, steroids, saponins, alkaloids, *etc*. (Barbosa et al., 2013). Excess production of oxidants is linked with the pathogenesis of many diseases, including inflammatory disorders, cardiovascular disease, cancer, and aging (Poulose, Miller, & Shukitt-Hale, 2014; Singh, Suman, & Shukla, 2014). The antioxidant properties of dietary phytochemicals are well documented (Wang, Chen, Xie, Ju, & Liu, 2013; Zhang et al., 2015) and explain the mechanisms mediated through these phytochemicals in mitigating morbidity associated with chronic diseases (Kruk, 2014; Kyro et al., 2013; Mursu, Virtanen, Tuomainen, Nurmi, & Voutilainen, 2014; Soobrattee, Neergheen, Luximon-Ramma, Aruoma, & Bahorun, 2005; Yamada et al., 2011). Human Studies have also

shown that consumption of phytochemical-rich food significantly increased the level of antioxidants in the body and provide better protection against chronic disease conditions (Cao, Russell, Lischner, & Prior, 1998; Murakami, 2013). Besides antioxidant properties, the anti-inflammatory and immunomodulatory properties of dietary phytochemicals are also implicated in protective effect against various pathological conditions (Aggarwal & Harikumar, 2009; Bak, Truong, Kang, Jun, & Jeong, 2013; Chen et al., 2012; Corbi et al., 2016; Garcia-Lafuente, Guillamon, Villares, Rostagno, & Martinez, 2009).

Cardiovascular diseases (CVD) are considered one of the most common causes of death in many developed countries, and epidemiological data have shown that a decreased mortality rate in the US and the UK can be correlated to high intake of flavonoids with diet (McCullough et al., 2012; Peterson, Dwyer, Jacques, & McCullough, 2012; Reuland, McCord, & Hamilton, 2013). Increased levels of oxidants associated with CVD are responsible for endothelial cell damage and poor vasodilator effects. It was reported that flavonoids obtained from *Flos chrysanthemi* exerted protective effects on arterial endothelial cells against oxidative stress in an *in vitro* model (He et al., 2012). These effects were comparable with another *in vitro* study which reported the athero-protective effect of flavonoids from *Euterpe oleracea* pulp (Costa, Garcia-Diaz, Jimenez, & Silva, 2013). Phytochemicals consumption against other chronic diseases are also well documented. The anti-diabetic activity of curcumin is mainly attributed to its anti-inflammatory and antioxidant potential (Meng, Li, & Cao, 2013). Resveratrol, a polyphenolic compound, showed protective effects against type-2 diabetes mellitus by enhancing insulin expression in pancreatic cells (Xie et al., 2013). Grapes are rich in polyphenols and reported to possess anti-inflammatory and antioxidant properties, making them effective against chronic

inflammation by reducing proinflammatory cytokine levels (Chuang & McIntosh, 2011). High tomato products in diet were reported to exert protective effects on endothelial function of CVD patients. Lycopene, a phytochemical present in tomato was attributed to its beneficial effects (Gajendragadkar et al., 2014; Weberling, Bohm, & Frohlich, 2011). Epidemiological data have shown that whole grain food has a favorable impact on metabolic homeostasis and delayed the development of type-2 diabetes (Belobrajdic & Bird, 2013).

Consumption of alternative medicines, changing lifestyle, and a well-balanced diet play a key role in cancer prevention. In both *in vitro* and *in vivo* models, fruits and vegetables rich in phytochemicals have prevented the development and progression of cancers effectively (Mittal, Elmets, & Katiyar, 2003; Sak, 2014; Sreelatha, Dinesh, & Uma, 2012). Free radicals play a major role in all stages of cancer development by causing DNA damage. Evidences suggest that the anti-inflammatory and antioxidant properties of many phytochemicals like green tea polyphenols, silymarin from milk thistle, and proanthocyanidins from grape seeds are responsible for their anti-carcinogenic properties in various types of cancer (Aziz, Kumar, & Ahmad, 2003; Jang et al., 1997; Katiyar, Korman, Mukhtar, & Agarwal, 1997; Mittal, Elmets, et al., 2003; Mittal, Piyathilake, Hara, & Katiyar, 2003; Nichols & Katiyar, 2010; Wang et al., 1992). Curcumin alone or in combination with catechin have exhibited significant anti-cancer properties in *in vitro* experiments (Li & Zhang, 2014; Manikandan et al., 2012). The neuroprotective effects of phytochemicals are also extensively studied. Role of dietary phytochemicals in protection against brain injury via immunomodulatory and anti-inflammatory properties have been explained by Corbi et al. in 2016 (Corbi et al., 2016). Curcumin, catechin, and resveratrol have shown significant protective effects against Alzheimer's disease (AD) (Davinelli et al., 2012). A report of population-based

study in Europe has indicated a correlation between flavonoid intake and neuroprotective effects in Alzheimer's and its related dementias (Beking & Vieira, 2010). These reports explain that phytochemicals offer various health benefits because of their antioxidant, anti-inflammatory, and anti-cancer properties.

1.3 Phytochemicals and IBD

IBDis a chronic inflammatory condition characterized by dysregulated immune response and defective intestinal epithelial barrier system. Food habits play a major role in the development and progression of IBD, and a deficiency of antioxidant dietary components has already been reported in UC patients (Sung & Park, 2013). Detailed literature searches have revealed that most IBD experiments performed on cell lines and animal models evaluated the inflammatory and intestinal permeability markers and uncovered that a significant amount of herbal-based bioactive molecules like terpenes, lignans, and other polyphenolic compounds hold a promising future in IBD treatment (Hossen et al., 2020). These phytochemicals demonstrated their anti-inflammatory properties by regulating NF-κB transcription factor mediated responses (Calixto, Campos, Otuki, & Santos, 2004). Herbal extracts rich in polyphenols like catechin, epicatechin, procyanidin B2 and procyanidin B1 have shown a significant reduction in colon damage *via* anti-inflammatory action mediated through inhibition of NF-κB phosphorylation in both *in vitro* models and *in vivo* models of IBD/inflammation (Andujar et al., 2011; Chiou et al., 2012; Paiotti et al., 2013; Yoshioka et al., 2008; Zhang et al., 2015). Further, dietary grape seed extract rich in proanthocyanidins (PAC) was reported to protect the intestinal barrier integrity by enhanced tight junction protein occludin expression in rats (Goodrich et al., 2012; Song et al., 2011). Reports have also linked polyphenol-rich green tea with a lower

incidence of colorectal cancer (Butt & Sultan, 2009; Murakami, 2014).

Previous reports on phytochemical research were focused mainly on its antioxidant properties. However, recently anti-inflammatory properties of phytochemicals exerted its action via the inhibition of NFκB signaling pathway and subsequently suppressed proinflammatory cytokines production (Boesch-Saadatmandi et al., 2011; Byun et al., 2013; Kao et al., 2009; Terra et al., 2007).

A few phytochemicals have entered the clinical trials with promising results among IBD patients. The beneficial effects of phytochemical rich diet in pediatric patients with IBD have already been well established (Beattie, Bentsen, & MacDonald, 1998; Escher & Taminiau, 2001). Curcumin is one of the most extensively studied phytochemical compound reported to be effective and safe among UC patients in a double-blind placebo controlled multicentric trials (Hanai et al., 2006; Singla et al., 2014). Curcumin and combination with mesalamine were reported as safe and effective in patients with mild-to-moderate ulcerative colitis conditions (Lang et al., 2015). It was also reported that polyphenol-rich diet successfully maintained remission in patients with CD for over two years (Chiba et al., 2010). Pycnogenol, a polyphenolic extract from maritime pine rich with procyanidins, was reported to induce a beneficial effect among CD patients (Kolacek et al., 2013). Catechins, another group of phytochemicals, are gaining importance in IBD research. Polyphenol E is present in green tea that contains 65% EGCG and 22% of other catechins, was shown to increase the rate of remission in mild to moderate UC patients with good safety profile (Dryden, Lam, Beatty, Qazzaz, & McClain, 2013). Moreover, polyphenol rich red wine consumption improved beneficial gut flora in human subjects (Clemente-Postigo et al., 2013). These reports indicate that dietary phytochemicals can offer a promising

alternative therapy in mitigating inflammation and improves IBD-associated clinical symptoms.

1.4 Oxidative stress and IBD

Recent decades of research have helped us to understand the physiological and pathological mechanisms that led to IBD pathogenesis to some extent. IBD is primarily an immunological condition also associated with the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). The elevated production of ROS/RNS is being contributed by the activated immune cells such as neutrophils and monocytes in the lamina propria (Babbs, 1992). An upregulation of ROS/RNS was documented in IBD patients and in experimental colitis (Arab, Al-Shorbagy, Abdallah, & Nassar, 2014; Borrelli et al., 2013; Ioannidis, Varnalidis, Paraskevas, & Botsios, 2011; Pravda, 2005; Sengul, Isik, Aslim, Ucar, & Demirbag, 2011). These ROS and RNS play a significant role in the transcriptional regulation of genes involved in the GI tract's innate and adaptive immune responses (Balmus, Ciobica, Trifan, & Stanciu, 2016; Pravda, 2005). ROS includes superoxide ($O2\cdot^{-}$), hydroxyl radicals ($HO\cdot$), peroxyl ($RO_2\cdot$), alcoxyl ($RO\cdot$), hydroperoxyl ($HO_2\cdot$) hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). RNS comprises nitric oxide ($\cdot NO$), nitrogen dioxide ($\cdot NO2$), peroxynitrite ($ONOO^{-}$), and dinitrogen trioxide (N_2O_3). ROS/RNS upregulation lead to significant intracellular damage, including damages to nucleic acids (Bedard & Krause, 2007; Tian, Wang, & Zhang, 2017). Further, when oxidative stress-induced DNA damage occurs in mitochondria, it predisposes into colorectal cancer progression from chronic ulcerative colitis (Nishikawa et al., 2005; Novak & Mollen, 2015). Proinflammatory mediators involved in the pathogenesis of UC and CD, such as myeloperoxidases (MPO), nitric oxide

synthase (NOS) and cyclooxygenases (COX) aid excessive ROS production (Kulkarni, Kuppusamy, & Parinandi, 2007; Swindle & Metcalfe, 2007).

In gut inflammation, intestinal epithelial cells (IEC), neutrophils, and macrophages activate inducible nitric oxide synthase (iNOS) via the proinflammatory cytokines release and uncontrolled ROS/RNS generation. Excess production of these factors can lead to tight junction protein rearrangement or destruction resulting in the disruption of intestinal epithelial barrier, thereby instigating the development and progression of IBD (Andersen, 2004; Banan, Choudhary, Zhang, Fields, & Keshavarzian, 1999; Rao, Baker, & Baker, 1999). Uncontrolled ROS production and receded antioxidant enzyme activity are reported to be significant risk factors of IBD. An efficient host antioxidant defense mechanism plays a major role in scavenging free radicals intracellularly. Various intracellular enzymatic antioxidants contribute to body's defense mechanism. These antioxidant system includes superoxide dismutase (SODs), glutathione peroxidase (GPX), and catalase (CAT) (Tian et al., 2017).

SOD acts by reducing $O_2\cdot^-$ into O_2 and H_2O_2. There are three isoforms of SOD: SOD-1 (Cu/ZnSOD, cytosol), SOD-2 (MnSOD, mitochondria), and SOD-3 (Cu/ZnSOD, extracellular). GPX present in cytosol, catalyzes hydrogen peroxide reduction into water and oxygen, and further converts/reduces lipid hydroperoxides into stable alcohols. CAT is predominantly seen in peroxisomes and an integral part of antioxidant defense mechanism by reducing H_2O_2 to H_2O and O_2 (Schrader & Fahimi, 2006) and impaired CAT activity was observed in IBD patients (Iborra et al., 2011). Furthermore, the resolved colitis symptoms were linked with enhanced CAT activity in experimental colitis (de Moreno de LeBlanc et al., 2008; LeBlanc et al., 2011).

Nuclear factor-erythroid 2-related factor 2 (Nrf-2), a transcription factor, suppresses excess free radical production in IBD and conserves intestinal mucosal homeostasis (Khor et al., 2006). Nuclear translocation of Nrf-2 is a key event regulating the transcriptional activation of various enzymatic antioxidants like SOD, CAT, HO-1. Nrf-2-knock out (KO) mice showed increased susceptibility to DSS-induced colitis than wild type. Elevated proinflammatory cytokines and remarkably declined antioxidant enzyme activity were observed in Nrf-2 KO mice (Khor et al., 2006; Khor et al., 2008). Therefore, Nrf-2 negatively modulates progression of IBD by enhancing cellular antioxidant defense mechanisms and downregulates the elevated oxidative stress seen in IBD pathogenesis.

NF-κB, a transcription factor, plays a critical role in IBD development and progression. The activated NF-κB mediates the transcription of genes responsible for proinflammatory responses (Andresen et al., 2005; Rogler et al., 1998; Visekruna et al., 2006). The downstream targets of NF-κB respond to oxidative stress and proinflammatory cytokines like TNF-α promote activation of genes such as NOX1 and NADPH oxidase organizer-1 (NOXO1). These genes are responsible for ROS generation in colon epithelial cells during inflammation (Kuwano et al., 2008). Enhanced antioxidant activity was shown to exert a protective effect on DSS-induced colitis in mice *via* inhibition of NF-κB signaling pathway. These reports suggested that oxidative stress activates NF-κB signaling and is one of the major pathways involved in the development of IBD pathogenesis (Naito et al., 2002).

1.6 Mitogen-activated protein kinase (MAPK) pathway and IBD

Reports have suggested that initiation and progression of inflammatory responses during chronic inflammation depend upon various signaling pathways inside

cells (Dong, Davis, & Flavell, 2002). During inflammation, intestinal epithelium secretes an enormous level of cytokines, which subsequently activate various intracellular kinases. MAP kinases (serine/threonine protein kinases) are responsible for modulating cellular responses to various stimuli, including mitogens, osmotic stress, heat shock, and proinflammatory cytokines. MAPK signal transduction pathways are one of the extensively studied mechanisms of eukaryotic cell regulation. Elevated proinflammatory cytokines during IBD activate MAPK signaling pathway, which mediates the transcriptional regulation of various genes involved in IBD progression (Coskun, Olsen, Seidelin, & Nielsen, 2011; Cuadrado & Nebreda, 2010; Kyriakis & Avruch, 2001; Roy, Rashid, Bragg, & Ibdah, 2008; Schieven, 2009; Weston & Davis, 2007; Yoon & Seger, 2006).

In mammals, the MAPK family of proteins comprises of three major kinases: the extracellular signal-related kinases ($ERK^{1/2}$ or p42/p44), the c-Jun N-terminal ($JNK^{1/2/3}$), and the p38 MAP kinases. Ligand binding to the receptor activate various MAP kinase kinase kinase kinases (MAPKKKK) leads to the subsequent phosphorylation and activation of MAP kinase kinase kinases (MAPKKK) (Broom, Widjaya, Troelsen, Olsen, & Nielsen, 2009; Chen et al., 2001) followed by MAP kinase kinases (MKK) activation. MKKs phosphorylate all the three major MAP kinases. For example, p38 MAP kinases are phosphorylated and activated by MKK3, MKK4, and MKK6. ERKs are activated by MKK and MKK2; JNK by MKK4 and MKK7. These activated MAPKs mediate the transcription of various genes responsible for inflammation. TNF-α is a major proinflammatory cytokine secreted by colonic mucosa and other inflammatory cells during IBD. TNF-α was documented to activate the transcription factor NF-κB that in turn regulated inflammatory responses in IBD (Ellis et al., 1998; Rogler et al., 1998; Schreiber, Nikolaus, & Hampe, 1998).

NF-κB has been reported as an important downstream target of MAPKs (Kyriakis & Avruch, 2012; Lee et al., 1994; Lee & Young, 1996). The p38 MAPK was shown to increase the phosphorylation of p65 residue of NF-κB and its subsequent nuclear translocation *via* mitogen- and stress-activated kinase 1 (MSK1) (Vermeulen, De Wilde, Van Damme, Vanden Berghe, & Haegeman, 2003). Once translocated into the nucleus, NF-κB mediates the transcriptional regulation of genes encoding the proinflammatory cytokines like TNF-α, IL-1β, IL-6, and inflammatory mediators such as cyclooxygenase-2 (COX-2) (Ulivi, Giannoni, Gentili, Cancedda, & Descalzi, 2008). The role of p38 MAPK in IBD is well documented and phosphorylation of p38 in IBD tissues induced excess TNF-α production (Dahan et al., 2008; Waetzig, Seegert, Rosenstiel, Nikolaus, & Schreiber, 2002). Signal transducers and activators of transcription (STATs) are a different family of transcription factors involved in the initiation and progression of IBD. Among STATs, STAT3 is shown to regulate gene transcription, involved in cytokine production, cell growth, anti- and pro-apoptosis, and cell motility. STAT3 role is well established in different animal models of experimental colitis (Sugimoto, 2008; A. Suzuki et al., 2001). Studies proposed that activation of STAT3 is subjected to phosphorylation and activation by p38, $JNK^{1/2}$, and $ERK^{1/2}$ MAPKs (Chung, Uchida, Grammer, & Blenis, 1997; Kovarik et al., 1999; Zhang, Liu, & Dong, 2001). These reports indicate the importance of MAPK signaling in UC and CD pathogenesis.

Another extensively studied downstream target of MAPKs is activator protein-1 (AP-1), a class of dimeric transcription factors, consists of Jun (c-Jun, Jun-B, and Jun-D) or Fos family of proteins (c-Fos, Fos-B, Fra-1, and Fra-2) (Matthews, Colburn, & Young, 2007). JNK regulates the transcriptional activation of AP-1 protein *via* direct c-Jun phosphorylation (Binetruy, Smeal, & Karin, 1991). In IBD, an active AP-1 was

found to enhance genes encoding for inflammatory responses, namely COX-2 and iNOS. Further, an enhanced AP-1 activation was documented in DSS-induced colitis as well (Takada et al., 2010). AP-1 complexes induce the expression of extracellular matrix proteins in colon mucosa, that shown to integrate growth factor and cytokine signaling pathways (Olsen et al., 2003; Olsen et al., 2000). According to previous reports, JNK inhibition in murine UC experimental model has shown reduced infiltration of inflammatory cells and proinflammatory cytokine secretion. Increased phosphorylation of JNK was also observed in the tissues of IBD patients (Assi, Pillai, Gomez-Munoz, Owen, & Salh, 2006; Dahan et al., 2008).

ERKs are one of the extensively studied MAPK kinases involved in immune responses of IBD. Studies using $ERK^{1/2}$ inhibitors have shown to prevent IBD pathogenesis in the experimental conditions indicated that can be a therapeutic target in IBD (Caruso et al., 2007; Kwon, Ohigashi, & Murakami, 2007). It has been reported that the activation of AP-1 family proteins, namely c-Jun and c-Fos are also regulated by $ERK^{1/2}$ (Gupta, Campbell, Derijard, & Davis, 1995). Isolated crypts obtained from human biopsies have reported to shown increased $ERK^{1/2}$ phosphorylation during active stages of IBD. (Dahan et al., 2008; Waetzig et al., 2002). Therefore, $ERK^{1/2}$, p38 and JNK of MAPK signaling are implicated in IBD pathogenesis.

1.7 Role of phytochemicals in modulating MAPK pathway in IBD

Many phytochemicals are shown to suppress the MAPK pathway to curtail inflammatory response, beneficial in IBD. Both *in vitro* and *in vivo* studies reveal that phytochemicals compounds such as apocyanin, curcumin, mangiferin, allin, resveratrol, and andrographolide inhibit the MAPK signaling pathway to attenuate the inflammatory responses (Camacho-Barquero et al., 2007; Dou et al., 2014; Hwang et

al., 2016; Jeong, Jang, Hyam, Han, & Kim, 2014; Kim et al., 2019; Sanchez-Fidalgo, Cardeno, Villegas, Talero, & de la Lastra, 2010; Shi et al., 2017). These reports suggest that suppressing the MAPK pathway by the phytochemicals could be a possible molecular therapeutic target -for IBD treatment.

1.8 Intestinal tight junctions and IBD

Intestinal epithelium comprises of a single layer of columnar epithelial cells (acts as a barrier between luminal contents) and the underlying lamina propria layer (Ulluwishewa et al., 2011; van der Flier & Clevers, 2009). Intestinal epithelium's primary function is to provide a dynamic and selectively permeable physical barrier that prevents the permeation of microbial pathogens, toxins, and antigens present inside the intestinal lumen into the layer of lamina propria and the circulatory system (Turner, 2009). Intestinal permeability is a key property, help to absorb nutrients from the intestinal lumen and facilitates fluid exchange between the lumen and tissues (Lee, 2015). A compromised intestinal barrier is a critical event associated with dysregulated immunological conditions that contributed the development and progression of IBD (Suenaert et al., 2002). The securely arranged junctional complexes existing between the epithelial cells lining the intestinal lumen are critical for the barrier integrity maintenance. These junctional complexes comprise tight junctions (TJs), gap junctions, adherens junctions, and desmosomes. TJs are multiple protein complexes localized at the apical end of the epithelial cells' lateral membrane and involved in regulating ions, solute, and water movements *via* the paracellular pathway across the surface epithelium of the intestine. Since TJs guard the paracellular spaces between adjacent epithelial cells and are constantly exposed to luminal microbes, food-derived antigens and other external stimuli make them dynamic and undergo constant

remodeling (Farquhar & Palade, 1963). There are four integral transmembrane proteins (1) occludin (2) claudins (3) junctional adhesion molecule (JAM), and (4) tricellulin. Zonula occludins (ZO) are intracellular proteins, primarily anchoring the transmembrane proteins into peri-junctional actin cytoskeleton (Furuse, Fujita, Hiiragi, Fujimoto, & Tsukita, 1998; Furuse et al., 1993; Gonzalez-Mariscal, Betanzos, Nava, & Jaramillo, 2003; Ikenouchi, Furuse, Furuse, Sasaki, & Tsukita, 2005; Martin-Padura et al., 1998). TJ proteins' interactions with the actin cytoskeleton are critical because the contraction of actin cytoskeleton regulates the opening of TJs and paracellular permeability. The phosphorylation of myosin light chain (MLC) by MLC kinases is largely responsible for actin cytoskeleton contraction, making MLCK, as one of the key kinases, implicated in maintaining the epithelial barrier integrity (Scott, Meddings, Kirk, Lees-Miller, & Buret, 2002; Shen et al., 2006; Turner et al., 1997). Elevated proinflammatory cytokines are a major factor responsible for the compromised intestinal epithelial barrier integrity in IBD. In IBD *in vitro* models, TNF-α and IL-1β have shown to disrupt barrier integrity and associated with enhanced expressions of MLCK and phosphorylated MLC (Al-Sadi, Ye, Dokladny, & Ma, 2008; Ma, Boivin, Ye, Pedram, & Said, 2005). Studies have also documented that involvement of barrier dysfunction observed in several intestinal and metabolic diseases, such as food allergies, obesity and pathogenic bacterial invasion including inflammatory bowel diseases (Cani et al., 2007; Jenkins et al., 1987; Ventura et al., 2006).

1.9 Role of phytochemicals in modulating intestinal tight junction

Phytochemicals, such as genistein, quercetin, myricetin, and EGCG have been shown to exhibit protective effects on the intestinal TJ barrier dysfunction observed in

IBD. Genistein significantly attenuated intestinal TJ barrier dysfunction induced by oxidative stress and protective effects against acetaldehyde-induced TJ barrier insult (Rao, Basuroy, Rao, Karnaky Jr, & Gupta, 2002; Sheth et al., 2007). Quercetin, a flavonoid found in vegetables and fruits, was shown to preserve TJ integrity in rats (*in vivo* studies) and in human intestinal epithelial cell lines (*in vitro* studies) in a dose-dependent manner (Suzuki & Hara, 2009). A similar beneficial effect on TJ integrity was reported by other flavonoids present in grapes and green tea, myricetin, and EGCG (Suzuki & Hara, 2009; Watson et al., 2004). However, these phytochemicals' effect at the molecular level remains unexplored and need to be established undoubtedly. It is well established that intestinal epithelial barrier dysfunction plays a critical role in many intestine-related diseases, including IBDs (Lee, 2015; Soderholm et al., 1999). Phytochemical supplemented diets might help in the prevention and treatment of IBD. Therefore, this holds a promising future if further investigations are carried out on these phytochemical-mediated protective effects. Hence, these studies are necessary to comprehend the precise cellular pathways modulated by phytochemicals in regulating TJ physiology.

1.10 Experimental models for ulcerative colitis

The precise pathophysiological mechanisms involved in the development of IBD are still not completely understood yet. However, reports have identified 'IBD' as a condition with multifactorial origin involving genetic, microbial, and environmental factors (Sairenji, Collins, & Evans, 2017; Zhang & Li, 2014). In this context, especially the mouse model has become an indispensable tool to investigate IBD pathogenesis (Low, Nguyen, & Mizoguchi, 2013). For a multifactorial disease, single experimental model may not be convincing in elucidating all aspects of its

pathogenesis. Instead, observations and conclusions from various experimental models provide more insight to establish the IBD pathogenesis (Hermiston & Gordon, 1995; Kiesler, Fuss, & Strober, 2015).

Moreover, animal models have always been an effective tool to investigate novel drugs for these inflammatory conditions. Researchers have proved that experimental colitis can be induced in laboratory animals using chemicals or bacterial infection. Many transgenic and gene knockout models have also been identified to study the genetic susceptibility towards colitis development (Kiesler et al., 2015; Marcus & Watt, 1969). An ideal experimental colitis model should exhibit more similar pathophysiology, clinical symptoms of human IBD, and respond to pharmacological treatment.

1.10.1 Genetic models of IBD

Genomic studies have identified several genes, which are associated with increased susceptibility to CD and UC. These genetic data prompted researchers to develop genetically engineered animals as models to understand IBD, accomplished either by a complete gene knockout in the whole animal or a conditional knockout in selected organs.

IL-10 knockout mice

This model is created by targeted knockdown of the IL-10 gene, resulted in mice lacking the anti-inflammatory cytokine IL-10. These animals developed acute colitis spontaneously due to uncontrolled immune response mediated by excessive infiltration of neutrophils, macrophages, and T-lymphocytes. Those inflammatory response is histologically characterized by abnormal crypt and villi structures and destruction of entire mucosal epithelium (Kuhn, Lohler, Rennick, Rajewsky, &

Muller, 1993). These animals' inflammatory responses are mainly mediated through Th-2 helper cells like human UC (Berg et al., 1996). Further, genetic polymorphisms on the IL-10 gene were associated with increased susceptibility to develop both forms of IBD (Franke et al., 2008; Franke et al., 2010).

Multiple drug resistance 1a (Mdr1a) KO mice

Mdr1a gene codes for P-glycoprotein transporter on the cell surface, which regulate the transport across the cell membrane. Mice lacking this gene do not have the ability to eliminate bacterial products. So, these products were accumulated in epithelial cells and presented to T-cells by antigen-presenting cells, which ends up in an excess activation of T-cells. This abnormal T-cell activation serves as the triggering factor for colitis, characterized by damaged mucosal epithelium, goblet cell depletion and partial or complete damage of crypts (Panwala, Jones, & Viney, 1998; Tanner, Staley, & Lorenz, 2013). A decreased level of Treg cells is also a characteristic feature of this model, associated with a decrease production of IL-10 (Tanner et al., 2013).

IL-2 KO mice

Deletion of IL-2 gene in mice resulted in the early development of colitis, and microscopic architecture analysis of colon exhibited very similar changes, identical to human UC, including intestinal epithelial destruction, crypt abscess, and loss of goblet cells. Symptoms of inflammation become prominent in those animals, when attain the age of 8-9 weeks. IL-2 (regulatory in nature) is secreted by the $CD4^+$ T cells and mediate the T-lymphocytes expansion (Baumgart et al., 1998; Sadlack et al., 1993).

$NOD2^{2939iC}$ mouse model

The NOD2 protein is an integral part of innate immunity that belongs to pattern recognition receptors (PRR). These receptors recognize microbial antigens and initiate

immune mechanisms by promoting the nuclear translocation of NF-κB (Inohara, Ogura, & Nunez, 2002). In human IBD, the susceptible allele of NOD2 is 3020insC (Ogura et al., 2001). It was reported that mice with a susceptibility allele (NOD22939iC) homologous to the human allele increased vulnerability to colitis with DSS administration compared to wild-type animals (Maeda et al., 2005). This report suggested that the mutation of $NOD2^{2939ic}$ alone was not sufficient to initiate a complete intestinal inflammation and required an additional colitic stimulant like DSS.

1.10.2 Adoptive transfer model

These models are generated by transferring $CD4^+$ T-cells or immune tissue from a donor animal into a histo-compatible immunocompromised SCID recipient host mice. These animals developed colitis within 5-10 weeks of post treatment. It was also reported that this model produced inflammation only when naïve T-cells were transferred and not with matured T-cells. Interesting fact behind the information was that mature T-cells can produce Treg cells, fighting and suppressing the inflammation (Leach, Bean, Mauze, Coffman, & Powrie, 1996; Powrie, Leach, Mauze, Caddle, & Coffman, 1993; Powrie et al., 1994; Read, Malmstrom, & Powrie, 2000). This is an ideal model to study different T-cells' role in IBD progression and mucosal immune regulation.

1.10.3 Chemically induced models for IBD

The chemical-induced ulcerative colitis model is the most widely accepted animal model, which is classified based on the name of chemicals used.

<u>Trinitrobenzene sulfonic acid-induced ulcerative colitis</u>

Trinitrobenzene sulfonic acid (TNBS) is an oxidizing nitroaryl acid (Neurath, Fuss, & Strober, 2000). After rectal administration, trinitrophenyl haptens of TNBS form covalent bonds with host proteins and trigger an immune-mediated inflammatory response. Ethanol is used to dissolve TNBS, and that mixture produces modified hapten self-antigens, which are detected by the host immune mechanism. The colonic damage induced by these responses is mainly attributed to high myeloperoxidase activity, a hallmark of neutrophil infiltration into the inflamed area (Elson, Sartor, Tennyson, & Riddell, 1995). Alcohol acts as a solvent for TNBS and enhances inflammatory responses by damaging the mucosal epithelium. The cytokine responses induced by TNBS are mostly Th-1 mediated, whereas human UC is Th-2 mediated (Fichtner-Feigl et al., 2007; Kawada, Arihiro, & Mizoguchi, 2007). Therefore, the TNBS colitis resembles more closely to Crohn's disease rather than UC.

Oxazolone induced ulcerative colitis

Oxazolone is another chemical agent like TNBS, which produce hapten like proteins (Boirivant, Fuss, Chu, & Strober, 1998). This chemical agent is dissolved in ethanol and when administered rectally, resulted in the superficial inflammation of the distal colon. The major physiological effects of oxazolone-induced murine colitis are bodyweight loss, diarrhea and ulcers (Boirivant et al., 1998; Heller, Fuss, Nieuwenhuis, Blumberg, & Strober, 2002; Olszak et al., 2012). Like human UC, the cytokine responses induced by oxazolone is Th-2 mediated with elevated levels of IL-5, IL-13 and IL-4. Many reports have substantiated the use of oxazolone as an effective inducer of acute inflammation in animals. However, its potency to induce chronic inflammation has not been investigated in detail (Gerlach et al., 2014).

Acetic acid-induced colitis

Rectal administration of diluted acetic acid is also a recognized rodent model of experimental colitis. Rectal acetic acid administration caused ulcerative lesions at the distal part of the colon dose-dependently (Low et al., 2013; MacPherson & Pfeiffer, 1978). Necrosis of mucosa and submucosa, vascular dilatation, and edema are the key features observed in acetic acid-induced colitis. Studies have documented that acetic acid releases proton, which causes intracellular acidification and that resulted in epithelial damage. The cost-effectiveness and easy administration are few advantages observed in the acetic acid-induced colitis model. (Elson et al., 1995). However, acetic acid-induced colitis is not displaying many immunological responses as in IBD, making it a less reliable model to investigate novel drugs.

Dextran sodium sulfate (DSS) induced ulcerative colitis

Numerous *in vivo* experiments over 30 years have identified and accepted dextran sodium sulfate (DSS) induced ulcerative colitis as the most reliable animal model of experimental colitis (Gaudio et al., 1999; Jurjus, Khoury, & Reimund, 2004; Randhawa, Singh, Singh, & Jaggi, 2014). The observed morphological changes and symptoms of DSS-colitis in the animal are similar to the mucosal epithelial damage seen in human UC. Extensive reports on DSS-induced colitis model have demonstrated that DSS does not directly cause inflammation. But DSS, a negatively charged sulfated polysaccharides act as a direct chemical toxin causing a focal injury on colon mucosal epithelium, which results in the destruction of mucosal epithelial barrier of the intestine. This allows the luminal microbes and other antigens to enter into the underlying lamina propria and thus triggering the host immune responses which are manifested as ulcerative lesions (Kiesler et al., 2015; Kim, Shajib, Manocha, & Khan, 2012; Okayasu et al., 1990; Perse & Cerar, 2012; Poritz et al., 2007; Samak et al., 2015; Wirtz, Neufert, Weigmann, & Neurath, 2007). Every day, freshly prepared 40-

50 kDa DSS dissolved in autoclaved drinking water is given to mice to induce colitis. The severity of inflammation depends on the DSS concentration administered (1-5% DSS). The advantage of the DSS colitis model over other chemical-induced colitis is that by changing the concentration and cycles of DSS administration, both acute and chronic colitis models can be generated. For acute model of UC, DSS is administered over a period of 6-10 days at desired concentrations. On the other hand, DSS is given to animals in repeated cycles and in different concentrations for certain periods, to induce chronic colitis in animals. Each cycle is followed by normal drinking water administration for 7-14 days (Cooper, Murthy, Shah, & Sedergran, 1993; Giner, Recio, Rios, & Giner, 2013; Okayasu et al., 1990; Randhawa et al., 2014).

DSS-induced colitis effects are manifested as loss of body weight, loose stool leading to diarrhea, and hematochezia. More than 20% loss from initial body weight indicates a substantial mucosal injury of intestinal lumen (Kim et al., 2012). Following the DSS administration, the mild symptoms of UC start from day 1, which is marked by alteration of tight junction protein expression (essential in maintaining the intestinal barrier integrity) (Ichikawa-Tomikawa, Sugimoto, Satohisa, Nishiura, & Chiba, 2011; Poritz et al., 2007). In human UC, rearrangement of tight junction proteins and apoptosis of epithelia eventually progresses into a leaky gut, responsible for consistent diarrhea and rectal bleeding. Tight junctions (TJ) are maintained by protein complexes, namely claudins, zonula occludins (ZO), occludin, and junctional adhesion molecules (JAMA) (Iwaya, Maeta, Hara, & Ishizuka, 2012; Kyoko et al., 2014; Luettig, Rosenthal, Barmeyer, & Schulzke, 2015; Poritz et al., 2007). Studies have revealed that any alterations of these protein expressions or their rearrangement are resulted in compromised intestinal barrier integrity and decreased transepithelial resistance; these are the critical events that precede UC development. Altered TJ protein expression

characterized by the depletion of various claudins, occludin, and ZOs reported in DSS-induced UC, resulted in breach of colon mucosal epithelial barrier (Iwaya et al., 2012; Kitajima, Takuma, & Morimoto, 1999; Luettig et al., 2015; Mahler et al., 1998; Mennigen et al., 2009; Poritz et al., 2007).

Epithelial barrier breach caused by DSS colitis is followed by the entry of luminal microbes into the underlying lamina propria. Innate immune system senses entered luminal microbes *via* various pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) and trigger a series of signaling cascades, including the activation of transcription factors like NF-κB phosphorylation. This is responsible for the transcriptional regulation of proinflammatory cytokines and other inflammatory mediators like COX-2 (Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014; Sartor, 2006). Elevated TNF-α, IL-6 and IL-1β levels are one of the indications of intestinal epithelial damage in DSS colitis. Increased MPO activity is found at mucosal layer due to neutrophil infiltration at the site of inflammation. Immune responses of acute phase UC are predominated by innate immunity. When colitis progresses into the chronic phase, then acquired immunity takes over. Chronic phase immune responses are Th-2 cells mediated, marked by elevated cytokine levels of IL-4, IL-5, IL-10, IL-12, and IL-13 (Alex et al., 2009; Perse & Cerar, 2012; Randhawa et al., 2014). Histological changes in DSS colitis can be classified into two types: early and advanced. The typical histological damages in DSS colitis are characterized by mucin depletion, epithelial erosion, crypt damage and edema, which could sometimes be a focal loss of crypts, along with granulocytes infiltration into lamina propria extending up to the submucosa layer. Transepithelial migration of neutrophils into mucosal epithelium is called as cryptitis. This migration of neutrophils into the crypt lumen lead to crypt abscesses (Melgar, Karlsson, & Michaelsson, 2005; Perse & Cerar, 2012).

The availability of different IBD models allow the researchers to choose the appropriate model as per specific research needs. Above mentioned models have helped scientists to figure out intracellular signaling mechanisms involved in IBD pathogenesis. However, considering genetic models, the initiation and extend of inflammation is highly variable due to environmental factors, like animal enteric flora, housed cages and its cleanliness, weather, stress, and feed. In some transgenic models, the induction of inflammation takes typically months, making them less reliable and time-consuming for drug screening (Wirtz & Neurath, 2007). Henceforth, chemical models are commonly used for investigating the therapeutic potential of novel drugs against IBD. Reproducibility and cost-effectiveness are other beneficial aspects of these models. DSS-induced colitis model elicits more identical human UC immunological responses coupled with compromised intestinal mucosal epithelial barrier function and histological parameters. So, researchers accepted and considered DSS-induced colitis to investigate the efficacy of novel anti-inflammatory compounds for UC treatment.

1.10.4 *In vitro* models to evaluate anti-inflammatory properties of compounds

There are various *in vitro* models available to investigate the effect of novel anti-inflammatory compounds in colon inflammation.

RAW 264.7 murine macrophages

Macrophages are mononuclear phagocytic cells that form an integral part of the innate immune system and act as a first-line defense mechanism against foreign antigens. In addition to phagocytosis, macrophages also secrete large amounts of cytokines, chemokines, and other proinflammatory mediators etc. These diverse properties of macrophages are implicated in many pathologic conditions including

rheumatoid arthritis, cancer, cardiovascular disease and IBD (Kuhl, Erben, Kredel, & Siegmund, 2015; Parameswaran & Patial, 2010).

RAW 264.7 cells are macrophage like cells transformed from Abelson leukemia virus induced tumor in BALB/c mice. These cells strongly resemble the bone marrow derived murine macrophages with the same cell surface receptor density and response to inflammatory stimuli (Berghaus et al., 2010; Raschke, Baird, Ralph, & Nakoinz, 1978). RAW cells serve as a prominent tool to evaluate the potency and efficacy of anti-inflammatory compounds. Bacterial lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, is a potent stimulator of these kinds of cells. When the RAW cells are exposed to LPS, it will cause a robust release of proinflammatory cytokines including IL-1β, IL-6, and TNF-α, similar as an inflammatory response of body (Akira, Taga, & Kishimoto, 1993; Dinarello, 1996; Tracey & Cerami, 1994). These findings propose that RAW macrophages is an effective model to evaluate the mechanisms involved in inflammatory responses. The important signaling pathway involved in LPS stimulation of macrophages is the TLR-4/NF-κB pathway, by enhancing NF-κB phosphorylation and nuclear translocation into nucleus (O'Neill, Bryant, & Doyle, 2009; Wertz & Dixit, 2010). Upon stimulation of RAW cells with LPS, the MAP kinases (p38, $ERK^{1/2}$ and JNK) are immediately phosphorylated (Hambleton, Weinstein, Lem, & DeFranco, 1996; Meng & Lowell, 1997; Weinstein, Sanghera, Lemke, DeFranco, & Pelech, 1992). These reports also suggested that the involvement of MAPK pathway is strongly implicated in inflammatory responses of RAW cells.

Caco-2 cell line

The epithelial cell line Caco-2 were cultured from human colorectal adenocarcinoma (Ponce de Leon-Rodriguez, Guyot, & Laurent-Babot, 2019). This cell line is widely accepted as an *in vitro* model to study intestinal absorption, transport, and permeability. It is proved that Caco-2 cells after reaching confluence during subculture can differentiate spontaneously and exhibited enterocyte characteristics. When Caco-2 cells are allowed to grow in to monolayer, they form tight junctions between the cells resembling an *in vivo* intestinal barrier. (Pinto, 1983). This property makes Caco-2 cells, an ideal model to investigate the epithelial barrier integrity and transport studies. It was shown that Caco-2 cultured on permeable trans-well filter supports tend to form a monolayer and that allow the transport of ions and nutrients across the monolayer, resembling a more physiological set up of human ion/nutrients transport (Artursson & Magnusson, 1990). The integrity of intestinal permeability can be assessed by measuring the trans-epithelial electrical resistance, while growing these cells in specially designed transwell supports and plates. Studies have also shown that Caco-2 cells show a better morphological and functional differentiation into enterocytes, when compared to other colon carcinoma cell lines (Matsumoto et al., 1990).

HT-29 cell line

HT-29 cell line (intestine cell line) isolated from colon adenocarcinoma serve as an enterocyte model (Andoh, Kinoshita, Rosenberg, & Podolsky, 2001; Yi et al., 2016). After reaching confluence, HT-29 cells do not undergo spontaneous differentiation like Caco-2 cells, because it is dependent on culture conditions and growth media (Huet, Sahuquillo-Merino, Coudrier, & Louvard, 1987; Zweibaum, Laburthe, Grasset, & Louvard, 2010). For example, the presence of glucose in culture media can inhibit the differentiation of HT-29 cells. These properties make them more

of a pluripotent intestinal cell line in nature. Therefore, these cells are better suitable for inflammatory experiments (when stimulated with TNF-α (McCracken et al., 2002) rather than a physiological model for evaluating the epithelial barrier integrity. The other major difference between HT-29 and Caco-2 cells is that HT-29 can transform into goblet like cells and produce mucus that forms a protective layer in the intestine, unlike Caco-2 cells (Zweibaum et al., 2010).

The present study investigates the role of nerolidol as an anti-inflammatory compound in *in vitro* and *in vivo* model of colon inflammation. Nerolidol (NED) [3,7,11-trimethyl-1,6,10-dodecatrien-3-ol], a naturally occurring sesquiterpene is present in various plants with a floral odor (Chan, Tan, Chan, Lee, & Goh, 2016) and in essential oils of many plants (Ferreira et al., 2012). Scientific reports propose that sesquiterpenes and its derivatives have shown to possess significant biological activities (Bartikova, Hanusova, Skalova, Ambroz, & Bousova, 2014). Reports over the years provide pieces of evidence for its therapeutic efficacy in neurodegenerative diseases and characterized as anti-microbial, anti-biofilm, anti-oxidant, anti-parasitic, skin-penetration enhancer, skin-repellent, anti-nociceptive, anti-inflammatory and anti-cancer effects (Chan et al., 2016; De Carvalho, De Almeida, Campelo, Lellis, & Nunes, 2018). High content of enhanced (*E*)-nerolidol are found in Oolong tea (a traditional semi-oxidized Chinese tea) and contributed the floral aroma to teas (*Camellia sinensis*) (Zhou et al., 2017). Madeira wines from Portugal are reported to contain NED (Alves, Nascimento, & Nogueira, 2005) and a reason behind kiwi fruit flavor (Green et al., 2012) and also found to be present in strawberry fruit (Yan et al., 2018). U.S. Food and Drug Administration (FDA) has also accepted the use of NED as a flavor enhancer in the food industry (Chan et al., 2016). Reports have indicated that NED possesses significant neuroprotective effects mediated through

its antioxidant and anti-inflammatory activities (Javed, Azimullah, Abul Khair, Ojha, & Haque, 2016). Further, a NED derivative was shown to prevent oxidative injury in human lung epithelial cells and identified as Nrf-2 activators (Nuclear factor erythroid-2-related factor-2 represents a central cellular defense mechanism) (Zhou et al., 2018). Besides, NED showed its potent anti-inflammatory properties by inhibiting IL-1β and TNF-α levels in an experimental mouse model of pain (Fonseca et al., 2016). Based on these scientific reports, well-controlled preclinical studies were designed to evaluate NED and its anti-inflammatory properties using *in vivo* and *in vitro* models of colon inflammation.

Research hypothesis

Based on extensive literature review it is indicated that, NED exhibited many biological properties such as anticancer, antioxidant and anti-inflammatory properties. Therefore, it is hypothesized that NED mitigates inflammatory responses observed in ulcerative colitis through its potent anti-inflammatory properties.

Chapter 2: Aims and Objectives

In the recent times, a major focus of nutrition research aims to develop functional food and nutraceuticals compounds with demonstrable health benefits. Therefore, investigating the role of the dietary phytochemical compound NED in colon inflammation is significant and novel. In this study, both *in vivo* and *in vitro* experimental models of colon inflammation are employed to investigate the role of NED as a putative anti-inflammatory compound. These preclinical studies will help us to understand the molecular mechanisms regulated by NED in the experimental model of colon inflammation and may help us pave a way to clinical settings to treat IBD in the future.

Therefore, the current study was carried out using the following specific aims.

1. To investigate the role of NED as a putative anti-inflammatory phytochemical using LPS-stimulated RAW 264.7 murine macrophages on the proinflammatory cytokines and mediator responses and their mRNA expression.
2. To investigate the role of NED in colon inflammation using dextran sodium sulfate (DSS)-induced (*in vivo* model) of colon inflammation on the proinflammatory cytokines/mediator's response, and colon epithelial nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf-2) antioxidant system.
3. To investigate the role of NED on colon inflammation using TNF-α stimulated HT-29 cells (*in vitro* model) on proinflammatory chemokines/mediator mRNA expression.

4. To investigate the role of NED on mitogen-activated protein kinase (MAPK) pathway and nuclear factor kappa-B (NF-κB) transcription factor involved in inflammation using *in vitro* and *in vivo* models of colon inflammation.
5. To investigate the role of NED on colon epithelial tight junction integrity and barrier function using LPS-stimulated Caco-2 cell monolayer as an *in vitro* model and DSS-induced colitis as an *in vivo* model of colon inflammation.

Chapter 3: Methods

3.1 Chemicals and reagents

Dextran Sulfate Sodium—DSS (Molecular weight 36,000–50,000 Da), FITC-dextran, LPS and NED were obtained from Sigma-Aldrich, (St. Louis, MO, USA). MPO, IL-6, IL-1β, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D systems (Minneapolis, MN, USA). The high capacity cDNA reverse transcription kit and SYBR green Real-Time PCR mastermix were supplied by Applied Biosystems (Foster City, CA, USA). Macrogen Inc. (Seoul, Korea) supplied primers used in qPCR. Antibodies were procured from Abcam (Cambridge, CA, USA), Santacruz Biotechnology (USA) and Thermo scientific (USA). Protease & phosphatase inhibitor cocktail tablets, Pierce BCA protein estimation kit, PVDF membrane, TRIZOL reagent, NE-PER nuclear and cytoplasmic extraction reagents and West Pico super signal chemiluminescent substrate were supplied by Thermo-Scientific (Waltham, MA, USA). RIPA buffer was purchased from Millipore (St. Louis, CA, USA). Dulbecco's modified Eagle's medium (DMEM) was bought from Pierce Hyclone (Fremont, CA, USA) and Gibco (Carlsbad, CA, USA). Heat-inactivated fetal bovine serum (FBS) and Penicillin-Streptomycin was obtained from Gibco (Carlsbad, CA, USA). Cell Titer-Glo® Luminescent Cell Viability Assay Kit was bought from Promega (Madison, WI, USA). Reagents used in the estimation of biochemical parameters were purchased from Sigma Aldrich (St. Louis, MO, USA) and Sisco Research Laboratories, India. RAW 264.7 murine macrophages, HT-29 cells and Caco-2 cells were obtained from American Type Culture Collection (ATCC), USA

3.2 Experimental design

Animals used

Male C57BL/6J (~ twelve weeks old) mice weighing around 25–30 g obtained from the Central animal facility, College of Medicine and Health Sciences, UAE University. The animals were housed in groups of 6 per cage at a constant temperature of 23 ± 1°C, a 12-h light–dark cycle, and a humidity of 50–60% maintained all the time. Food and water provided *ad libitum*. Studies were approved by the Institutional Animal Ethics Committee of UAE University (Approval # ERA_2017_5599).

Experimental design - *in vivo model*

After a week of acclimatization, the animals were randomly allocated into 6 groups having 10 animals each (2 animals per cage during treatment period).

Group 1: Control or vehicle treated group.

Group 2: DSS + vehicle treated group.

Group 3: DSS + NED at the dose of 50 mg/kg bodyweight.

Group 4: DSS + NED at the dose of 100 mg/kg bodyweight

Group 5: DSS + NED at the dose of 150 mg/kg bodyweight.

Group 6: DSS + Sulfasalazine (SAZ) at the dose of 50 mg/kg bodyweight.

Drugs and vehicle were administered via oral gavage. Sunflower oil was used to dissolve NED, hence acted as vehicle in this study. DSS (3%) was prepared freshly everyday using autoclaved drinking water. Sulfasalazine (SAZ) was used as the positive control drug. The whole treatment period was seven days. At the end of treatment, animals were sacrificed using a lethal dose of pentobarbital (100 mg/kg body weight) administered as an intraperitoneal injection. After laparotomy, the colon was excised & measured using graduated scale along with caecum. Then caecum

removed, the excised colon was flushed with ice cold saline to remove fecal contents and cut into 1 cm pieces, which were immediately snap frozen using liquid nitrogen and stored at −80°C freezer for protein and RNA analysis. For histopathological analysis, the colon pieces near the junction between proximal and distal colon were taken and fixed in 10% formalin.

Experimental design - *in vitro* models

Three cell lines were used for *in vitro* experiments.

i. RAW 264.7 murine macrophages
ii. Human colorectal adenocarcinoma (HT-29) cells
iii. Human colon adenocarcinoma (Caco-2) cells

General methodology

Only pre-sterilised plastic wares were used for *in vitro* studies. Cell culture experiments were performed in a biosafety hood adhering to aseptic techniques already in place, which included use of pre-sterile cell culture tools/consumables, frequent 70% (*v/v*) ethanol in water spray, UV lamp to avoid any sort of contamination by microorganisms in working area.

Generally, cells were maintained at 37°C and 5% CO_2 in a humidified incubator using high-glucose DMEM, supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 10% (*v/v*) FBS. Cells received from ATCC (cryovials) were transferred and stored in a liquid nitrogen tank until reviving. Revival of cells were done by keeping the frozen vial in a water bath at 37°C. Once thawed, cells were transferred into a 15 ml falcon tube and centrifuged at 1000 *rpm* for 5 minutes. Then, cell pellet was suspended in fresh medium and transferred in to a T25 flask to grow. Grown (80% confluent) cells were sub-cultured into another flasks. During

propagation, HT-29 and Caco-2 cells were detached from flask by using trypsin, whereas macrophages detachment performed by scraping (sterile cell scraper used). Detached cells, then transferred into a 15 ml falcon tube and centrifuged at 1000 *rpm* for 5 min to collect cell pellet and the supernatant discarded. Collected cell pellet was re-suspended in fresh media containing 10% FBS and allowed to grow in T75 culture flask for subsequent experiments.

Cell viability assay:

Cell viability was calculated using Cell Titer-Glo® Luminescent Cell Viability Assay kit from Promega.

Principle

Cell viability assay is a homogeneous method to determine the number of viable cells present in culture, based on quantitation of ATP present in viable metabolically active cells. The Cell Titer-Glo® assay relies on the properties of thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which generates a stable "glow-type" luminescent signal, that directly proportional to the number of viable cells present.

Procedure

Cells were seeded on 96 well plates at 5000 cells/well and allowed to adhere to well surface for 24 hrs. The next day, the cells were incubated with various concentrations of NED (200, 100, 50, 25, 12.5, 6.25 and 3.125 µM). After incubation period (24 hrs and 48 hrs), cells were processed for cell viability assay using Cell Titer-Glo(R) Luminescent Cell Viability kit according to manufacturer's protocol. Briefly, 100 µl cell titre glow reagent was added to wells (containing cells already incubated with various concentrations of NED) and incubated for 10 minutes at room

temperature. The luminescence was read using Tecan multi-mode plate reader instrument.

Experimental inflammation studies using HT-29 cells:

HT-29 cells were maintained under standard conditions. For inflammatory experiments, cells were seeded in 6-well plate at 1.5×10^5 cells/well and allowed to adhere for 24 hrs, DMEM media containing 2% FBS was used for seeding the cells. After 24 hrs, cells were treated with NED at two different concentrations - 25 and 50 µM (desired concentration were chosen as per cell viability results - 80% viability and above considered safe) for another 24 hrs. To induce inflammatory conditions, the cells were challenged with TNF-α at a concentration 1 ng/ml prepared with PBS. On completion of 24 hrs incubation, cells were collected for mRNA analysis.

Inflammatory experiments using RAW 264.7 murine macrophages:

RAW 264.7 murine macrophages were seeded as same way as HT-29 cells. Macrophages were stimulated with LPS at 1µg/ml concentration prepared with PBS. The cells were co-treated with two different doses of NED - 30 and 60 µM (doses chosen from cell viability experiments). After incubation period (24 hrs), the supernatant media was collected for ELISA estimation of proinflammatory cytokines TNF-α, IL-1β and IL-6. The cells were lysed in specific buffers for future analysis.

Caco-2 – LPS experiments

To investigate NED role on TJ proteins, caco-2 cells were seeded and allowed to grow on 6-well plates. After forming confluent monolayer, cells were incubated with LPS at 100 µg/ml concentrations. NED was co-administered at 30 and 60 µM concentrations. After 24 hrs, cells were lysed in Trizol / RIPA buffer and collected for real time PCR and western blot experiments, respectively.

3.3 Evaluation of clinical score for colitis and construction of disease activity index (DAI)

Disease activity index (DAI) score indicates intestinal inflammation severity in experimental animals. After DSS administration, animals started losing weight, symptoms like diarrhea and hematochezia observed. Weight loss of more than 20% of initial weight was considered as a significant stress sign.

During DSS treatment, mice weight, stool consistency and rectal bleeding recorded every day. Heme occult is a term used to describe the presence of blood in stool. Bloody fecal matter of mice was assessed using Hemospot kit (Standard Guaiac Method). Principle of the test is that if blood is present, the hematin of hemoglobin molecule catalyses the release of oxygen from hydrogen peroxide. That in turn, oxidizes colorless phenolic components of gum guaiac in to colored quinines. First, thin layer of stool was spread across the reactive surface of test card provided with Hemospot kit. The smeared samples were allowed to dry and turned over to the other side. One drop of developer solution was added on reactive surface of result window of test card. If reactive surface turned blue, that indicated presence of blood in sample. No blue color appeared means absence of blood.

Scores were constructed based on the parameters highlighted in Table 1. The scores for weight loss, diarrhea, and bleeding were used to compile DAI.

Table 1: Disease activity index score

Weight loss	Score	Stool consistency	Score	Rectal bleeding	Score
No loss	0	Normal	0	No Blood	0
1 - 5%	1	Loose stool	2	Heme occult + ve & visual pellet bleeding	2
5 - 10%	2	Diarrhea	4	Gross bleeding & blood around anus	4
10 - 20%	3				
>20%	4				

3.4 Histopathological evaluation

After saline wash, the proximal segment of each colon was placed in a tissue cassette and immersed in 10% formaldehyde overnight. For preparing thin slice sections for histological examinations, the tissue needed to be embedded in paraffin wax. Since, wax is not soluble in water or alcohol, to dehydrate the tissue, the cassettes were processed through increasing concentrations of ethanol. After dehydration, the alcohol present in the tissues was removed by xylene, because xylene is used as a solvent for paraffin as well. The tissue cassettes were further processed at wax station for paraffin embedding. When tissues were placed in warm paraffin wax, the melted wax entered the spaces occupied by xylene. The paraffin filled tissues were then allowed to cool and stored for future use. The paraffin blocked colon tissues were mounted on microtome to trim paraffin blocks and 2 μm sections were cut and mounted on a microscopic slide. Since staining solutions were aqueous, the sections were rehydrated which was done in a reverse manner to that of dehydration. The sections were passed through xylene and then decreasing concentrations of alcohol and finally water. After staining with hematoxylin and eosin, the sections were dehydrated once again, because the mounting medium is only soluble in xylene. After mounting,

cover slip was placed on top and allowed to dry. Histologic scoring of each sample was graded by a pathologist, who was blinded to the experimental protocol. Samples were rated for colonic inflammation and crypt damage based on the grading system highlighted in Table 2 and Table 3.

Table 2: Crypt distortion score

Crypt grade		Quantification on percentage of crypt change		Crypt distortion-graded based on extent of involvement	
Grade 0:	Intact crypt	1	1-25%	0	no crypt distortion
Grade 1:	Shortening and loss of basal 1/3 of crypts	2	26-50%	1	1-25%
Grade 2:	Loss of basal 2/3 of crypts	3	51-75%	2	26-50%
Grade 3:	Loss of entire crypt with intact surface epithelium	4	76-100%	3	51-75%
Grade 4:	Loss of both entire crypt and surface epithelium (erosion)			4	76-100%

Table 3: Colon tissue histological examination for inflammation Score

Inflammation Graded	Percentage of inflammation involvement of mucosal surface area		Hyperplastic epithelium-graded based on extent of involvement	
None	0	no inflammation	0	none
Mild	1	1-25%	1	1-25%
Moderate	2	26-50%	2	26-50%
Severe	3	51-75%	3	51-75%
	4	76-100%	4	76-100%

3.5 Total protein quantification

Total protein content of colon homogenate and cell lysate was quantified using Pierce™ BCA Protein Assay Kit.

Principle

The principle of this method is based on the biuret reaction, in which peptide bonds of protein react with copper ions to produce a violet or purple complex, which can be detected colorimetrically. Briefly, Cu^{+2} is reduced to Cu^{+1} by protein in an alkaline medium using a reagent containing bicinchoninic acid (BCA). The violet or purple-colored end product is formed by the chelation of two molecules of BCA with one cuprous ion.

Reagents/Kit components

1. BCA reagent A: 1000 mL, containing sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M sodium hydroxide.
2. BCA reagent B: 25 mL, containing 4% cupric sulfate
3. Albumin standard ampule: Bovine Serum Albumin (BSA) (2000 µg/ml) prepared with 0.9% saline and 0.05% sodium azide.

Preparation of diluted albumin (BSA) standards

One BSA ampule was diluted into labelled sterile microcentrifuge vials in order of decreasing concentration, using the same diluent of homogenate/cell lysate sample (RIPA buffer). The details of standard preparation are explained in Table 4. Dilution Scheme for Microplate Procedure (Working Range = 20 - 2,000 µg/ml)

Table 4: Preparation of BSA standards

Vial	Vol. of Homogenization buffer (µl)	Volume and Source of BSA (µl)	Final BSA Concentration (µg/ml)
S1	0	300 of Stock	2000
S2	125	375 of Stock	1500
S3	325	325 of Stock	1000
S4	325	325 of vial S3 dilution	500
S5	325	325 of vial S4 dilution	250
S6	325	325 of vial S5 dilution	125
S7	400	100 of vial S6 dilution	25
Blank	400	0	0

Preparation of the BCA working reagent (WR)

1. The following formula was used to determine the total volume of WR required: (# standards + # unknowns) × (# replicates) × (volume of WR per sample) = total volume WR required

2. The WR was prepared by mixing 50 parts of Reagent A with 1 part of Reagent B (50:1, Reagent A: B from BCA kit)

When Reagent B was first added to Reagent A, turbidity was observed that quickly disappeared upon vortex, yielded the clear green WR.

Procedure:

Each samples and standards (25 µl) were added in duplicates in a 96 well microplate and followed by prepared WR addition (200 µl). The plate was shaken thoroughly using plate shaker for 30 seconds. The plate was then covered and incubated at 37°C for 30 minutes. After allowing the plate to cool at room temperature, absorbance was read at 570 *nm* using Tecan microplate reader. Blank absorbance value was subtracted from the values of standards and samples. Standard curve was generated by plotting absorbance readings of BSA standards against its concentration (µg/ml). Test sample concentrations were calculated using obtained standard curve equation.

3.6 Measurement of superoxide dismutase, catalase activities and tissue nitrate concentration

Superoxide dismutase (SOD)

SOD was assayed by the method previously described (Kakkar, Das, & Viswanathan, 1984).

Principle

The assay was based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazan. The reaction was initiated by the addition of NADH and incubated for 90 sec. Glacial acetic acid was added to arrest the reaction. The color developed at the end of the reaction was extracted into the n-butanol layer and the absorbance was read in a spectrophotometer at 560 *nm*.

Reagents

1. Sodium pyrophosphate buffer: 0.052 M, pH 8.3.
2. Absolute ethanol
3. Chloroform
4. n-butanol
5. Phenazine methosulphate (PMS): 186 μM
6. Nitroblue tetrazolium (NBT): 300 μM
7. Reduced nicotinamide adenine dinucleotide (NADH): 780 μM
8. Glacial acetic acid

Procedure

Tissue was homogenized using sodium pyrophosphate buffer (0.025 M, pH). To 500 μl tissue homogenate, 1ml of water was added followed by the addition of 2.5

ml ethanol and 1.5 ml chloroform (all reagents pre-chilled). This mixture was shaken for 90 sec at 4°C and then centrifuged.

SOD enzyme activity of supernatant was determined as follows. The assay mixture contained 1.2 ml sodium pyrophosphate buffer, 100 µl PMS and 300 µl NBT and appropriately diluted enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of 200 µl NADH. After incubation at 30°C for 90 sec, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken with 4 ml n-butanol. The contents were left aside for 10 min, centrifuged and the n-butanol layer was separated. Color intensity of the chromogen in n-butanol layer was measured using spectrophotometer at 560 *nm*. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit.

The specific activity is expressed as 50% NBT reduction/min/mg protein for tissues.

Catalase (CAT)

The activity of CAT was determined by the method previously described (Sinha, 1972).

Principle

Dichromate in acetic acid when heated in the presence of H_2O_2 was converted to per-chromic acid and then to chromic acetate. The chromic acetate formed was measured at 620 *nm*. The enzyme CAT was allowed to split H_2O_2 for various periods of time. The reaction was stopped at different time intervals by the addition of dichromate-acetic acid mixture and remaining H_2O_2 as chromic acetate was measured.

Reagents

1. Phosphate buffer: 0.01 M, pH 7.0
2. H_2O_2: 0.2 M
3. Potassium dichromate: 5%
4. Dichromate-acetic acid reagent: 1:3 ratio of potassium dichromate and glacial acetic acid.
5. Standard H_2O_2: 100 µl /100 ml in distilled water.

Procedure

Tissue homogenate was prepared by using phosphate buffer (0.01 M, pH 7.0). 900 µl phosphate buffer was mixed with 100 µl tissue homogenate and 400 µl H_2O_2. The reaction was arrested after 15, 30, 45 and 60 sec by adding 2 ml of dichromate-acetic acid reagent. The tubes were kept in a boiling water bath for 10 min, cooled and color developed, the absorbance was read at 620 *nm* using spectrophotometer. Standards concentration range of 20-100 µmoles were processed for the test.

The specific activity of CAT is expressed as µmoles of H_2O_2 utilized/min/mg protein for tissues.

Tissue nitrate estimation

Tissue nitrite (NO_2^-) concentration in colon homogenate is measured using Griess reaction (Lu, Wu, Lin, & Chang, 2009).

An increased tissue nitrite concentration in samples is an indicator for the formation of nitric oxide (NO) which is an important physiological messenger. The Griess reagent assay is based on the reaction between Sulfanilamide and N-1-napthylethylenediamine hydrochloride under acidic (phosphoric acid) conditions.

Reagents

1. Ortho phosphoric acid (H_3PO_4)
2. Sulfanilamide
3. Napthaethylene diamine hydrochloride
4. Sulfosalicylic acid
5. Sodium nitrite ($NaNO_2$)

Procedure

Griess reagent was prepared by making up 2.35 ml of H_3PO_4 to 100 ml with distilled water. Sulphanilamide 1 g and 100 mg of Napthaethylene diamine hydrochloride was added and mixed thoroughly.

First, samples were deproteinized by adding 100 µl of 5% sulfosalicylic acid into 100 µl test samples and vortexed immediately. The samples were then incubated in ice for 10 mins and centrifuged at 3000 *rpm* to collect the supernatant. Standards were prepared by serially diluted solutions of sodium nitrite ($NaNO_2$). The standards and samples (100 µl) were added into 96 well plate followed by adding 100 µl Griess reagent and incubated at RT for 5-10 mins for the color development. The microplate was read at 490 *nm* and quantification of samples were calculated using the standard curve generated with standards and its absorbance.

3.7 Enzyme-linked immunosorbent assay (ELISA)

The concentrations of myeloperoxidase (MPO), TNF-α, IL-1β, and IL-6 of mice colon homogenate and collected supernatant media from *in vitro* samples were measured using R&D brand ELISA kits according to manufacturer's instructions.

Principle

ELISA is an immunological method used for detection and quantification of proteins or antibodies. Since many types of ELISA assays in practice, sandwich ELISA (used in the present study) captures target antigen (antibody) in samples using specific antibody or antigen and of specific target molecule detection using an enzyme reaction with its substrate. The unbound antibodies are removed by wash buffer in between each step. The enzyme activity is measured colorimetrically and converted into numerical values.

Reagents required

1. Phosphate buffered saline (PBS)
2. Wash buffer: 0.05% Tween 20 in PBS, pH 7.2 - 7.4
3. Reagent diluent: 1% BSA in PBS, pH7.2 - 7.4
4. Substrate solution
5. Stop solution: 2N H_2SO_4

Plate preparation

Capture antibody was diluted using PBS into working concentration and immediately coated (100 µl/well) in a 96 well microplate. The plate was sealed and incubated overnight at room temperature. The next day, each well was washed with wash buffer (400 µl) for three times using ELISA plate washer. After wash, plates were blocked by adding (300 µl) reagent diluent to each well and incubated at room temperature for 2 hrs. This was followed by another set of washes and the plates were ready for sample addition.

Assay procedure

Test samples and standards were prepared in reagent diluent (100 µl/well) were added in duplicates. The plates were then covered with an adhesive strip and incubated

overnight at room temperature. Next day, plates were washed 3 times using ELISA washer and then detection antibody (100 µl/well) prepared with reagent diluent added in all wells. After 2 hrs incubation, plates were washed (3 times) and 100 µl of working concentration Streptavidin-HRP (prepared in reagent diluents) added to each well and incubated at room temperature for 20 minutes. After washing the plates (3 times), substrate solution (100 µl/well) was added to each well and incubated for 20 minutes at room temperature. After 20min, the reaction was stopped by adding stop solution (50 µl/well). Optical density (OD) was measured immediately after stopping the reaction using Tecan microplate reader absorbance set at 450 *nm*.

Calculation of results

The average OD value of blank is subtracted from average of each standards and samples in duplicates. The concentration of target proteins in samples was calculated from the equation obtained from the standard curve, which was generated by plotting the mean absorbance of standards on Y-axis and concentration on X-axis. The concentrations calculated from the standard curve were multiplied with dilution factor, in case the samples were diluted. Target protein concentration in the samples were normalized with the total protein content of homogenates (quantified using Pierce™ BCA Protein Assay Kit).

3.8 Preparation of cytosolic and nuclear Fractions

To separate cytoplasmic and nuclear fractions, the Thermo Scientific™ NE-PER nuclear extraction kit was used. Extraction reagents helped stepwise separation and preparation of cytoplasmic and nuclear extracts from mammalian cultured cells or tissue.

Reagents:

1. Cytoplasmic Extraction Reagent I (CER I)
2. Cytoplasmic Extraction Reagent II (CER II)
3. Nuclear Extraction Reagent (NER)
4. Phosphate-buffered saline (PBS)

Procedure:

The mouse colon tissue (20 µg) was cut into small pieces, placed in a microcentrifuge tube and washed with PBS. Then centrifuged at 500 x *g* and the supernatant was discarded. CER I (200 µl) was added to that tissue cuts and homogenized using zirconium beads (Precellys 24 homogenizer). The tube was vortexed vigorously for 15 seconds to fully suspend the tissue contents. The tube was incubated on ice for 10 minutes. To this tissue suspension, ice cold CER II (11 µl) was added and mixed by vortex. Then, homogenate was centrifuged at 16000 x *g* at 4°C and the supernatant (cytoplasmic extract) was immediately transferred and stored in a pre-chilled tube.

Insoluble pellet fraction (after collecting supernatant) contains the nuclear contents and suspended in ice cold NER (100 µl) and placed on ice. The cell suspension was then vortexed vigorously for 15 seconds for every ten minutes in total of 40 minutes. The cell suspension was again centrifuged at 16000 x *g* for 10 min at 4°C and the supernatant (nuclear fraction) was stored in pre-chilled tube. Protein quantification of both fractions were done using Pierce BCA protein assay kit.

3.9 Western blot

Separation of proteins

Proteins were separated by SDS-Polyacrylamide gel electrophoresis as described by Laemmli (1970).

Principle:

SDS-PAGE involves the separation of protein based on molecular weight. By heating the sample under denaturing and reducing conditions, proteins become unfolded and coated with SDS detergent molecule, acquiring a high net negative charge that is proportional to its polypeptide chain length. When loaded on to the gel matrix and once connected to an electric field, the negatively charged protein molecules tend to migrate towards the positively charged electrode and being separated by molecular sieving effect of gel matrix. Molecular weight protein marker that produces bands of known size helped to identify molecular weight of proteins (of interest).

Reagents:

1. 40 % Acrylamide/Bis solution 37.5:1 (Bio Rad, USA)
2. 10% (*w/v*) SDS: 10 g SDS was dissolved in 90 ml water with gentle stirring and made up to 100 ml with deionized water.
3. 1.5M Tris - HCl (pH 8.8): 18.15 g Tris base was dissolved in 80 ml deionized water, adjusted to pH 8.8 with 6N HCl and made up to total volume of 100 ml with deionized water and stored at 4ºC.
4. 0.5 M Tris - HCl (pH 6.8): 6 g Tris base was dissolved in 60 ml deionized water, adjusted to pH 6.8 with 6N HCl and made up to total volume to 100 ml with deionized water and stored at 4ºC.
5. 10% Ammonium persulfate APS (Fresh daily): 100 mg APS was dissolved in 1ml of deionized water freshly before use.
6. N′ N′ - Tetramethyl ethylene diamine (TEMED): Commercially available

7. 6X Sample Buffer (SDS Reducing buffer): 12% SDS, 40% glycerol, 30% β-mercapto-ethanol, 300 mM DTT, 120 mM EDTA, 1 mg/ml BPB, 375 mM Tris HCl pH 6.8.
8. 10X Electrophoresis buffer (2.5MTris, 1.92M Glycine and 1% SDS) (pH8.3): 30.3 Tris base, 144.0 g Glycine and 10 g SDS were dissolved in 800 ml deionized water and made up to total volume 1 litre with deionized water and stored at RT for further use. Usage: 50 ml of 10X buffer diluted with 450 ml of deionized water for each electrophoresis run.
9. Prestained protein molecular weight markers – Spectra multicolour broad range protein ladder (Thermo Fischer Scientific: Catalogue # 26634)
10. 10X Transfer buffer (250 mM Tris and 1.92 M Glycine) Transfer buffer (250 mM Tris and 1.92M Glycine) (pH7.6) 3.03 g Tris, and 14.4 g glycine were dissolved in 800 ml of double distilled water and made up to 1000 ml and stored at RT. Usage: 10 ml + 20 ml methanol + 70 ml double distilled water were added freshly in that ratio, just before use.
11. Polyvinylidene difluoride (PVDF) membrane (Thermo Scientific, USA Catalog no. 88518).
12. TBS (Tris-buffered saline) (150 mM NaCl and 10 mM Tris, pH7.6) 4. 383 g of NaCl and 10 ml of 1M Tris HCl (pH7.6) were dissolved in 450 ml of distilled water, adjusted the pH to 7.6 and made up to 500ml with distilled water.
13. TBS-T (Tris-buffered saline Tween-20) 500 µl of Tween 20 was dissolved in 500 ml of TBS and adjusted to pH 7.6.
14. Blocking solution: 5% BSA in TBS-T

Preparation of running gel

12% separating gel (16 ml) was prepared by mixing the reagents as shown below.

Deionized H_2O	–	6.9 ml
40% acrylamide/bis	–	4.8 ml
1.5M Tris - HCl (pH8.8)	–	4 ml
10% (*w/v*) SDS	–	160 µl
10% (*w/v*) APS	–	160 µl
TEMED	–	16 µl

6 % stacking gel was prepared by mixing the reagents as given below.

Deionized H_2O	–	6 ml
40% acrylamide/bis	–	4.8 ml
1.5M Tris - HCl (pH 8.8)	–	4 ml
10% (*w/v*) SDS	–	160 µl
10% (*w/v*) APS	–	160 µl
TEMED	–	16 µl

Separation of proteins

Protein samples from colon homogenate / cell lysate (20 µg) was diluted 5:1 with 6x sample buffer, heated at 95ºC for 10 mins and then cooled on ice for 5 min. Samples and prestained protein molecular markers were loaded to 8-12% SDS-PAGE in electrophoresis buffer in a Bio-Rad mini gel apparatus. Electrophoresis of protein was performed at 100 *V* (constant) until the dye front reaches the bottom of the running gel.

Transfer of proteins to the membrane and immunoblotting

After separation of proteins by SDS-PAGE, the stacking gel was cut and discarded; the separating gel was briefly rinsed in distilled water for 2-3 min and then equilibrated in cold transfer buffer under gentle agitation for 5 min. The proteins in gel transferred to PVDF membrane using Trans-Blot® Turbo™ transfer system (Bio-

Rad). The system is allowing rapid transfer of protein in 25 min. PVDF (0.45 µm pore size) was used. Bottom layer of transfer paper pack placed at center of cassette base followed by PVDF membrane, then gel and top layer of transfer paper pack to form a sandwich kind of packing. The roller was used to get rid of bubbles between gel and membrane. After that cassette closed by cassette lid and placed in Trans-Blot Turbo instrument.

After transfer around 20-30 min, the PVDF blot was removed from the transfer system, transferred into a box with 5% BSA blocking solution for 1 hrs (to block the areas where proteins are not attached in membrane). After 1 hrs of blocking, decanted the blocking solution and rinsed/washed the membrane in TBS-T and incubated in 5% BSA-TBS-T containing primary antibody at appropriate dilutions overnight. Following day, the blot was washed for four times (10 min each) with TBS-T. After washing, blot was incubated for 1 hrs with horseradish peroxidase (HRP)-conjugated (mouse/rabbit/rat) secondary antibody, diluted around 1:10000 with TBS-T. After secondary incubation, the blot was washed for four times (10 mins each) with TBS-T. Super Signal West Pico Plus chemiluminescent substrate was used as a detection reagent system to develop the blots. Briefly, the blots were incubated with that substrate for 3-4 min and excess reagent drained off. Sapphire Biomolecular Imager (Azure Biosystems, Dublin, California, USA) was used to capture the protein bands through the chemiluminescent detection of HRP along with the color image of the protein ladder. The captured protein bands were subjected to densitometric analysis using Image J software.

<u>Stripping and re-probing the membrane</u>

To confirm, equal quantity of protein was loaded on to each wells of the gel. Membrane stripping step was performed using commercially available Stripping Buffer (Restore, Thermo Scientific, USA) for 1 hrs followed by washing with TBS-T six times in every ten mins interval. Then, the blots were re-blocked in 5% BSA for 1 hrs followed by overnight incubation with GAPDH re-probing (internal control for western experiments).

3.10 Gene expression analysis

Isolation of total RNA

Total RNA was isolated from control/experimental samples using Trizol extraction, 100 mg fresh tissue was homogenized with 1ml Trizol reagent with zirconium beads using Precellys 24 Homogenizer. Homogenate was transferred immediately to a fresh microcentrifuge tube. For cell samples, cells are lysed with Trizol for RNA extraction. Then, 0.2 ml chloroform was added, vortexed for 1 min and placed on ice at 4°C for 5 min. The homogenates were centrifuged at 12,000 x *g* for 15 min at 4°C. The aqueous phase was carefully transferred into a fresh microcentrifuge tube and an equal volume of isopropanol was added, vortex for 15 sec and placed on ice for 10 min. The samples were centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was discarded, and RNA pellet was washed with 1 ml of 75% ethanol by vortex and subsequently centrifuged for 5 min at 7,500 x *g* (4°C). The supernatant was removed, and RNA pellets were dissolved using 50 µl autoclaved Milli-Q water and allowed to dissolve completely, by placed in hot water bath for 10 min at 60°C.

Quantification of RNA

Diluted RNA sample was quantified spectrophotometrically (Nanodrop 2000/2000c Spectrophotometers – Thermo-Scientific were used) by measuring the absorbance (A) at 260/280 *nm*. 40 μg of RNA in 1 ml gives one absorbance at 260 *nm*. Therefore, the concentration of RNA in the given sample can be determined by multiplying its A260 by 40 and dilution factor. The purity of RNA can be calculated using the ratio between its absorbance at 260 and 280 *nm*. A ratio of absorbance at 260/280 *nm* > 1.8 is generally considered as good quality RNA.

Real-time polymerase chain reaction (RT-PCR)

RT-PCR is a molecular biology technique used to monitor the amplification of the target DNA sequence and amplification of DNA monitored in real time as it occurs.

Principle:

RT-PCR is a method used to amplify cDNA copies of RNA (enzymatic conversion of mRNA into a single cDNA template). A specific oligodeoxynucleotide primer hybridized into mRNA and is then extended by RNA dependent DNA polymerase to create a complementary DNA copy (cDNA). In principle, PCR amplifies DNA exponentially, doubling the number of target molecules with each amplification cycle. In real-time PCR, DNA amount is measured after each cycle using fluorescent dyes yield increasing fluorescent signal, directly proportional to number of PCR product (amplicons) generated. If a specific sequence (DNA or RNA) is abundant in test sample, amplification can be observed in much earlier cycles; if amount is scarce, amplification observed only in later cycles.

Conversion of RNA to cDNA

High-capacity cDNA reverse transcription kit from Applied Biosystems was used for the conversion of RNA into cDNA.

Reagents:

10X RT buffer

25X dNTP Mix (100 mM)

10X RT Random Primers

Multiscribe™ Reverse Transcriptase enzyme

Nuclease free water

Procedure:

The kit components allowed to thaw on ice and 2X cDNA conversion master mix was used. Total reaction volume of 20 µl was prepared as per Table 5.

Table 5: Preparation of cDNA concversion master mix

Component	Volume/Reaction (µl)
10X RT buffer	2
25X dNTP Mix (100 mM)	0.8
10X RT Random Primers	2
Multiscribe™ Reverse Transcriptase	1
Nuclease free water	4.2
Total per reaction	10

After thawing of kit components, master mix was prepared as above-mentioned table and placed on ice and 10 µl needed for each tube (Kit capable to convert up to 2 µg RNA). 2 µg total RNA was taken and made up to the volume of 10 µl using nuclease free water. cDNA conversion master mix (10 µl) was added into tube containing 2 µg total RNA (10 µl), thus making the total reaction volume into 20 µl. The tubes were briefly centrifuged to remove air bubbles and loaded in a thermal cycler (kit specific

temperature cycles) for cDNA synthesis. The synthesized cDNA was diluted accordingly and stored at -20°C.

Steps involved in RT-PCR

There are three major steps that make up each cycle in a real-time PCR reaction. Reactions are generally run for 40 cycles.

1. Denaturation: High temperature incubation is used to 'melt' double-stranded DNA into single strands and loosen secondary structure of single-stranded DNA. The highest temperature that the DNA polymerase can withstand is typically used (usually 92-95°C).

2. Annealing: During annealing, complementary sequences have an opportunity to hybridize on that single strands with proper temperature, that temperature calculation based on melting temperature (Tm) of primers (5°C below the Tm of primer).

3. Extension: At 70 - 72°C, the activity of DNA polymerase is optimal, and primer extension ensued at rates of up to 100 bases per second.

Procedure

The PCR assays were carried out using SYBR green dye-based PCR. SYBR green is a commonly used fluorescent DNA binding dye that binds into double stranded DNA. Detection is monitored by measuring the increasing fluorescence throughout the cycle. 40 ng cDNA were used in PCR assays and the mastermix is prepared as per Table 6. The primer details used for assays are listed in Table 7.

Table 6: PCR master mix preparation of for sample/well

SYBR® Select Master Mix (2X)	10 µl
Forward primer	0.4 µl
Reverse primer	0.4 µl
Nuclease-free water	7.2 µl
Total volume	18 µl

18 µl master mix prepared as per gene of interest and then 2 µl corresponding cDNA were added in duplicates. The plate was then sealed and briefly centrifuged at 1000 *rpm* to spin down the components to the well bottom and eliminated air bubbles. Real-time polymerase chain reaction (PCR) was performed using Quant Studio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, MD, USA). The obtained data were normalized against 18s RNA (used as a reference gene), and the comparative CT ($2^{-\Delta\Delta CT}$) method was used to calculate relative quantification for mRNA expression. CT is the cycle number at which the fluorescent signal of reaction crosses the threshold limit. Threshold limit of RT-PCR refers to the signal reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish relevant amplification signal from the background.

Table 7: Primer details

Gene	Forward	Reverse	PMID No	Gene Accession Number
CXCL-2 (mouse)	5'-GGATGGCTTTCATGGAAGGAG-3'	5'-TTGCTAAGCAAGGCACTGTGC-3'	22326488	NM_009140.2
CCL2 (mouse)	5'-CAGCCAGATGCAGTTAACGC-3'	5'-GCCTACTCATTGGGATCATCTTG-3'	10953027	NM_011333.2
IL-6 (mouse)	5'-ACAAGTCGGAGGCTTAATTACACAT-3'	5'- TTGCCATTGCACAACTCTTTTC-3'	21735552	X06203
TNF-α (mouse)	5'-AGGCTGCCCCGACTACGT -3'	5'-GACTTTCTCCTGGTATGAGATAGCAAA-3'	21705622	NM_013693.2
IL-1β (mouse)	5'-TCGCTCAGGGTCACAAGAAA-3'	5'-CATCAGAGGCAAGGAGGAAAC -3'	21735552	NM_008361.4
COX-2 (mouse)	5'-AACCGCATTGCCTCTGAAT -3'	5'-CATGTTCCAGGAGGATGGAG -3'	22158945	NM_011198.4
iNOS (mouse)	5'-CGAAACGCTTCACTTCCAA -3'	5'- TGAGCCTATATTGCTGTGGCT -3'	22158945	BC062378.1
NRF-2(mouse)	5'-GAGCTAGATAGTGCCCCTGG-3'	5'-CAGGACTCACGGGAACTTCT-3'	29162986	U20532.1
HO-1 (mouse)	5′-AAGCCGAGAATGCTGAGTTCA-3′	5′-GCCGTGTAGATATGGTACAAGGA-3′	25112868	BC010757.1
SOD-3 (mouse)	5'-TTCTACGGCTTGCTACTGGC-3'	5'-GCTAGGTCGAAGCTGGACTC-3'	26513461	NM_011435.3
18S (mouse)	5'-CCCCTCGATGACTTTAGCTGAGTGT -3'	5'-CGCCGGTCCAAGAATTTCACCTCT -3'	22427817	NR_003278
CXCL-1 (human)	5'- GCGGAAAGCTTGCCTCAATC – 3'	5'- GGTCAGTTGGATTTGTCACTGT – 3'	25938459	BC011976.1
IL-8 (human)	5'-CTGATTTCTGCAGCTCTGTG-3'	5'-GGGTGGAAAGGTTTGGAGTATG-3'	20150959	BC013615.1
CXCL2 (human)	5'- TTTATTGTGGGCTTCACACG-3'	5'- GATTTGCGCACACAGACAAC-3'	17591792	NM_004591.3
COX-2 (human)	5'- ACAGTGTGTGGTCAACATTTCTC – 3'	5'- TCGAAACCTCTCTGCTCTAACAC – 3'	25938459	BC015753.1
18S (human)	5'-GTGGAGCGATTTGTCTGGTT-3'	5'-AACGCCACTTGTCCCTCTAA-3'	25369870	NR_003286

3.11 Intestinal permeability assay using FITC-dextran in mice

A disruption of epithelial barrier integrity leads to an increased intestinal permeability, a key event involved in UC pathogenesis. The permeation of FITC dextran through the paracellular pathways of intestinal epithelium is an indicator of compromised epithelial barrier integrity.

Materials and reagents required

1. Fluorescein isothiocyanate–dextran (FITC-dextran)
2. Sterile 1x phosphate buffered saline (PBS) – pH - 7.4
3. BD Microtainer blood collection tubes.

4. Syringes
5. Spectrophotoflurometer – (Tecan multimode plate reader used)
6. Opaque 96 well microplates

Procedure

Colitis was induced in C57BL/6J male mice by the same way already explained in experimental design section. Penultimate day evening of experiment, water bottles were removed from all group's animal cages, to make mice water starve overnight. On experiment day (final day), FITC-dextran prepared (dose of 60 mg/100 g body weight) in PBS was administered to each mouse by oral gavage. After 4 h, the animals were anesthetized and blood was collected and transferred to a microtainer SST tube for serum collection. The blood was mixed by inverting the tube for 3-4 times and stored in dark. Separated serum was diluted with an equal volume of PBS, and then diluted serum (100 µl) is added in to a black 96-well plate in duplicates. The fluorescence intensity of serum was determined by a spectrophotoflurometer with set an excitation wavelength of 485 *nm* and an emission wavelength of 528 *nm*. The concentration of FITC-dextran in serum is calculated by comparing with standards prepared by the serial dilution of FITC-dextran at 0, 125, 250, 500, 1000, 2000, 4000, 6000, 8000 ng/ml concentration by standard curve method. To calculate background emission value, serum from a mouse which was not administered FITC-dextran was used.

3.12 TEER experiments using Caco-2 cells

Principle

Transepithelial electrical resistance (TEER) is the measurement of electrical resistance across a cellular monolayer. This method is utmost sensitive and reliable to assess the integrity and permeability of cell monolayer. TEER is measured in ohms

and represents the ionic conductance of paracellular route in epithelial monolayer. For TEER measurements, cellular monolayer was allowed to form on a semipermeable filter insert (transwell), which defines a partition for apical (or upper) and basolateral (or lower) chambers. Two electrodes were used to measure TEER values, one electrode is placed in apical chamber and other in basolateral chamber and the readings are taken using epithelial volt/ohm meter (Figure 1), (Model: EVOM^2, WPI, Sarasota County, Florida, United States). The pair of electrodes of EVOM is called as STX2/chopstick electrodes. Each stick of the electrode pair has a silver/ silver-chloride pellet for measuring voltage and a silver electrode for passing current. TEER measurement of semipermeable insert containing media only (without cells) is considered as value of blank resistance (R_{BLANK}) and resistance measured from transwell containing monolayer is taken as (R_{TOTAL}). The cell specific resistance (R_{TISSUE}) in units of Ω, is calculated by subtracting R_{BLANK} from $R_{TOTAL.}$ The resistance recorded by EVOM is inversely proportional to the transwell surface area. Hence, final TEER values are calculated using the following method and is always expressed as Ω.cm^2. $TEER_{REPORTED} = R_{TISSUE}\ (\Omega) \times M_{AREA}\ (cm^2)$

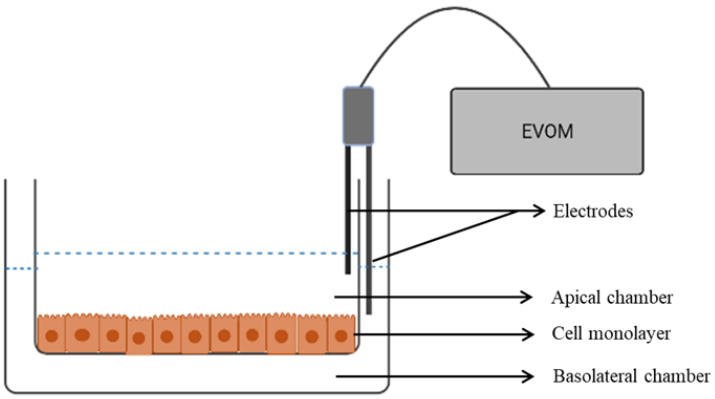


Figure 1: TEER measurement with chopstick electrodes

Cell seeding method for TEER measurements

Caco-2 cells were utilized to evaluate intestinal epithelial barrier integrity. Cells were seeded (density of 1.5 x 10^5 cells/well) into the apical chamber (transwell insert - 1.12 cm^2 size), with polyester membrane (0.4 μm pore size) attached in the bottom, can be placed in a 12-well (can hold transwell insert) plate (basolateral chamber). Cells were allowed to proliferate and differentiate for 14-16 days to form a confluent monolayer in that transwell insert. DMEM medium supplemented with 2% (*v/v*) FBS were added (1 ml each) in apical chamber and basolateral chamber. One well per plate was maintained without cells for blank reading. Supplemented DMEM media was replaced every two days.

Measurement of TEER

Caco-2 monolayer integrity was determined by measurement of TEER values using an EVOM instrument and denoted as Ω.cm^2, calculated by multiplying the resistance (Ω) with grown monolayer surface area (1.12 cm^2), as explained before. Monolayers insert with TEER values around 1200 - 1400 Ω.cm^2 were selected for the experiment. Once desirable values of TEER reached, those transwells were treated with NED at 30 and 60 μM concentrations. After 24 h, the monolayers of apical chamber were incubated with LPS at 100 μg/ml to induce inflammation. The control and inflamed monolayer TEER values were recorded every 3 hrs for 24 hrs period. The resistance observed in unseeded well (approximately 60 Ω.cm^2) served as blank, were subtracted from all experiment values for background correction.

3.13 Statistical Analysis

All statistical analysis was carried out using SPSS Software (Armonk, New York, USA). Comparisons between groups were performed by one-way analysis of

variance (ANOVA), followed by Tukey's post hoc test for multiple comparisons. Data are plotted as mean ± SEM in the figures. *P* values <0.05 were considered statistically significant.

Chapter 4: Results and Discussion

4.1 Study 1: Role of NED as an anti-inflammatory compound in LPS-stimulated RAW 264.7 macrophages

The anti-inflammatory effect of NED was evaluated using RAW 264.7 macrophages, a well-established *in vitro* model for inflammatory experiments. These cells were stimulated with LPS to cause an inflammatory condition and then NED role in modulating the proinflammatory cytokines protein expression of iNOS and COX-2 were investigated.

Results:

4.1.1 Effect of NED on cell viability of RAW 264.7 macrophages

Cell viability of macrophages after NED exposure were performed to find out any cytotoxic effect of NED and the safe dose range. Cells were treated with different concentrations of NED ranging from 0 µM to 100 µM for 24 and 48 hrs. NED was observed to be safe in all the dose ranges studied at both 24 and 48 hrs (Figure 2a and Figure 2b). Based on these observations, 30 and 60 µM dose of NED were selected for subsequent studies on macrophages.

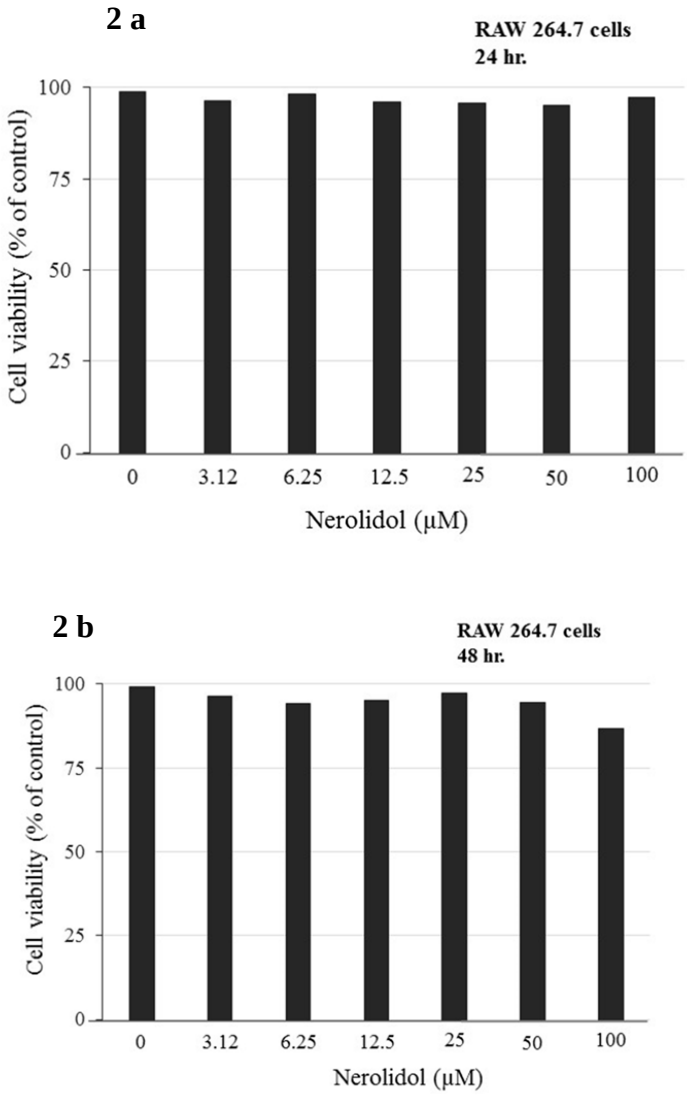


Figure 2: Effect of NED on cell viability of RAW macrophages

The concentration-dependent cytotoxic effects of NED were investigated using RAW 264.7 macrophages cells treated with different concentrations of NED for 24 hrs and 48 hrs.

4.1.2 Effect of NED on proinflammatory cytokine release and mRNA expression in RAW 264.7 macrophages stimulated with LPS

LPS-stimulated macrophages showed a significant (all $p < 0.001$) increase in the release of proinflammatory cytokines; TNF-α, IL-1β, and IL-6 (Figure 3a, 3b, and 3c). NED co-treatment significantly ($p < 0.01$ for TNF-α at NED 30 µM and $p < 0.001$ at NED 60 µM; For IL-1β, $p < 0.05$ at NED 30 µM and $p < 0.01$ at 60 µM; For IL-6,

$p < 0.01$ at NED 30 µM and $p < 0.001$ at NED 60 µM) decreased the secreted proinflammatory cytokines in the supernatant (Figure 3). LPS treatment significantly ($p < 0.001$) increased mRNA expression of these cytokines. NED co-treatment significantly ($p < 0.01$ for TNF-α at NED 30 µM and 60 µM. For IL-1β, $p < 0.05$ at NED 30 µM and at 60 µM. For IL-6, $p < 0.01$ at NED 30 and at NED 60 µM) prevented the increased mRNA expression of these cytokines (Figure 3d, 3e, and 3f). However, NED (60 µM) alone did not show any significant effect compared to untreated control. Therefore, NED alone treatment group was removed from the subsequent experiments.

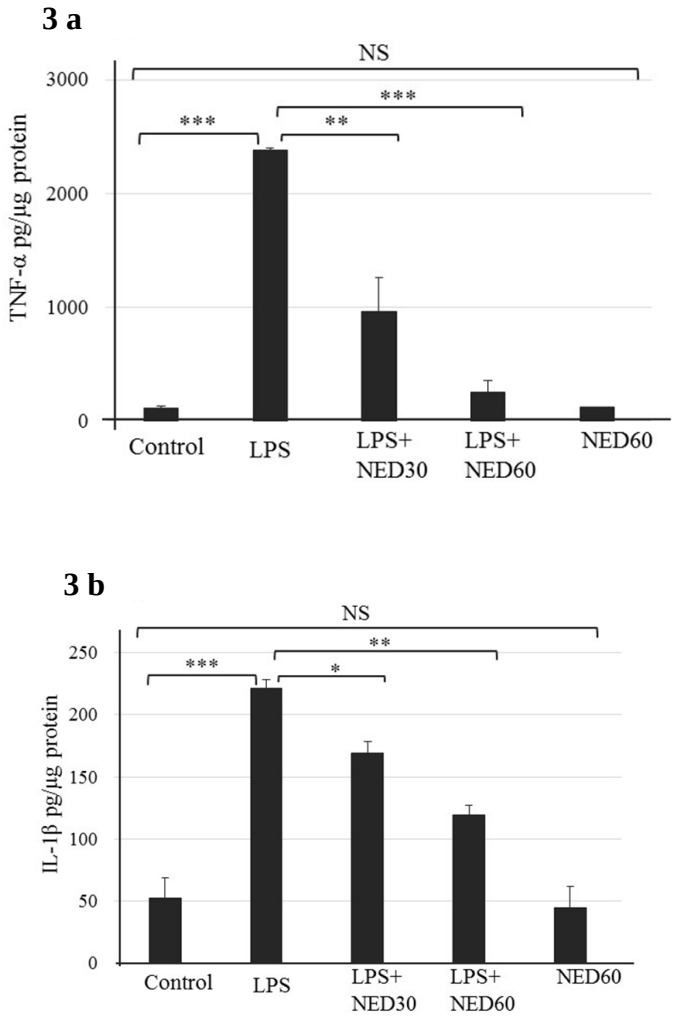
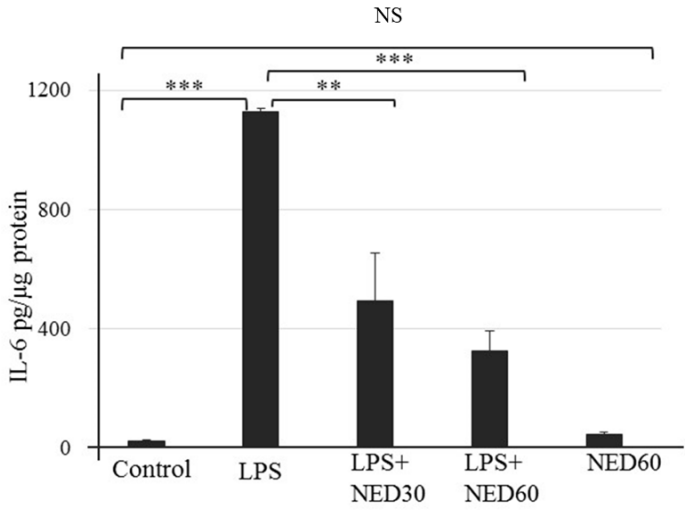


Figure 3: Effect of NED co-treatment on the proinflammatory cytokine release and mRNA expression in RAW 264.7 macrophages stimulated by LPS.

3 c



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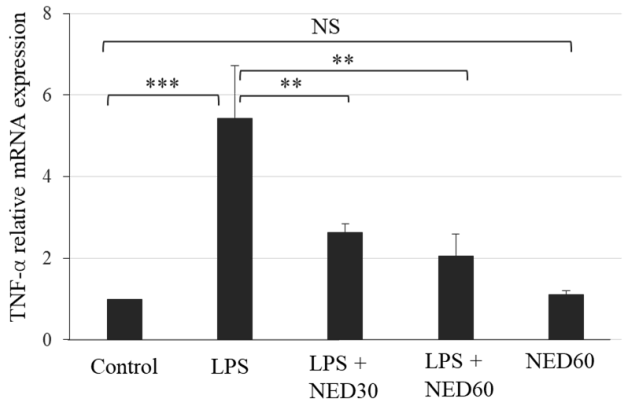

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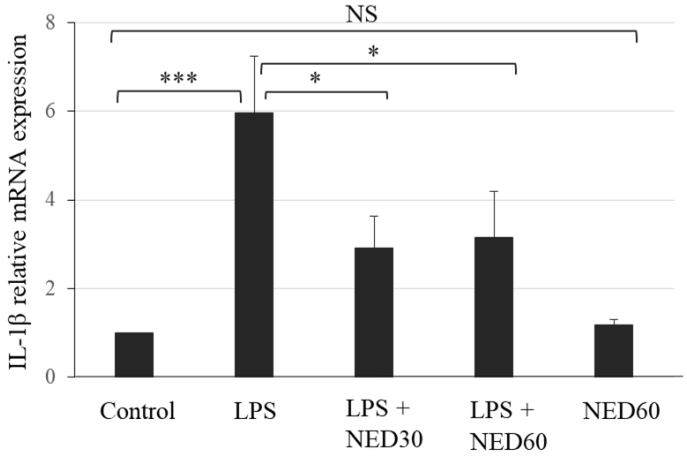


Figure 3: Effect of NED co-treatment on the proinflammatory cytokine release and mRNA expression in RAW 264.7 macrophages stimulated by LPS (continued).

3 f

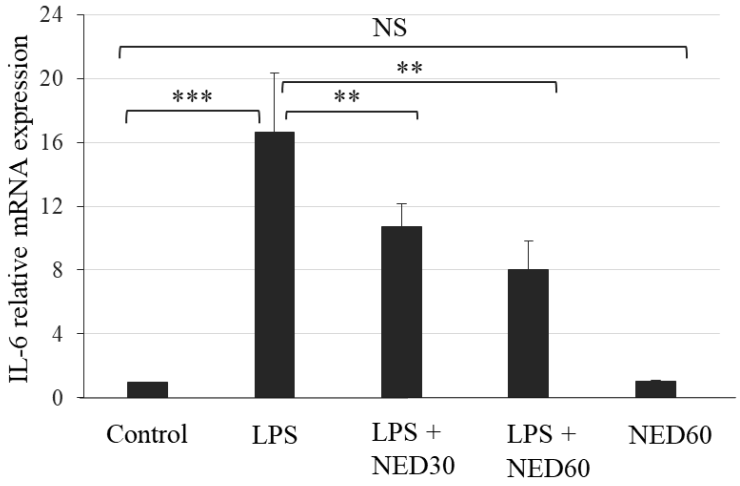


Figure 3: Effect of NED co-treatment on the proinflammatory cytokine release and mRNA expression in RAW 264.7 macrophages stimulated by LPS (continued).

NED-treatment at 30 and 60 µM significantly decreased the LPS induced increase in the release of proinflammatory cytokines TNF-α, IL-1β and IL-6 (Figure. 3a, 3b and 3c) and their mRNA expression (Figure. 3d, 3e and 3f). Data were obtained from n = 6 samples for ELISA and mRNA expression. The results expressed as mean ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant. NS indicate not significant.

4.1.3 Effect of NED on the expression of COX-2 and iNOS protein in RAW 264.7 macrophages stimulated with LPS

The effect of NED on the COX-2 and iNOS protein expression in LPS stimulated RAW 264.7 macrophages were evaluated using Western blot. LPS stimulation significantly increased COX-2 and iNOS protein expression; ($p < 0.01$) (Figure 4a and b). NED treatment prevented the further rise of COX-2 protein expression induced by LPS in a dose-dependent manner, ($p < 0.05$ at NED 30 µM and $p < 0.01$ at NED 60 µM) (Figure 4a). Similarly, NED decreased iNOS protein expression at both doses ($p < 0.05$ at 30 µM NED and $p < 0.01$ at 60 µM NED) (Figure 4b).

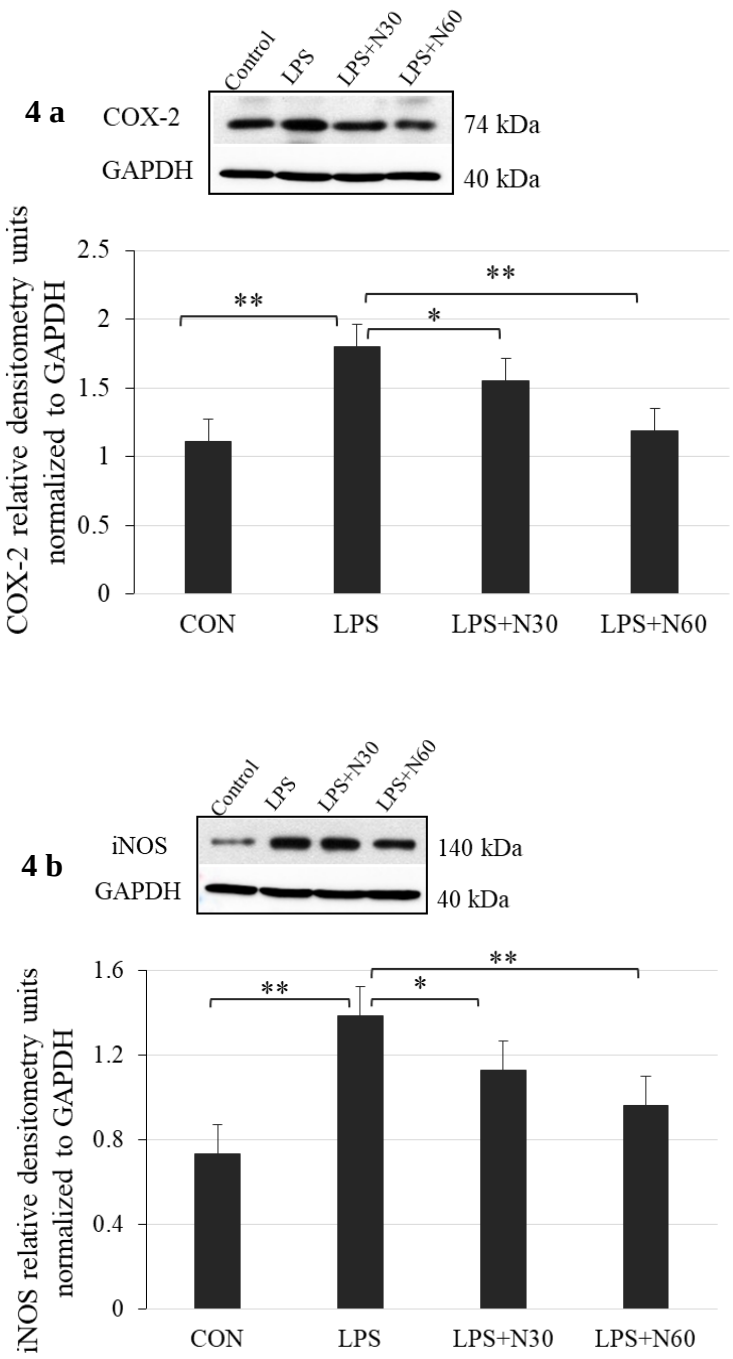


Figure 4: Effect of NED on the expression of COX-2 and iNOS protein in RAW 264.7 macrophages stimulated with LPS.

Co-treatment with NED significantly decreased the LPS induced expression of COX-2 and iNOS proteins in RAW macrophages (Figure 4a and 4b). Data were obtained from $n = 4$ animals and expressed as means ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.1.4 Discussion

Inflammation generally occurs as body's defense mechanism against any infections. Sometimes, these immediate inflammatory responses go unchecked and may lead to chronic inflammation, which in turn results in tissue injury/damage, if uncontrolled (Medzhitov, 2010). Chronic inflammation plays a critical role in the progression of various pathological conditions including type-2 diabetes, Alzheimer's disease, cardiovascular diseases, and IBD (Elisia et al., 2018). Further, chronic inflammation is the leading cause of tumorigenesis and its progression (Limtrakul, Yodkeeree, Pitchakarn, & Punfa, 2015). The beneficial effects of dietary phytochemicals in these chronic inflammatory conditions are well established (Casas, Sacanella, & Estruch, 2014; Ricker & Haas, 2017; Steckhan et al., 2016). Plant extracts' therapeutic effects have been exploited for centuries and identifying and characterizing these components may provide a better alternative treatment option for inflammatory conditions and consumption of plant-based diet is always linked with a lesser risk of developing chronic inflammatory diseases (Aune et al., 2017). Therefore, it is imperative to investigate plant-based bioactive phytochemicals' therapeutic potential against inflammation.

Macrophages are inflammatory cells, constitute an important part of the innate immune system that acts on foreign pathogens by the phagocytosis method (Hou et al., 2015). Once a foreign antigen enters the body, and its pathogen-associated molecular pattern (PAMPs) is recognized by the pattern recognition receptors (PRR) of macrophages during inflammation. Upon activation by foreign antigens, macrophages secrete proinflammatory cytokines/mediators like nitric oxide (NO) and PGE_2 derived from iNOS and COX-2 as an immediate response (Alderton, Cooper, & Knowles,

2001; Rajakariar, Yaqoob, & Gilroy, 2006). These cytokines/mediators act on these toxic/harmful pathogens (bacteria), mitigate the inflammation, and protect the host by facilitating healing processes. However, an extended period of elevated cytokines progress in to chronic inflammation, which is detrimental to normal physiological conditions and immune responses (Muniandy et al., 2018). Toll-like family receptors (TLR) belong to the PRR family, TLR-4 in particular, recognize the lipopolysaccharides (LPS), a cell wall component of gram-negative bacteria and activate the NF-κB pathway, that in turn regulated the transcription of genes involved in various biological processes, including inflammation (Limtrakul et al., 2015; Tan, Arulselvan, Karthivashan, & Fakurazi, 2015). Therefore, LPS isolated from *E.coli* serves as macrophages stimulant, caused the elevated proinflammatory mediators/cytokines production.

RAW 264.7 murine macrophages are a well-accepted *in vitro* model to investigate novel compounds for their anti-inflammatory properties. When challenged with LPS, as an inflammatory stimulus, these cells (tremendously sensitive) initiate a robust inflammatory response by secreting large amounts of proinflammatory cytokines/mediators involved in the inflammation (Berghaus et al., 2010). RAW macrophages can grow continuously in culture media due to perpetual changes made in their genes and cause bacterial ligand-activated signaling events (Hartley et al., 2008). The extensive use of macrophages as an *in vitro* model for inflammation is mainly attributed to its commercial availability and easy culture and maintenance practices.

Stimulation with bacterial LPS can trigger via TLR-4 signaling (Figure 5). The binding of LPS to TLR-4 causes the dimerization of TLR and recruitment of adaptor

molecules to its Toll/Interleukin-1 receptor (TIR) domain. Following that recruitment of MyD88, adaptor protein inducing IFN-β (TRIF) to TIR domain, resulted in the synthesis of proinflammatory cytokines and type-I IFNs. The binding of MyD88 to TLR-4, further recruits IL-1R associated kinase-4 (IRAK-4), and in turn, phosphorylates IRAK-1 and binds with TNFR associated factor-6 (TRAF-6). TRAF-6, along with ubiquitin-protein ligase (E3), catalyzes the formation of TGF-β-activated kinase-1(TAK1) and TAK1-binding proteins (TAB-1, -2, -3). This activation of TAK/TAB triggers MAPKs and NF-κB signaling cascades. IKK-β, accompanied by IKK-α and NEMO (IKK-γ), constitute the IKK complex are known to initiate IκB-α (an NF-κB inhibitory protein that prevents NF-κB nuclear translocation) phosphorylation. Upon phosphorylation, IκB-α undergoes ubiquitination and allows NF-κB to translocate into the nucleus and activate transcription of inflammatory cytokines and other inflammatory mediators like COX-2 and iNOS by binding to gene promoter regions. Inhibition of NF-κB signaling has been shown to suppress those inflammatory responses indicated by reduced levels of proinflammatory cytokines like TNF-α, IL-1β, and IL-6 (Ham et al., 2015; Hsieh, Chang, Teng, Chen, & Yang, 2011). TAK1 also phosphorylates MAP kinase kinase kinases (MKK6) by activating the MAP kinase pathway (Akira, Uematsu, & Takeuchi, 2006). Thereby, a compound that can inhibit this target pathway could be a potential anti-inflammatory agent.

In this present study, the anti-inflammatory effect of NED was investigated on RAW 264.7 macrophages stimulated with LPS (an endotoxin, can mimic a real case of bacterial invasion (Limtrakul et al., 2015). Cell viability assay of NED indicated that doses up to 100 μM at both 24/48 hrs were not toxic to the cells. LPS stimulated RAW macrophages secreted an elevated amount of proinflammatory cytokines TNF-α, IL-1β, and IL-6, as documented by ELISA/RT-PCR. These cytokines (proteins) act

as messengers between cells and play a significant role in the innate immune responses against inflammation (Schett, Elewaut, McInnes, Dayer, & Neurath, 2013).

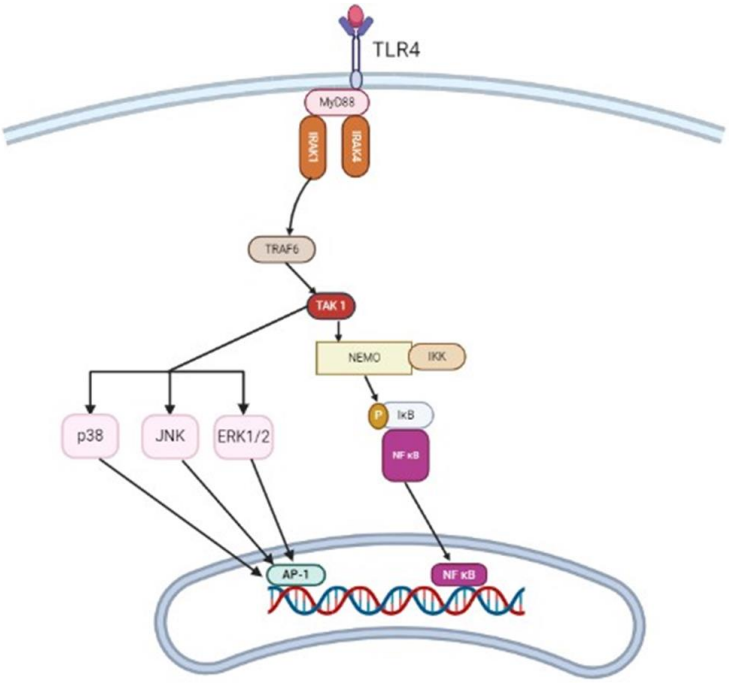


Figure 5: LPS induced TLR-4 signaling - MyD88 dependent pathway

These cytokines' interdependency caused amplification of the inflammatory response, resulting in cytokines secretion in large quantities. This means that these cytokines are a part of a cascade mechanism, where one cytokine can influence release of other cytokines. For example, IL-1 can stimulate the production of TNF-α and IL-6 (Knofler, Kiss, Mosl, Egarter, & Husslein, 1997; Schulte, Bernhagen, & Bucala, 2013; Tanabe et al., 2010). Therefore, a compound that can prevent the production of any of these cytokines, invariably affects the production of other ones.

NED treated group recorded a reduction in proinflammatory cytokines levels in LPS stimulated RAW macrophages and a higher dose of NED (60 µM) exerted its

maximum effect. However, NED-treated control group did not demonstrate any significant effect, when compared to the control. Subsequently, the NED alone treated group was omitted for further protein expression analysis of this study. The proinflammatory cytokines mRNA expressions were also increased by LPS treatment and NED treatment prevented this increase in a dose-dependent manner. These observations can be correlated with a previous study, where NED was shown to prevent the elevated secretion of IL-6 and IL-1β in LPS stimulated peritoneal macrophages (Fonseca et al., 2016). Increased secretion of cytokines for a more extended period may result in chronic inflammation. So, regulating cytokine synthesis is a critical step for any anti-inflammatory therapeutic compound and considered as an accepted therapeutic intervention.

iNOS (an inflammatory mediator) is expressed by many inflammatory cells, including macrophages, after recognizing pathogens (Kobayashi, 2010; Leppanen, Tuominen, & Moilanen, 2014). LPS is known to enhance iNOS expression on macrophages involved in nitric oxide (NO) production from L-arginine. During inflammation, NO increases the vascular permeability and recruits more and more inflammatory cells into the inflamed area and deteriorates the condition (Muniandy et al., 2018). Cycloxygenase-2 (COX-2) is another proinflammatory mediator, shown to be upregulated in macrophages upon LPS-stimulation. Upregulated COX-2 initiated PGE_2 formation, which causes localized vasodilatation and allowed entry of neutrophils and macrophages (Limtrakul et al., 2015; Muniandy et al., 2018). LPS treatment induced the increased expression of both iNOS and COX-2, and that increase was prevented significantly by NED co-treatment. These results indicated that NED orchestrated its effects by suppressing the production of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) and associated inflammatory mediators (iNOS and COX-2).

Based on these *in vitro* results, further evaluation of the NED's effects on colon inflammation were carried out using *in vivo* and *in vitro* models of colon inflammation.

4.2 Study 2: Role of NED as an anti-inflammatory and antioxidant compound in *in vivo* and *in vitro* models of colon inflammation

In the preliminary investigations, NED showed a potent anti-inflammatory effect in LPS-stimulated RAW macrophages. Based on these results, further studies were designed to investigate the anti-inflammatory and antioxidant properties of NED in *in vivo* and *in vitro* models of colon inflammation. TNF-α-stimulated HT-29 cells were used to examine the effects of NED on proinflammatory chemokines expression.

Results:

4.2.1 Effect of NED on Disease Activity Index (DAI)

In C56BL/6J mice, 3% DSS in drinking water induced distinct features of ulcerative colitis. The DAI score was very high in the DSS animals compared to the control group ($p < 0.001$). NED treatment of DSS-colitis animals at the higher doses improved the DAI scores significantly (100 mg and 150 mg/kg bd wt; $p < 0.01$). However, at the lowest dose (50 mg/kg bd wt), NED treatment had shown no significant effect. SAZ group animals, as a positive control showed significant ($p < 0.05$) improvement in DAI score (Figure 6).

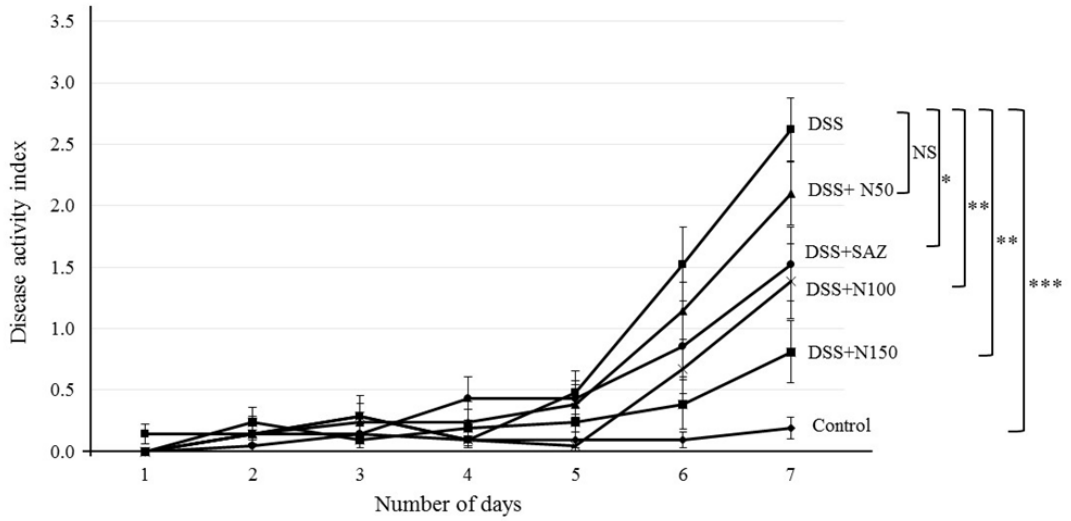


Figure 6: Effect of NED on disease activity index (DAI). DSS-treatment significantly increased the DAI score.

SAZ and the higher concentrations of NED significantly prevented the increase in DAI scores induced by DSS. However, the lowest dose of NED (50mg/kg) treatment had no effect. Data were obtained from $n = 10$ animals in each group and are expressed as mean ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, P value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ was considered statistically significant.

4.2.2 Effect of NED on colon length, myeloperoxidase (MPO) concentration, and CXCL-2 and CCL2 mRNA

DSS-induced colitis displayed ($p < 0.01$) a marked decrease in colon length. SAZ and NED treatment prevented the shortening of colon length at higher doses of NED (100 mg and 150 mg/kg, $p < 0.05$, (Figure 7a & 7b). However, the lowest dose of NED was not effective in preventing the colon length shortening.

MPO is a marker to assess the level of neutrophil infiltration into submucosa, indicating the degree of inflammation. DSS treatment ($p < 0.01$) increased MPO concentrations compared to untreated control group significantly (Figure 7c). NED treatment at the two higher doses and SAZ treatment significantly decreased MPO

concentration ($p < 0.01$, Figure 7c). However, the lowest dose (50 mg/kg) of NED had no significant effect.

Chemokines CXCL-2 and CCL2 (markers of macrophage infiltration) mRNA expression were analyzed. DSS treatment significantly increased the expression of both CXCL-2 (Figure 7d and 7e) ($p < 0.01$) and CCL2 ($p < 0.001$), was substantiated by macrophage infiltration observed in the following histoarchitecture analysis. NED treatment (100 mg and 150 mg/kg) and SAZ treatment significantly decreased the expression of both CXCL-2 and CCL2 (Figure 7d and 7e). However, the lowest dose (50 mg/kg) of NED did not induce any effect on CXCL-2 and CCL2 expression significantly.

7a

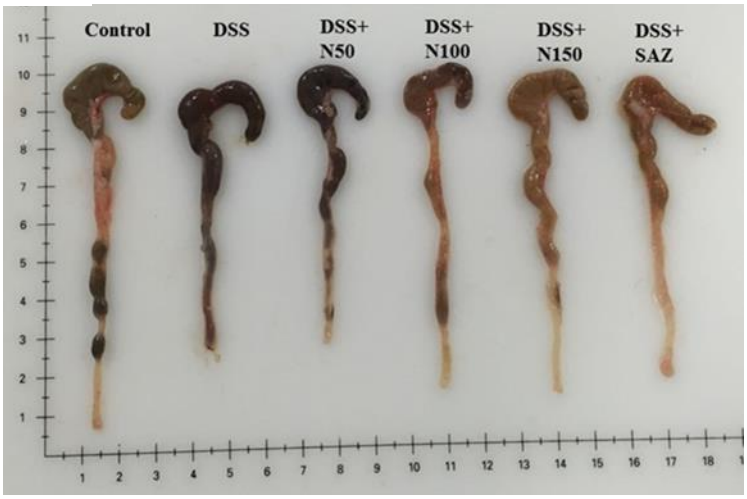
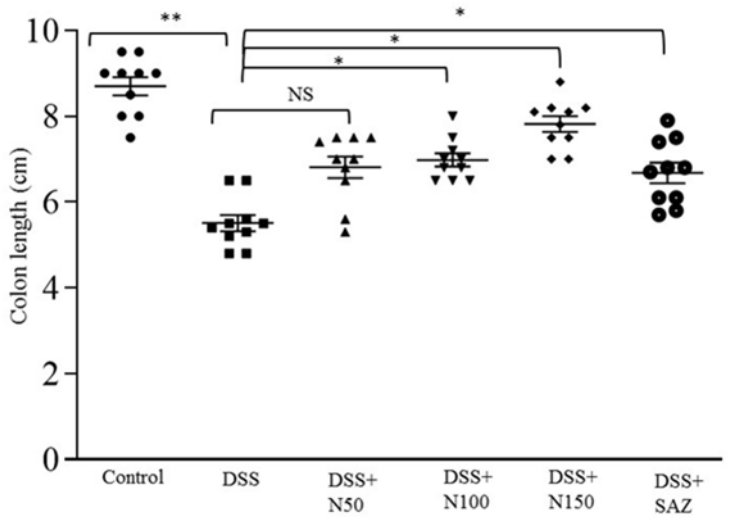
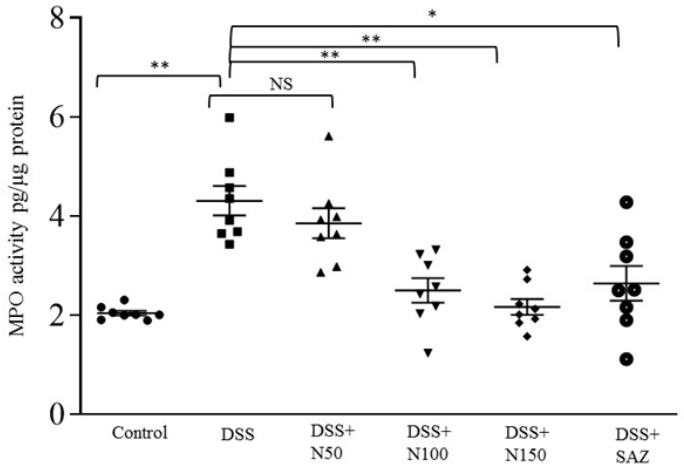


Figure 7: Effect of NED on colon length, myeloperoxidase (MPO) concentration, CXCL-2 and CCL2 mRNA macrophage markers.

7b



7c



7d

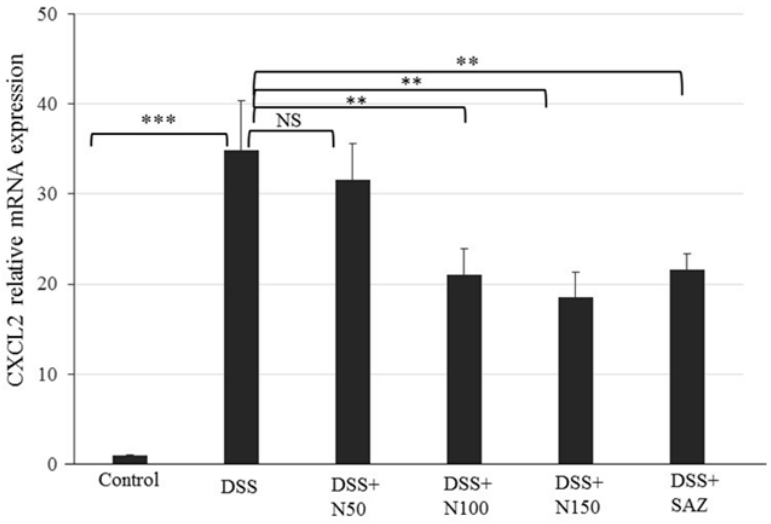


Figure 7: Effect of NED on colon length, myeloperoxidase (MPO) concentration, CXCL-2 and CCL2 mRNA macrophage markers (continued).

7e

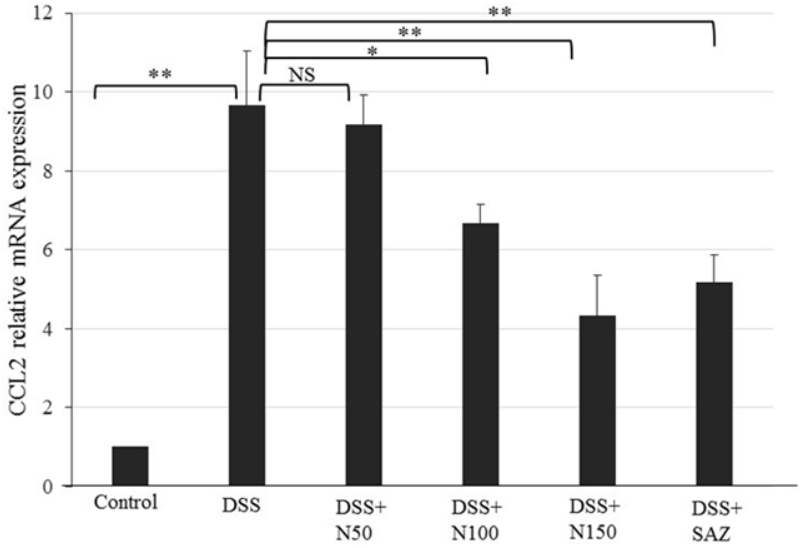


Figure 7: Effect of NED on colon length, myeloperoxidase (MPO) concentration, CXCL-2 and CCL2 mRNA macrophage markers (continued).

The mean colon length was significantly decreased in the DSS-treated group. The higher doses of NED treatment and SAZ-treatment significantly prevented the decrease in colon length compared to DSS alone group. The lowest concentration of NED had no effect Data were obtained from n = 10 animals in each group and are expressed as means ± SEM. * $p \leq 0.05$. DSS-treatment significantly increased MPO concentrations compared to untreated controls. The higher doses of NED and SAZ reduced MPO concentrations compared to DSS treatment alone However, the lowest dose of NED had no effect Data were obtained from $n = 10$ animals in each group and are expressed as means ± SEM. ** $p \leq 0.01$ * $p \leq 0.05$. DSS-treatment significantly increased colonic CXCL-2 and CCL2 mRNA levels compared to controls. The higher concentrations of NED and SAZ-treatment prevented the increase of both CXCL-2 and CCL2 mRNA concentrations compared to DSS alone However, the lowest concentration of NED had no effect. Data were obtained from $n = 8$ animals in each group and are expressed as means ± SEM: *** $p \leq 0.001$ ** $p \leq 0.01$, and * $p \leq 0.05$ considered statistically significant, and NS indicate not significant.

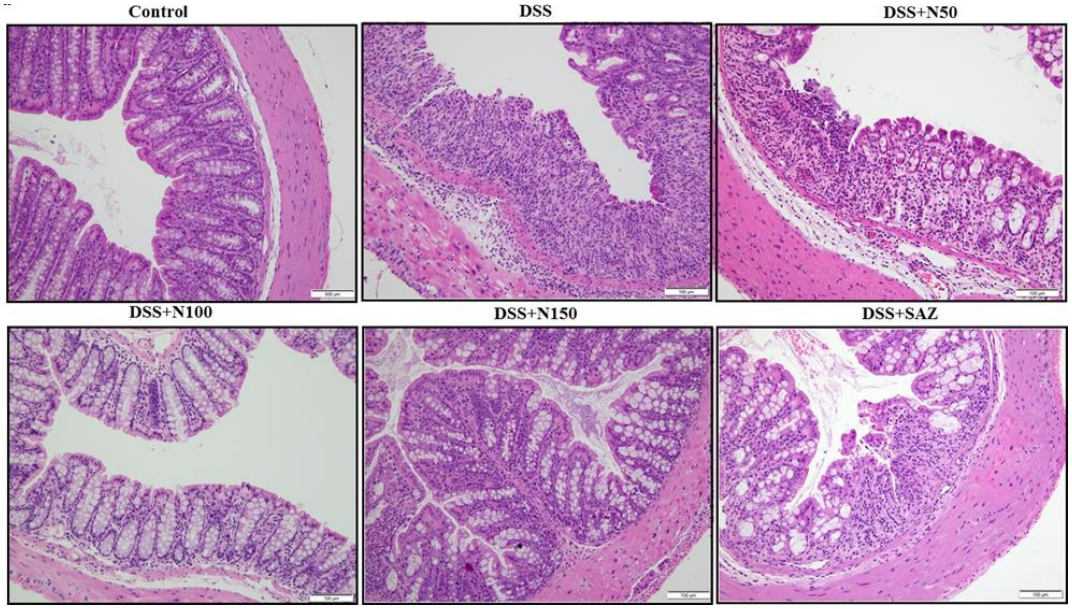
4.2.3 Effect of NED on colon microscopic architecture

Colon sections of control animals depicted typical architecture with normal thickness of the submucosa and muscle layers, as well as uniformly spaced crypt structure and density in the mucosa. The most notable histological finding in the DSS-treated group was excessive crypt damage with edema and complete destruction of

colon surface epithelium (Figure 8a). In contrast, the two higher doses of NED treated, and SAZ-treated animals showed intact epithelium with minimum loss of crypt and inflammation. SAZ-treated DSS animals also showed protection of colon microarchitecture.

The parameters used for grading the score of colonic inflammation and crypt damage are highlighted in Tables 2 & 3. The colonic inflammation score was statistically high ($p < 0.001$) in the DSS-treated group, compared to untreated control (Figure 8b). NED treated DSS-animals at the two higher doses ($p < 0.01$) showed a better colonic inflammation score significantly than DSS animals (Figure 8b). As expected, SAZ treatment also reduced DSS-induced inflammation. However, the lowest dose of NED did not reduce that inflammation significantly. The crypt damage score was higher in the DSS animals compared to the control group ($p < 0.001$, Figure 8c). NED treatment protected crypt damage at the two higher doses ($p < 0.01$, Figure 8c). Significant protection of crypt damage was also observed in the SAZ-treated DSS group as well ($p < 0.05$). However, the lowest dose (50 mg/kg) of NED treatment did not provide any protection from crypt damage.

8a



8b

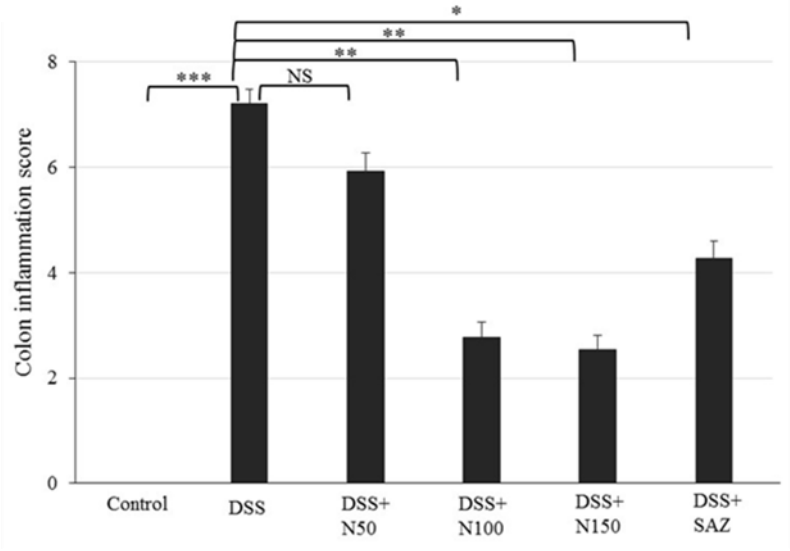


Figure 8: Effect of NED on colon histology.

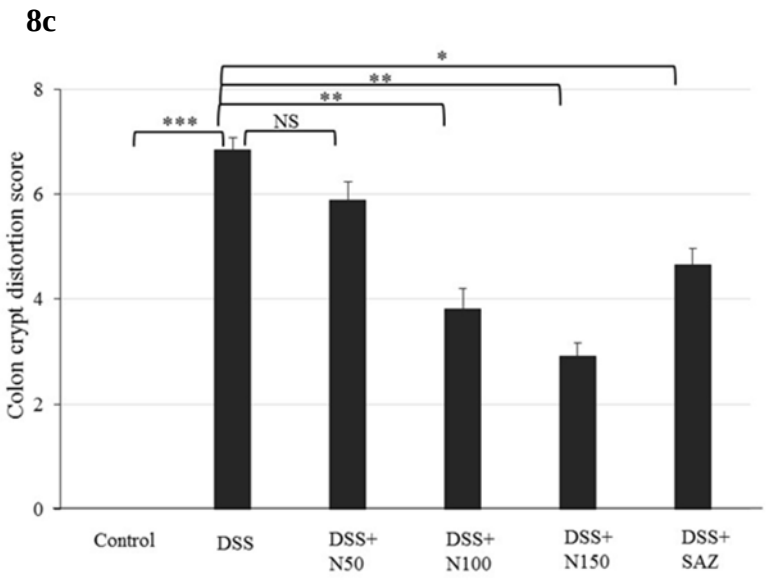


Figure 8: Effect of NED on colon histology (continued).

Microscopic analysis shows typical architecture of the colon with normal thickness of the submucosa, muscle layer and regular crypt and villi structure in the mucosa in the control samples (Scale Bars: 100 µM). The DSS-induced colitis colon section shows focal loss of crypts and surface epithelium with inflammation reaching up to the submucosa. NED treatment, at 100 and 150mg/kg, protected the microscopic architecture in DSS-induced colitis (Figure 8a). Crypt distortion score and colon inflammation scores were high in DSS-induced colitis group. NED treatment at 100 and 150mg/kg significantly reduced both colon inflammation scores (Figure 8b) and crypt distortion (Figure 8c). The lowest concentration of NED (50mg/kg) had no effect. Data were obtained from $n = 8$ animals in each group and are expressed as means ± SEM. *** $p \leq 0.001$ ** $p \leq 0.01$, and *$p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant and NS indicate not significant.

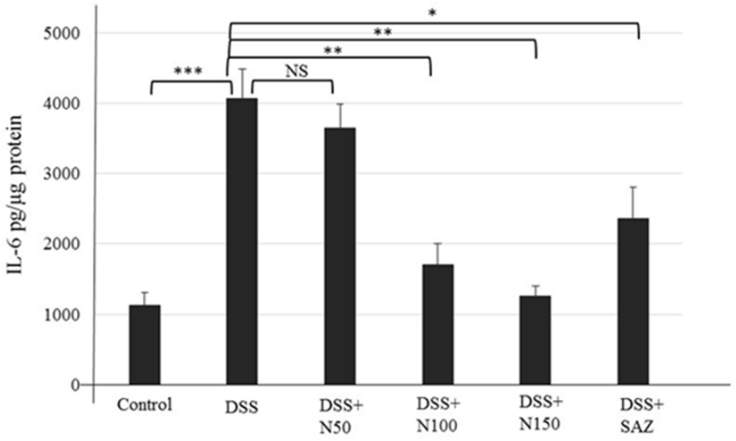
4.2.4 Effect of NED on proinflammatory cytokines and proinflammatory mediators

DSS caused the rise in concentrations of proinflammatory cytokines statistically: IL-6, IL-1β, and TNF-α, (all $p < 0.001$, Figure 9a, 9b, and 9c). Concomitant increase in the relative expression of mRNA of these respective cytokines ($p < 0.001$) was also observed, when compared to control group (Figure 9d, 9e, and 9f). NED treated animals had shown significantly less concentrations of these cytokines than the DSS-treated control group ($p < 0.01$ for IL-6 and TNF-α at 100 mg

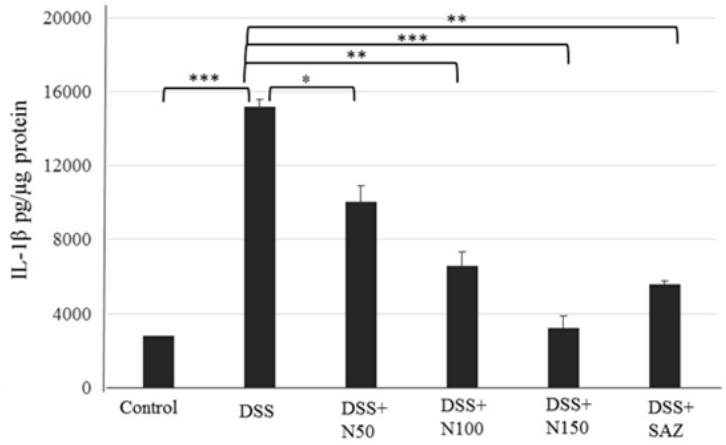
and 150 mg/kg NED) (Figure 9a, 9c); for IL-1β ($p < 0.01$ at 100 mg and $p < 0.001$ at 150 mg/kg) (Figure 9b). However, the lowest dose of NED did not provide any protection for IL-6; for IL-1β and TNF-α ($p < 0.05$) at 50 mg/kg NED. SAZ treatment reduced the cytokines IL-6 ($p < 0.05$), IL-1β and TNF-α ($p < 0.01$) significantly (Figure 9a, 9b, 9c). Similarly, NED treatment caused a significant decrease in mRNA expressions of these cytokines. For IL-6 and IL-1β ($p < 0.05$) at 100 mg/kg NED; ($p < 0.01$) 150 mg/kg NED. For TNF-α ($p < 0.01$) at 100 mg/kg NED; ($p < 0.05$) at 150 mg/kg NED. SAZ treatment also cause a significant decrease in the expression of these cytokines ($p < 0.05$) (Figure 9d, 9e, 9f). These results indicated that the lowest dose of NED (50 mg/kg) was found to be not effective as the other higher doses of NED in limiting DSS-induced inflammatory responses; this group was omitted from subsequent experiments. Similarly, the SAZ group was also removed from further studies.

The effect of NED in DSS treatment was also evaluated on COX-2 and iNOS at both the protein and mRNA levels. DSS treatment significantly increased COX-2 and iNOS mRNA and protein expression (COX-2; $p < 0.01$; COX-2 mRNA $p < 0.001$; iNOS; $p < 0.01$and iNOS mRNA; $p < 0.001$, Figure 10a, 10b). NED treatment showed a significant decrease in COX-2 protein / mRNA expression ($p < 0.05$ at 100 mg/kg NED and $p < 0.01$, at 150 mg/kg NED) (Figure 10a and 10b). Similarly, NED decreased iNOS protein and mRNA expression as well ($p < 0.05$ at both 100 and 150 mg/kg NED) (Figure 10c and 10d). The decrease observed at both protein and mRNA levels of iNOS upon NED treatment can be correlated with decreased tissue nitrate levels also observed in the present study ($p < 0 .05$ at 100 mg/kg and $p < 0 .01$ at 150 mg/kg NED) (Figure 10e).

9 a



9 b



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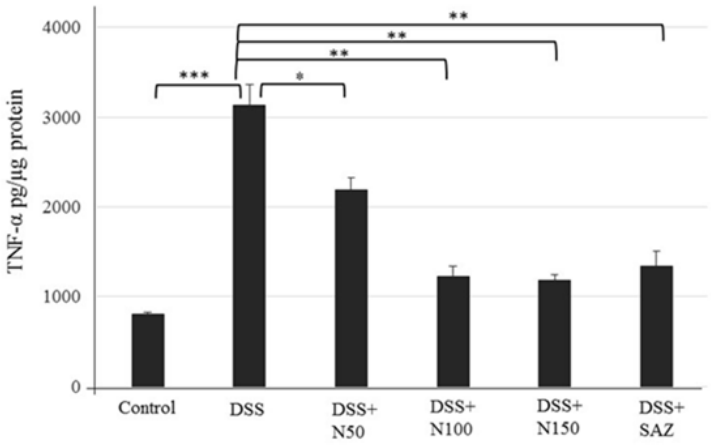


Figure 9: Effect of NED on proinflammatory cytokine protein and mRNA expression.

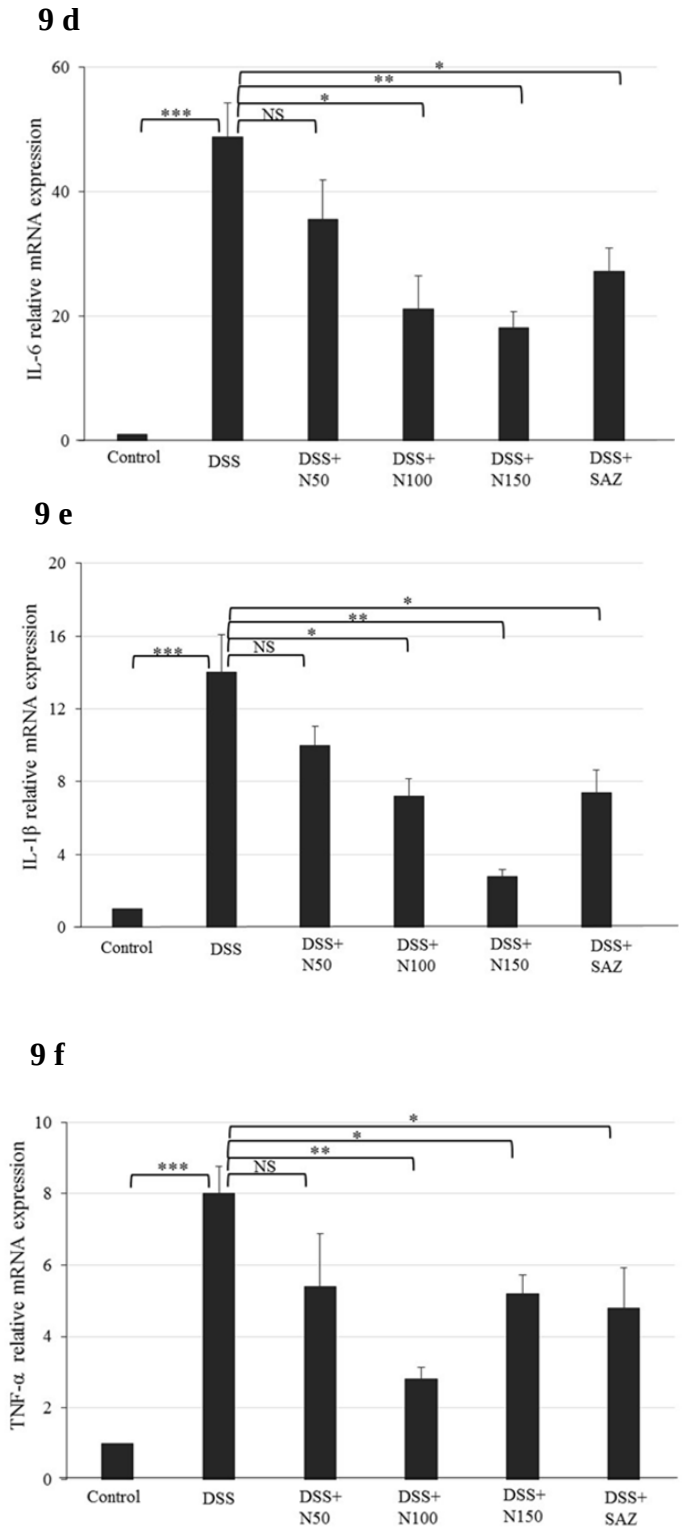


Figure 9: Effect of NED on proinflammatory cytokine protein and mRNA expression (continued).

NED treatment at 100 and 150mg/kg, significantly inhibited the DSS-induced increase in concentrations of proinflammatory cytokines, at both the protein level [IL-6 (Figure 9a), IL-1β (9b), & TNF-α (9c)] and mRNA expression levels [IL-6

(Figure 9d), IL-1β (9e), & TNF-α (9f)] in DSS-induced colitis group. Data were obtained from $n = 8$ animals for ELISA and $n = 6$ animals for mRNA expression studies in each group and expressed as means ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant and NS indicate not significant.

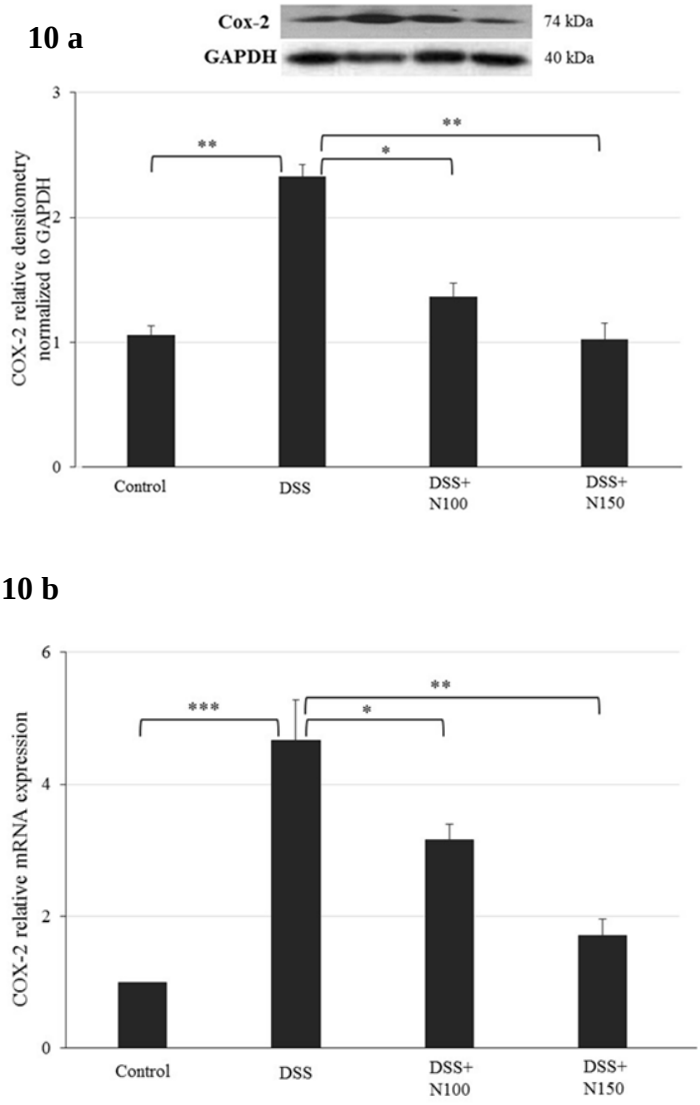
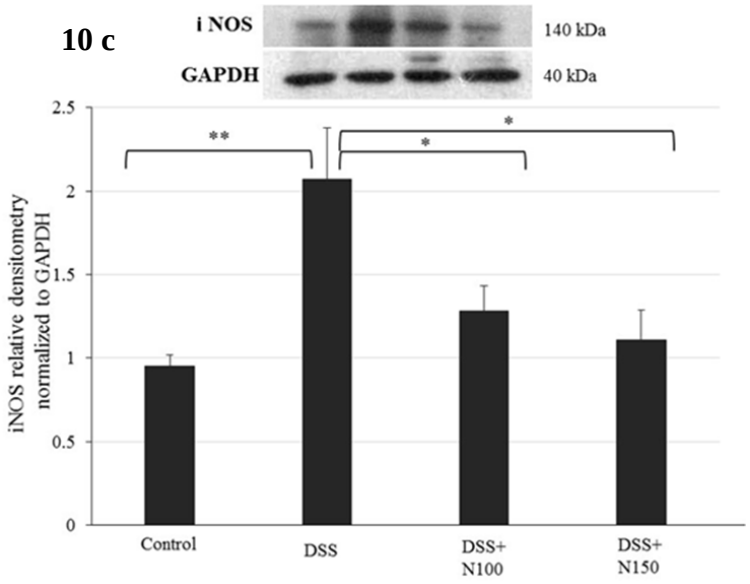
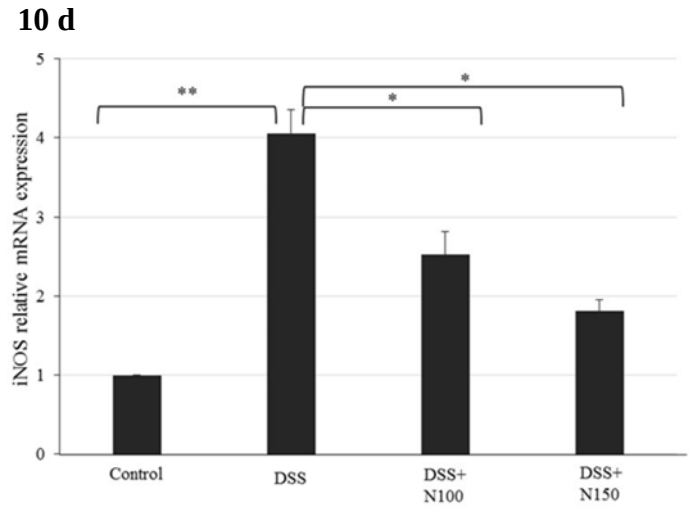


Figure 10: Effect of NED on inflammatory mediator COX-2 and iNOS protein and mRNA expression, and tissue nitrate levels.





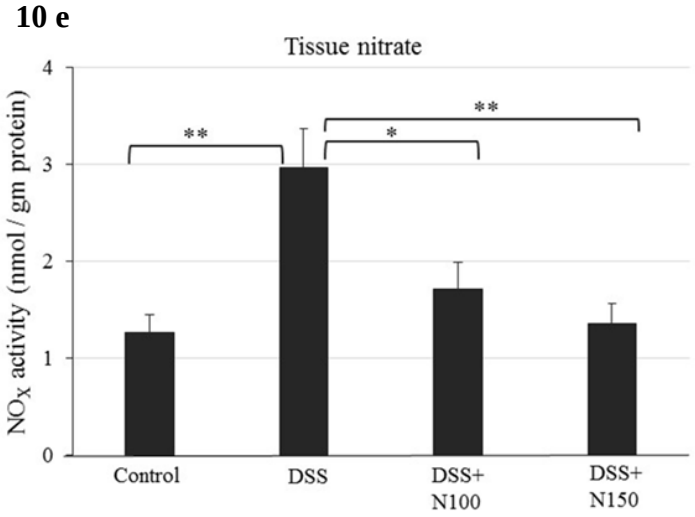


Figure 10: Effect of NED on inflammatory mediator COX-2 and iNOS protein and mRNA expression, and tissue nitrate levels (continued).

NED treatment significantly inhibited the DSS-induced expression of COX-2 and iNOS at both the protein (Figure 10a and 10b), and mRNA levels (Figure 10c and 10d); and also reduced the tissue nitrate levels (Figure 10e). Data were obtained from $n = 4$ animals for Western blot and $n = 6$ animals for mRNA expression and tissue nitrate level studies in each group, and expressed as means ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.2.5 Effect of NED on antioxidant Keap-1 and Nrf-2 transcription factor protein, SOD, CAT activity and downstream target mRNA expression

Nrf-2 is the major regulator for genes encoding antioxidant enzymes. Keap-1 acts as a repressor, that binds to Nrf-2 and promotes its degradation via the ubiquitin proteasome pathway. NED effect was investigated on the Keap-1 and Nrf-2 system. DSS treatment increased Keap-1 protein expression in the cytoplasm, and NED treatment significantly decreased the same ($p < 0.05$) (Figure 11 a). Under basal conditions, normally Keap-1 always attached with Nrf-2 in the cytoplasm; however, upon activation, Nrf-2 detaches from Kaep-1 and translocate into the nucleus and bind to the antioxidant response element, then transcription initiated. Therefore, Nrf-2 expression were analyzed in both the cytoplasm and nuclear fraction of colonic epithelial cells. In the cytoplasm, DSS treatment did not affect the Nrf-2 protein level compared to control; however, high dose of NED treatment significantly ($p <0.05$) decreased the Nrf-2 level (Figure 11 b). In contrast, the concentration of Nrf-2 in the nuclear fraction was modestly increased by DSS treatment ($p <0.05$) and was statistically increased in a concentration-dependent manner by NED ($p <0.05$ at 100 mg/kg and $p <0.01$ at 150 mg/kg) (Figure 11 c). Numerous cytoprotective genes are shown to be regulated by Nrf-2, when it is translocated into the nucleus (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). Therefore, tissue superoxide dismutase (SOD) and catalase enzyme activities were determined in NED treated DSS

colitis animals along with expression of Heme Oxygenase-1 (HO-1) and SOD-3 mRNA. NED treatment significantly increased colon mucosal SOD activity (p <0.05 at 100 mg/kg and p <0.01 at 150 mg/kg) (Figure 11 d). However, the catalase activity was significantly increased only at higher dose of NED (p <0.01 at 150 mg/kg) (Figure 11 e). Nrf-2 nuclear translocation significantly correlate with the increase of HO-1 mRNA expression in a dose-dependent manner (p <0.05 at 100 mg/kg and p <0.01 at 150 mg/kg) (Figure 11 f). SOD-3 mRNA expression was significantly increased (p <0.05 at 100 mg/kg and p <0.01 at 150mg/kg) associated with Nrf-2 nuclear translocation, which was induced by NED treatment in DSS colitis colon (Figure 11 g).

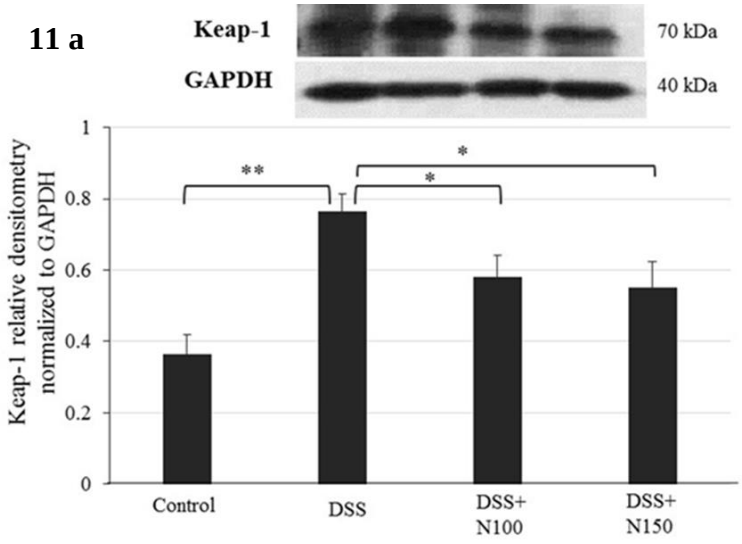


Figure 11: Effect of NED on Keap-1 and Nrf-2 and antioxidant system.

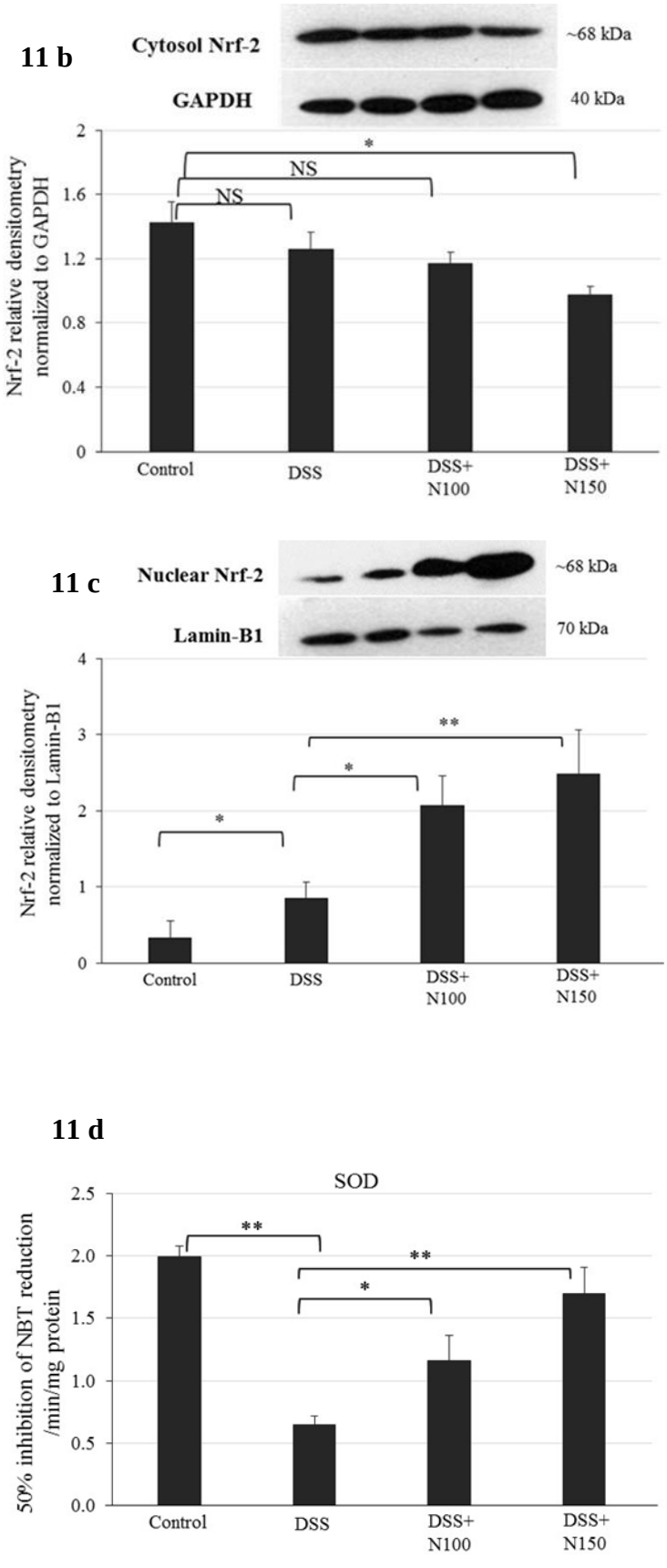
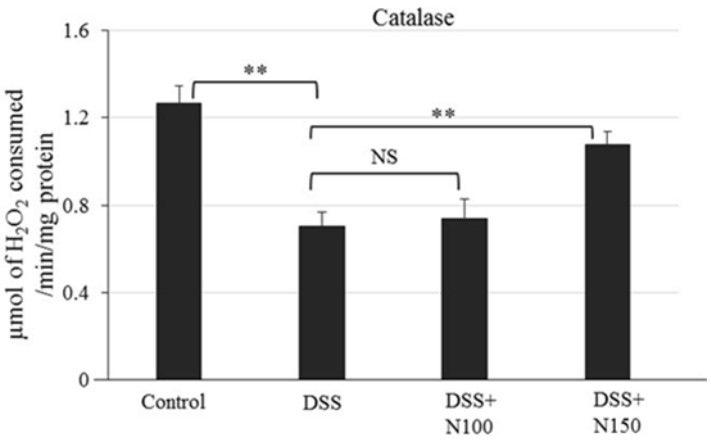


Figure 11: Effect of NED on Keap-1 and Nrf-2 and antioxidant system (continued).

11 e



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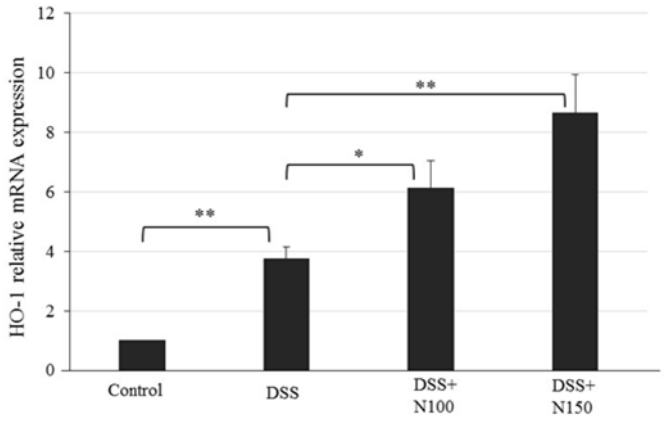


11 g

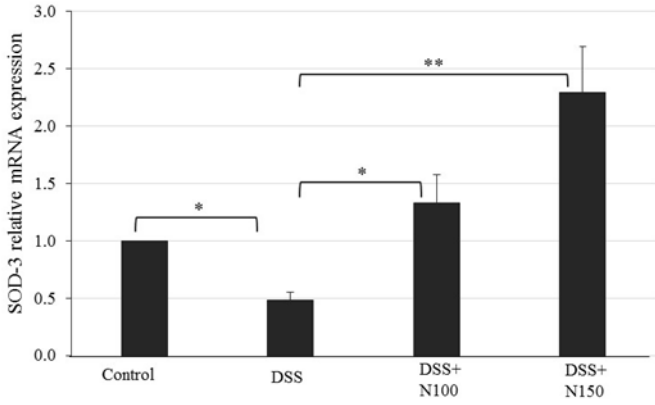


Figure 11: Effect of NED on Keap-1 and Nrf-2 and antioxidant system (continued).

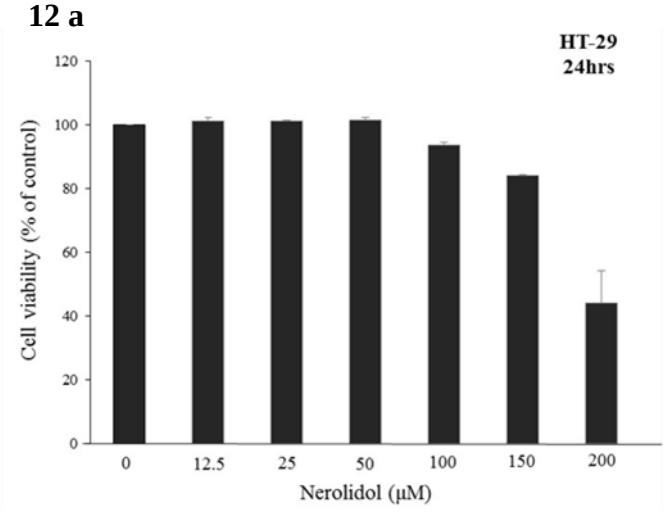
At the two higher concentrations, NED prevented the DSS-induced increase in Keap-1 protein expression. The nuclear translocation of the Nrf-2 protein was

enhanced by NED treatment in DSS-induced colitis (Figure 11b and c). Superoxide dismutase (SOD) and catalase enzyme activities were significantly increased in NED treated DSS colitis animals (Figure 11d and e). Heme Oxygenase-1 (HO-1) and SOD-3 mRNA expression were also significantly increased in NED treated DSS colitis mice (Figure 11f and g). Data were obtained from $n = 4$ animals for the western blot studies, $n = 6$ for enzyme activity and mRNA level. Data expressed as means ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.2.6 Effect of NED on TNF-α stimulated HT-29 colon cells

Anti-inflammatory effect of NED were evaluated in a disease-relevant cellular context by using TNF-α stimulated HT-29 colon cancer cells, a well-established model for inflammatory process of gut (Wilson & Browning, 2002). To eliminate the possibility of cytotoxicity of NED in HT-29 colon cells and to optimize treatment dose, cells were treated with different concentrations (in micromolar range) of NED for 24 hrs. No cytotoxic effect observed up to 150 µM concentration. Only at 200 µM a slight, but significant ($p < 0.05$) cytotoxic effect was observed (Figure 12 a). Therefore, the subsequent experiments carried out using 25 to 50 µM NED, and these concentrations were well within the non-cytotoxic range. HT-29 cells stimulated with TNF-α showed a significant increase in the mRNA of the inflammatory marker genes, CXCL-1, IL-8, CXCL-2 ($p < 0.001$), and COX-2 ($p < 0.01$). All these proinflammatory gene markers were significantly down regulated upon NED treatment in TNF-α stimulated HT-29 cells in a dose dependent manner (Figure 12b, 12c, 12d, and 12e). However, treatment with NED alone did not induce any changes in gene expression profile compared to the control treatment. These results further confirm the potent anti-inflammatory properties of NED.

12 a



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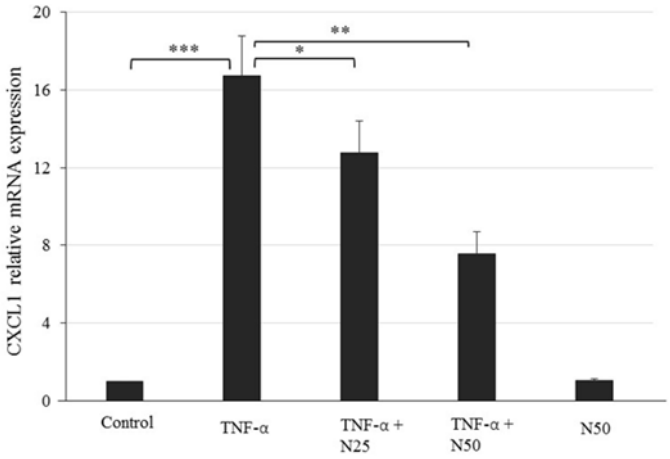

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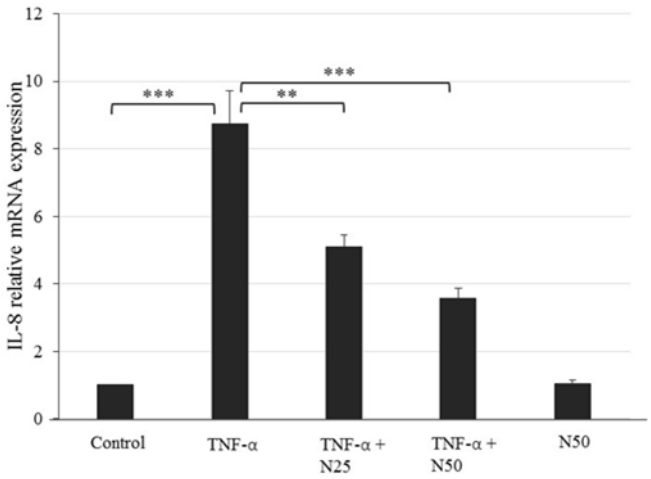
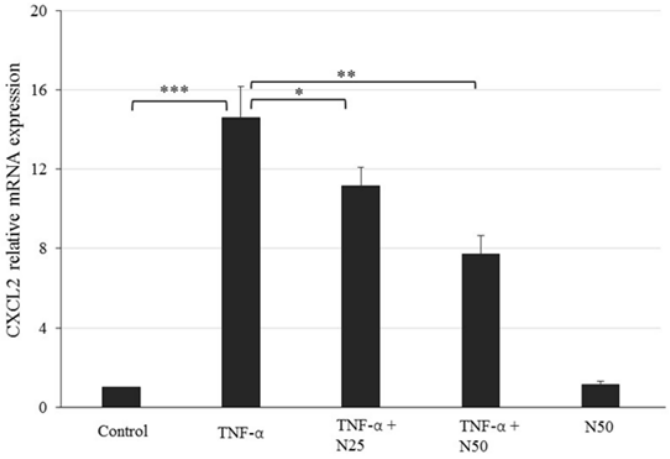


Figure 12: Effect of NED on cell viability and proinflammatory cytokine mRNA expression in TNF-α-treated HT-29 human colonic cancer cells.

12 d



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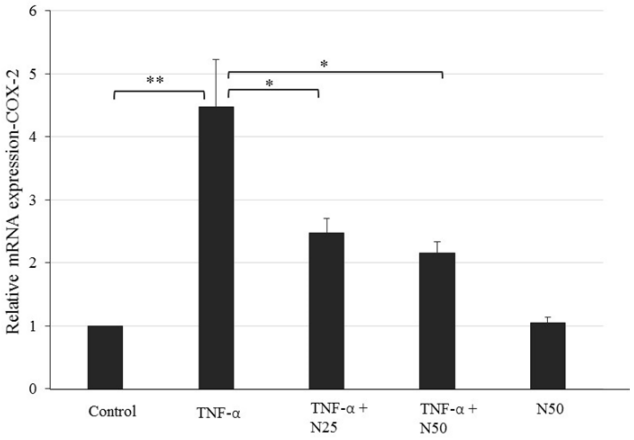


Figure 12: Effect of NED on cell viability and proinflammatory cytokine mRNA expression in TNF-α-treated HT-29 human colonic cancer cells (continued).

The concentration-dependent cytotoxic effects of NED were investigated treatment of HT-29 cells with different concentrations of NED for 24 hrs. NED had no effect on HT-29 cell viability at concentrations up to 100 µM, so its anti-inflammatory effects were examined at concentrations of 25 and 50 µM in the subsequent experiments. To investigate the effect of NED on inflammatory cytokine production, cells were treated with 1 ng/ml TNF-α for 24 hrs. Real-time PCR was carried out to detect gene expression of the inflammatory markers CXCL-1, CXCL-2, IL-8, and COX-2. Data are shown relative to DMSO vehicle-treated cells. Data expressed as mean ± SEM ($n = 6$). *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant and NS indicate not significant.

4.2.7 Discussion

UC is a type of IBD, where chronic inflammation is confined to the colon with varying severity and can make a major impact on emotional and social well-being. Prolonged inflammation of the gastrointestinal tract can also lead to colon cancer, when unchecked (Chung et al., 2007; Cuzzocrea, 2003). Adverse effects and relapse rates associated with prescription drugs are one of the main reason for the shift in focus towards alternative medicines (Langmead et al., 2002; Sands, 2000). Many reports have demonstrated that the role of phytochemicals implicated for its anti-inflammatory and antioxidant activities in experimental colitis (Cho et al., 2011; Kumar, Dhamotharan, Kulkarni, Honnegowda, & Murugesan, 2011; Marquez et al., 2010; Yoshioka et al., 2008).

In the present study, NED role was investigated DSS-induced colitis in mice; a well-accepted ulcerative colitis model resembles the human UC, in terms of epithelial damage and other associated conditions. The symptoms include weight loss, diarrhea, rectal bleeding or bloody stool and colon shortening. The sulfate moiety of colitic chemical DSS is toxic to the inner mucosal epithelium and disrupts intestinal epithelial integrity (Eichele & Kharbanda, 2017). After the disruption of colon epithelial layer, the T-cells and macrophages get activated to secrete many proinflammatory cytokines (Okayasu et al., 1990; Takizawa, Asakura, Sasakawa, Bannai, & Nomoto, 1995). NED administration prevented DSS-induced weight loss and clinical symptoms as reflected by the DAI score in mice, and these observations were substantiated by macroscopic, histological, and biochemical evaluations. DSS-treated colon displayed a severe form of inflammation with mucosal damage and partial/complete loss of crypt. High DAI scores and a shortened colon indicated its

pathological state and colon severity in DSS colitis. Both the concentrations of NED (100 & 150 mg/kg) were more effective than the standard drug, sulfasalazine in reducing inflammation. However, the lowest dose of NED (50mg/kg) had no significant effect on any inflammatory markers.

NED significantly prevented the DSS-induced reduction in colon length, and a dose-dependent decrease in the DAI score were also observed with NED treatment. Histopathological findings were also comparable to that of the DAI score. The DSS colitis mice colons exhibited marked histopathological changes, including epithelial erosion and ulceration, crypt abscess formation, mucosal erosion and edema, and substantial infiltration of macrophages. NED administration was able to preserve near normal microscopic architecture of colon at the higher doses, but the lowest dose of NED could not prevent these aberrations.

Myeloperoxidase (MPO) enzyme belongs to heme peroxidase–cyclooxygenase superfamily and abundantly expressed in neutrophils, macrophages, and other monocytes but to a lesser extent. The infiltration of these leukocytes has been attributed to tissue damage and necrosis (Malle, Furtmuller, Sattler, & Obinger, 2007). DSS-colitis animals exhibited neutrophils and macrophages infiltration and served as specific markers of inflammation. Leukocytes act as a major source of ROS. MPO is normally stored in azurophilic granules of neutrophils and monocytes. During inflammation, neutrophils are activated, and the degranulation releases MPO, catalyse the formation of oxidants responsible for tissue injury, and the progression of acute and chronic inflammatory conditions (Y. W. Liu, Su, Ong, Cheng, & Tsai, 2011). Thus, MPO acts as a significant marker for neutrophil infiltration at the inflamed area. Results from the present study indicated that colonic MPO was increased in DSS

colitis mice statistically, implicated the tissue damage and ROS generation. Upregulated MPO lead to weaken the antioxidant defence mechanisms, accelerated the oxidative injury of colon (Gautam et al., 2013). NED remarkably reduced the inflammation-induced neutrophil infiltration. CXCL-2 and CCL2 are secreted by neutrophils and macrophages as chemoattractant chemokines, mostly involved in immune responses (Al-Alwan et al., 2013; Deshmane, Kremlev, Amini, & Sawaya, 2009). NED significantly prevented the DSS-induced increase in mRNA levels of these chemo attractants in a dose dependant manner.

Infiltration of neutrophils into colonic tissue exerted mucosal damage by releasing reactive oxygen species (ROS), resulting in proinflammatory cytokines release (Williams & Parkos, 2007; Zhou & Liu, 2017). Cytokines are proteins secreted by the active immune cells and initiate inflammatory responses. Elevated levels of proinflammatory cytokines have already been reported in IBD patients and in experimental animals (Dieleman et al., 1998; Pullman, Elsbury, Kobayashi, Hapel, & Doe, 1992). TNF-α is a primary mediator of the inflammatory response and closely linked to colonic inflammation (Kany, Vollrath, & Relja, 2019). Once released, TNF-α induces apoptosis of intestinal epithelial cells and disruption of intestinal epithelial barrier integrity. TNF-α has also been shown to induce cytokines/chemokines secretion from intestinal epithelial cells. After the initial phase of inflammation, the adaptive immune system takes control of the immune responses, and TNF-α role is implicated in this via the recruitment and activation of neutrophils and macrophages in huge quantities at the site of inflammation (Bischoff et al., 1999; Cho et al., 2011; Guy-Grand, DiSanto, Henchoz, Malassis-Seris, & Vassalli, 1998). The significance of TNF-α in UC can be understandable because anti-TNF-α mouse monoclonal antibodies like infliximab were proven to be beneficial against UC in reported studies

(Levin & Shibolet, 2008; Sandborn et al., 2014). Besides TNF-α, IL-6 and IL-1β are other proinflammatory cytokines involved in UC progression. Suppression of neutrophils infiltration and other inflammatory cytokines have been documented in IL-1β receptor antagonist treated colitis animals (Dionne, D'Agata, Hiscott, Vanounou, & Seidman, 1998; Tountas et al., 1999). Another study have reported that the anti-murine IL-1β antibody effectively reduced the IL-6 gene expression in DSS-colitis (Kwon, Murakami, Hayashi, & Ohigashi, 2005). Moreover, excessive cytokine production due to immune response is implicated in extraintestinal manifestations in IBD as well (Neurath, 2014). Therefore, preventing excess production of these cytokines are reported to be an effective approach in UC treatment. In the present study, higher doses of NED treatment significantly reduced the elevated levels of IL-6, IL-1β, and TNF-α in colon tissue of DSS-colitis animals. These findings can be correlated with the previous report, where NED was shown to decrease the influx of polymorphonuclear cells in carrageenan-induced peritonitis, and showed reduction in TNF-α levels in the peritoneal lavage, and suppressed the IL-1β production in LPS-stimulated, peritoneal macrophages (Fonseca et al., 2016). These observations indicated that this sesquiterpene has a potential anti-inflammatory property.

Proinflammatory cytokines have shown to induce COX-2 expression, which triggered elevated prostaglandins synthesis and amplification of mucosal inflammation in IBD patients' intestinal epithelium (Jurjus et al., 2016). IL-6 has already been reported to upregulate COX-2 expression (Tsatsanis, Androulidaki, Venihaki, & Margioris, 2006). In the present study, COX-2 mRNA and protein expression was elevated significantly in DSS-treated animals. Enhanced COX-2 expression was significantly attenuated by NED treatment. In a previous study, NED was shown to suppress TNF-α and IL-6 secretion (Zhang et al., 2017); therefore, the

observed suppression of COX-2 activity may be attributed to the reduced cytokines level in the present study.

Inducible nitric oxide synthase (iNOS) is expressed in response to bacterial/proinflammatory stimuli and resulted in nitric oxide (NO) production, which provides cyto-protection. Excessive iNOS expression initiate upregulated NO turnover, implicated in IBD pathogenesis (Minhas, Bansal, & Bansal, 2019). Increased iNOS expression was initiated by proinflammatory cytokines in IBD (Soufli, Toumi, Rafa, & Touil-Boukoffa, 2016). It was reported that iNOS knockout and iNOS inhibitor treatment have shown to decrease the severity of inflammation in DSS-colitis, thus explains its vital role in UC pathophysiology (Krieglstein et al., 2001). Macrophage activation marker, iNOS mRNA, and protein expression were found to be increased in DSS-colitis animals. NED suppressed these COX-2 and iNOS expressions at both mRNA and protein levels significantly. NED containing *Lindera erythrocarpa* essential oil was reported to suppress iNOS and COX-2 expression and reduced the subsequent NO and prostaglandin E_2 production, respectively in LPS-stimulated RAW macrophages model of inflammation (Ko et al., 2017). Similarly, NED was found to inhibit iNOS and COX-2 protein expression in a rat model of neuroinflammation as well (Javed et al., 2016). To address the role of iNOS-derived NO in colitis, studies have been carried out using various iNOS specific inhibitors in *in vivo* colitis models and revealed conflicting results (Kolios, Valatas, & Ward, 2004).

Oxidative stress has long been recognized as a major player in many autoimmune disorders, neurodegenerative disorders, gastroduodenal ulcers, IBD and colorectal cancer (Chiurchiu & Maccarrone, 2011; Grisham, 1994; Inokuma, Haraguchi, Fujita, Tajima, & Kanematsu, 2009). ROS and RNS play a significant role

in tissue damage that occurred in IBD. Excessive production of superoxide anion, hydrogen peroxide, hypochlorous acid, and hydroxyl radical are detrimental to intestine's mucosal epithelium. Reports have substantiated the effective role of antioxidant agents in the prevention of IBD progression (Tian et al., 2017). Nrf-2 signaling pathway is an important molecular mechanism involved in the body's antioxidant defence mechanism. Under physiological conditions, Nrf-2 and Keap1 exist as a dimer in the cytoplasm. In response to electrophiles or ROS, Nrf-2 dissociates from Keap1, a negative modulator of Nrf-2, and resulted in translocation of Nrf-2 into the nucleus. Once entered in to nucleus, Nrf-2 forms a heterodimer with Maf or Jun and binds to the antioxidant response element (ARE), resulting in the activation/transcription of encoding genes involved in antioxidant defence mechanisms (Yu, Wei, Xu, Pan, & Chen, 2018). Increased Nrf-2 expression initiated the antioxidant responsive element (ARE) activation, caused transcription of Phase II detoxifying enzymes and antioxidant proteins like GSH, SOD, HO-1, *etc* (Vasconcelos, Dos Santos, Scavone, & Munhoz, 2019). It was reported that Nrf-2 deficient mice were more susceptible to developing colitis (Khor et al., 2008; Osburn et al., 2007). Activation of Nrf-2 by bioactive compounds has been reported to exert protective effects by antioxidant defence mechanisms and down-regulated the inflammatory mediators, and apoptosis (Kim, Cha, & Surh, 2010; Xu et al., 2017). Therefore, Nrf-2 plays a critical role in host defence against many types of tissue injury.

Experimental studies have reported that phytochemicals were found to activate the Nrf-2/Keap1 pathway and induce many antioxidant genes expression (Qin & Hou, 2017). NED promoted the Nrf-2 nuclear translocation, and a decrease of Keap-1 expression was observed from cytosolic fractions. Few other studies have shown that

sesquiterpenes activated Nrf-2 nuclear translocation and modulated antioxidant enzyme expression (Liu et al., 2018; Park, Choi, Ju, Pae, & Park, 2015; Peng, Hou, Yao, & Fang, 2019). Heme oxygenase-1 (HO-1) and superoxide dismutase-3 (SOD-3) are among the downstream targets of Nrf-2. Heme oxygenase exists as two isoforms, HO-1 and HO-2 (Grochot-Przeczek, Dulak, & Jozkowicz, 2012). HO-1 is inducible, and a rate-limiting enzyme involved in heme metabolism, which catalyzes the conversion of heme to carbon monoxide. It also mediates the catabolism of several heme-containing proteins, including hemoglobin, myoglobin, and cytochrome p450. It has already been reported that the substrate of heme, hemin enhances HO-1 activity, thereby causes attenuation of inflammation in various animal models (Grochot-Przeczek et al., 2012; Pae, Lee, & Chung, 2008; Xia, Zhong, Meyrowitz, & Zhang, 2008). The immunomodulatory effect of HO-1 in IBD is also well established using multiple animal models (Sheikh et al., 2011; Takagi et al., 2011; Varga et al., 2007). Hence, an activation of HO-1 using pharmacological agents can serve as a potential therapeutic strategy to reduce inflammation associated with UC. HO-1 is highly expressed in response to stressful stimuli than normal physiological conditions (Intagliata et al., 2019; Loboda et al., 2016). Increased HO-1 expression offers protection during intestinal inflammation (Takagi et al., 2018; Takagi et al., 2008). Dose-dependently increased HO-1 mRNA expression observed in the NED-treated group may have provided protection from inflammation. SOD and CAT are major enzymes involved in the antioxidant defence mechanism. SOD converts the superoxide radicals (generated due to oxidative stress) to hydrogen peroxide, subsequently removed by CAT and peroxidases (Zelko, Mariani, & Folz, 2002). In the present study, SOD-3 mRNA expression was decreased in DSS group animals, but dose-dependently increased by NED treatment than the control group. There are three

different isoforms of SOD: cytoplasmic CuZnSOD (SOD1), mitochondrial MnSOD (SOD2), and extracellular SOD (EC-SOD or SOD-3). Among these isoforms, SOD-3 was shown to be decreased in intestinal epithelial cells (IECs) (Kruidenier, Kuiper, van Duijn, et al., 2003), and increased SOD-3 mRNA expression triggered apoptosis and death of cancer cells (Laukkanen, 2016). NED was reported to maintain cellular SOD, CAT, and GSH levels and significantly suppressed malondialdehyde (MDA) levels in Rotenone-induced Parkinson's disease in rats (Javed et al., 2016). Further, NED was effective in reducing MDA levels and maintained GSH and SOD activities in surgically induced endometriosis rat model (Melekoglu, Ciftci, Eraslan, Cetin, & Basak, 2018). These results can be correlated with NED's role in preserving SOD and CAT activities.

The anti-inflammatory activity of NED was also evaluated using TNF-α-induced HT-29 cells. HT-29 cells are human colonic adenocarcinoma cells exhibiting epithelial morphology. During inflammation, proinflammatory cytokines like TNF-α sensitized the intestinal epithelial cells resulted in increased CD14 expression and PRR recognized bacterial cell wall components (McCracken et al., 2002). An increased TNF-α receptor expression was also reported in the intestinal epithelial cells during IBD (Mizoguchi et al., 2002; M. F. Neurath et al., 1997; Van Deventer, 1997). The binding of TNF-α to its receptor leads to form an assembly of death-inducing signaling complexes, DISC-1 and DISC-2 intracellularly. DISC-1 is membrane-bound and acts via NF-κB and MAPK pathway, whereas DISC-2 is found in the cytoplasm and activates caspase-8 and caspase-3 (Micheau, Lens, Gaide, Alevizopoulos, & Tschopp, 2001). TNF-α stimulated HT-29 cells have been reported to activate NF-κB pathway via DISC-1 and antagonized the caspase signaling via DISC-2, thus inhibited HT-29 cells apoptosis, which was induced by the caspases (Micheau et al., 2001; Micheau et

al., 2002). In the present study, the NED role was evaluated in the expression of COX-2, CXCL-1, CXCL-2, and IL-8 mRNA expression in HT-29 cells challenged with TNF-α, to mimic inflammatory state. Chemokine ligand-2 (CXCL-2) is a chemotactic chemokine produced by colonic epithelial cells and macrophages in response to any infection/injury (Ohtsuka, Lee, Stamm, & Sanderson, 2001); IL-8 is a neutrophil chemoattractant, found to be upregulated during inflammation (Fuhr, Rousseau, Plauth, Schroeder, & Sauer, 2015; Wilson & Browning, 2002). Neutrophil recruitment mediated by IL-8 is a critical step involved in tissue injury observed in IBD patients. During inflammation, bacterial antigens or cytokines stimulate the intestinal epithelial cells leading to IL-8 production. Significant elevation of IL-8 levels was observed in primary intestinal epithelial cells isolated from IBD patients as well (Daig et al., 1996; Rogler et al., 1998). In the present study, an increased level of IL-8 was found in TNF-α-stimulated HT-29, and that was significantly suppressed by NED treatment. Chemokine ligand-1 (CXCL-1) plays a role in inflammation and tumorigenesis (Ogata et al., 2010). In colonic mucosa of IBD patients, increased IL-8 and CXCL-1 levels can be correlated with disease severity (Imada et al., 2001). It was confirmed from these results that NED-treated TNF-α stimulated HT-29 cells has shown a potent anti-inflammatory property, which could be mediated through suppression of proinflammatory chemokine levels and its response. Reduction of mRNA expression by NED may be partly attributed to the study in which *Liquidambar formosana* leaves containing NED were shown to suppress inflammatory response LPS-induced macrophages (Hua, Yang, Chiu, & Ho, 2014). These results indicated that NED exhibited significant anti-inflammatory effects in *in vivo* and *in vitro* models of colon inflammation. It was notable that the effects of NED were as efficacious as

sulfasalazine, a drug commonly used in treatment of Crohn's disease and ulcerative colitis.

Based on these results, further experiments were designed to investigate the intracellular signaling pathways that NED could possibly target to exert its anti-inflammatory effects.

4.3 Study 3: Role of NED in modulating MAPK/NF-κB signaling pathway involved in inflammation

Evidence from the first two studies indicated the anti-inflammatory potentials of NED in both *in vivo* and *in vitro* models. Based on these results, studies were planned to investigate the intracellular signaling mechanisms that are believed to play a significant role in the inflammatory responses including MAPK and NF-κB signaling. NED's effect was investigated on the phosphorylation of p38, JNK, $ERK^{1/2}$ MAP kinases and NF-κB. These investigations were carried out in both DSS-induced colitis model and in LPS stimulated RAW 264.7 murine macrophages.

Results:

4.3.1 Effect of NED on phosphorylation of p38, JNK, $ERK^{1/2}$ MAP kinases and NF-κB in DSS colitis model

DSS administration in mice caused enhanced phosphorylation of p38, JNK and $ERK^{1/2}$ MAP kinases; ($p < 0.001$ for JNK) (Figure 13a), ($p \leq 0.01$ for $ERK^{1/2}$) (Figure 13b) and ($p \leq 0.0001$ for p38 MAPK) (Figure 13c). But NED treatment significantly suppressed the phosphorylation of these MAP kinases; ($p < 0.01$ for JNK at NED 100 mg/kg dose and $p < 0.001$ at NED 150 mg/kg dose); ($p < 0.05$ at NED 100 mg/kg and 150 mg/kg doses for $ERK^{1/2}$); ($p < 0.01$ at NED 100 mg/kg dose and $p < 0.001$ at NED 150 mg/kg dose for p38) (Figure 13a, 13b and 13c). Nuclear translocation of NF-κB is a key event that regulates the production of proinflammatory cytokines and

mediators. Phosphorylation/activation of NF-κB precedes the nuclear translocation of NF-κB. DSS treatment have increased the phosphorylation of NF-κB as well; ($p < 0.01$ compared to the control). NED treatment (both doses) significantly prevented the NF-κB phosphorylation; ($p < 0.05$ at 100 and 150 mg/kg dose) (Figure 13d). This activation of MAPKs coupled with the nuclear translocation of NF-κB is responsible for the elevated levels of proinflammatory cytokines. These results indicate that NED significantly decreases the phosphorylation and activation of p38, JNK ERK$^{1/2}$ MAP kinases, and NF-κB. However, the expression of the total non-phosphorylated proteins was not altered.

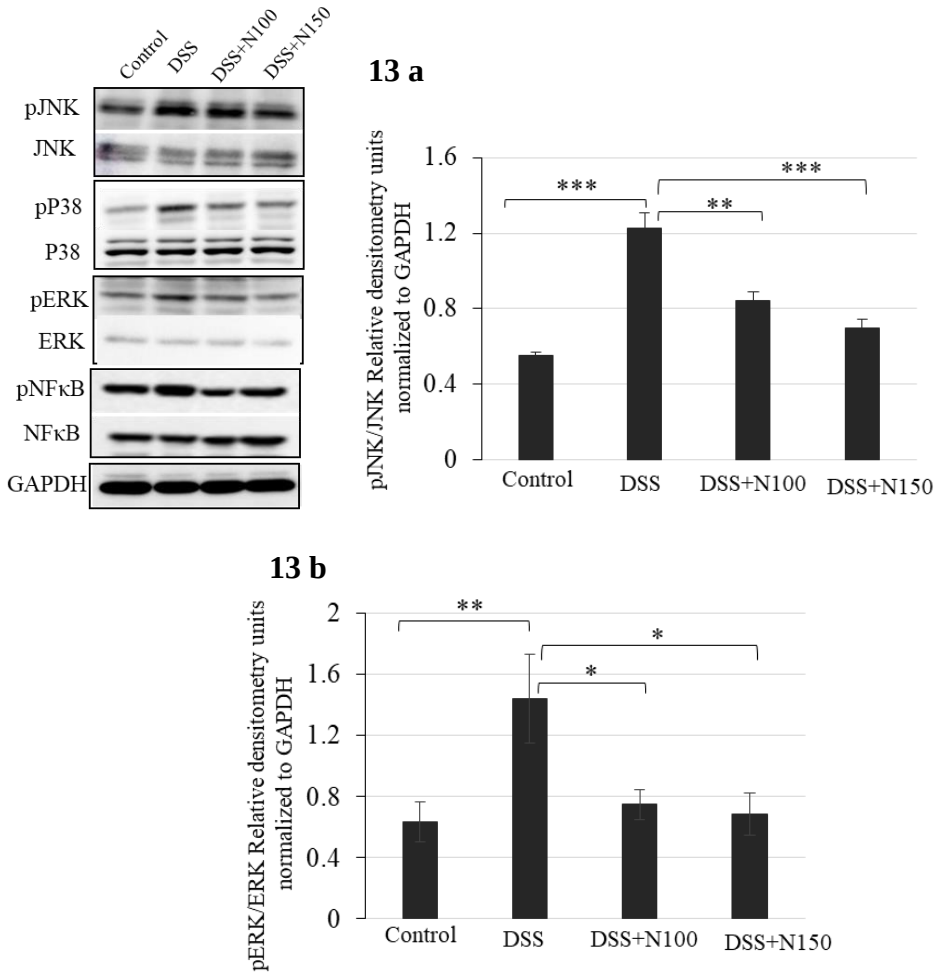
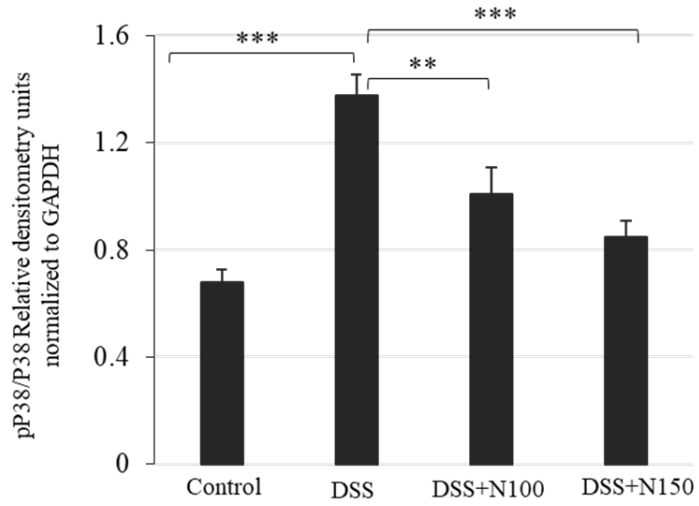


Figure 13: Effect of NED on the phosphorylation of MAPK proteins and NF-κB in DSS treated mice.

13 c



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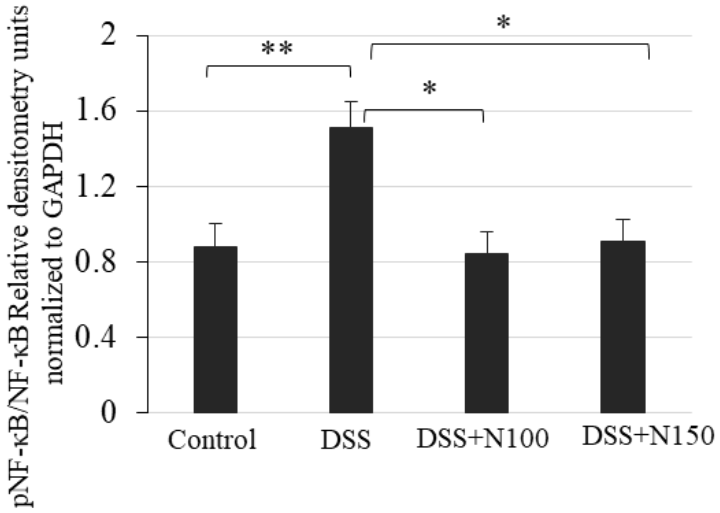


Figure 13: Effect of NED on the phosphorylation of MAPK proteins and NF-κB in DSS treated mice (continued).

Western blot experiments showed that NED treatment significantly inhibited the DSS induced phosphorylation of JNK, $ERK^{1/2}$ and p38 MAP kinases and NF-κB in mice (Figure 13a, 13b, 13c and 13d). Data obtained from $n = 4$ animals. Densitometry values are expressed as means ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.3.2 Effect of NED on the phosphorylation and activation of NF-κB, p38, JNK and ERK$^{1/2}$ MAP kinases in RAW 264.7 macrophages stimulated with LPS

Western blot experiments showed that LPS stimulation has significantly increased the phosphorylation of p38, JNK and ERK$^{1/2}$ MAP kinases in RAW macrophages; ($p < 0.01$ for JNK, $p < 0.05$ for ERK$^{1/2}$ and $p \leq 0.0001$ for p38). However, treatment with NED significantly reduced the phosphorylation of all these MAP kinases; ($p < 0.01$ at NED 60 µM and $p < 0.05$ at NED 30 µM dose for JNK and p38; $p < 0.05$ at NED 60 µM for ERK$^{1/2}$) (Figure 14a, 14b and 14c). NED treatment at 30 µM dose did not show any significant effect on ERK$^{1/2}$. Similarly, stimulation of RAW macrophages with LPS resulted in a significantly enhanced phosphorylation of NF-κB ($p < 0.05$). NED at 60 µM concentration inhibited the phosphorylation and activation of NF-κB to a significant level ($p < 0.05$) (Figure 14d). No significant effect on the phosphorylation of NF-κB was observed at NED 30 µM dose. These results indicate that NED significantly decreases the phosphorylation and activation of p38, JNK and ERK$^{1/2}$ MAP kinases and NF-κB. However, no significant changes were observed in non-phosphorylated protein expression.

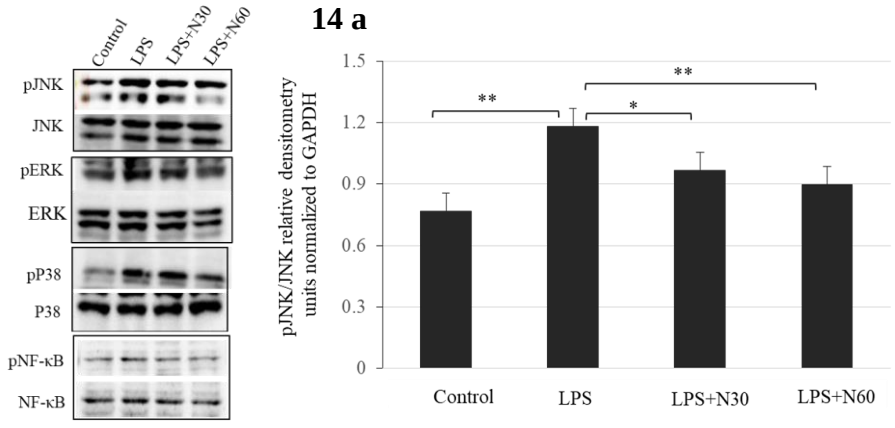
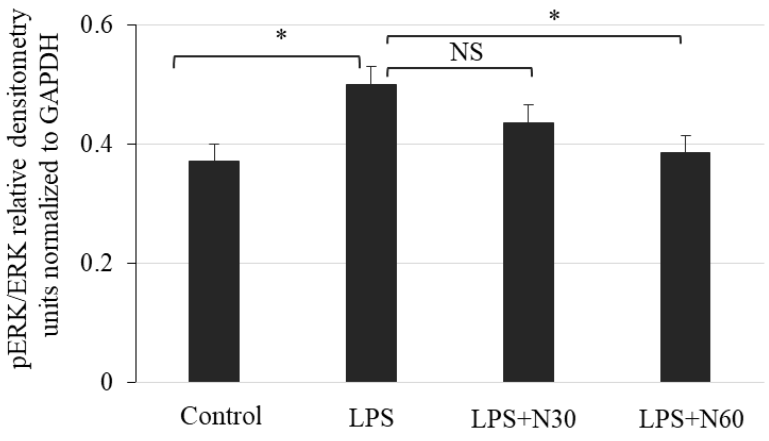
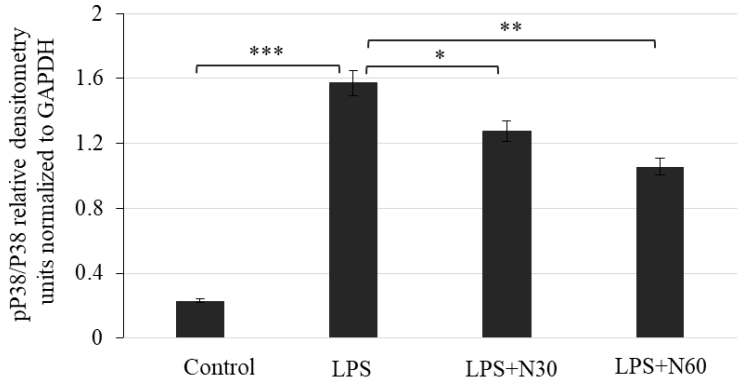


Figure 14: Effect of NED co-treatment on the phosphorylation of NF-κB and MAPK proteins in LPS stimulated RAW Macrophages.

14 b



14 c



14 d

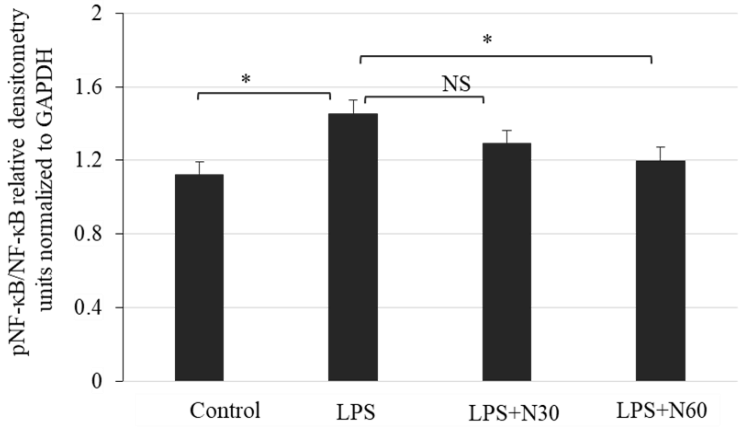


Figure 14: Effect of NED co-treatment on the phosphorylation of NF-κB and MAPK proteins in LPS stimulated RAW Macrophages (continued).

NED significantly inhibited the phosphorylation and activation of p38, JNK and ERK$^{1/2}$ MAP kinases and NF-κB induced by LPS in RAW macrophages (Figure 14a, 14b, 14c and 14d). Data obtained from n = 4 animals. Densitometry values are

expressed as means ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.3.3 Discussion

IBD research has highlighted the importance of various signaling pathways involved in cytokine response and maintenance of intestinal epithelial barrier integrity. Studies have established that TLR-4 signaling leads to NF-κB phosphorylation and subsequent nuclear translocation, regulated transcriptional activation of proinflammatory cytokines, and other inflammatory mediators like COX-2, and iNOS (Atreya, Atreya, & Neurath, 2008; Vukelic et al., 2018; Wullaert, Bonnet, & Pasparakis, 2011). Reports established that positive co-relationship demonstrated between NF-κB activation and intestinal inflammation-associated immune responses from IBD patients (Rogler et al., 1998). Further, NF-κB signaling plays a significant role in abnormal immune responses observed in UC (Tatiya-Aphiradee, Chatuphonprasert, & Jarukamjorn, 2018).

MAPKs comprise ERKs, JNKs, and p38 MAPKs are found to be activated by various stimuli and play a significant role in the immune responses (Hommes, Peppelenbosch, & van Deventer, 2003). Report established that MAPK activation was found to enhance the proinflammatory cytokines release and implicated in UC pathogenesis (Cargnello & Roux, 2011). Further, DSS treatment was shown to increase phosphorylation and activation of JNKs, ERKs, and p38 MAP kinases (Dou et al., 2014; Vukelic et al., 2018). Pieces of evidence suggested that activation of MAP kinases observed in IBD were suppressed by MAPK inhibitors (Broom et al., 2009; Kaminska, 2005; Waetzig et al., 2002). Inhibition of p38 MAPKs was resulted in the

inhibition of proinflammatory cytokine release and decreased NF-κB phosphorylation in DSS-colitis (Hollenbach et al., 2004). JNK inhibitors were also shown to alleviate murine UC colitis by suppression of TNF-α levels (Assi et al., 2006). In another study, JNK inhibitor SP600125 was also found to protect murine colitis by downregulating the proinflammatory cytokines release (Mitsuyama et al., 2006). The MAPK inhibition in an *in vitro* colon inflammation model showed a similar decrease in the proinflammatory cytokines IL-6 and IL-8 levels (Garat & Arend, 2003). But fewer studies have investigated the role of ERKs in IBD. It is reported that $ERK^{1/2}$ phosphorylation was found to be increased in IBD and DSS-induced IL-1β release, also mediated by $ERK^{1/2}$ activation (Kwon et al., 2007; Waetzig et al., 2002). It was also reported that enhanced IL-6 production observed in UC was shown to be mediated by $ERK^{1/2}$. The inhibition of $ERK^{1/2}$ phosphorylation significantly reduced IL-6 production (Leonard, Ryan, Watson, Schramek, & Healy, 1999). Another study reported that luteolin was found to ameliorate DSS-colitis via inhibition of $ERK^{1/2}$ (Vukelic, Detel, Baticic, Potocnjak, & Domitrovic, 2020). These reports indicate that novel anti-inflammatory compounds that can modulate MAPK/NF-κB pathways hold a promising future as a therapeutic drug for IBD.

NED's role on MAPK/NF-κB signaling cascade was designed to evaluate its efficacy on this intracellular signaling pathway involved in the inflammatory responses seen in IBD pathogenesis. Increased NF-κB phosphorylation observed in DSS-colitis was implicated in elevating proinflammatory cytokines levels and mediators at both protein and mRNA levels. In the present study, both doses of NED have suppressed the inhibition/activation of NF-κB phosphorylation, and the maximum response observed in a higher dose of NED. DSS treated animals have shown enhanced

phosphorylation of JNK, ERK$^{1/2}$, and p38 MAPKs, but NED was found to restore to near-normal levels, thereby exerted its significant anti-inflammatory effect.

Reports documented that LPS-stimulated macrophages were involved in phosphorylation/activation of MAPKs, including p38, ERK$^{1/2}$, and JNK (Gantke, Sriskantharajah, Sadowski, & Ley, 2012; Hambleton et al., 1996; Hwang, Ma, Park, Jung, & Park, 2019). Phosphorylation/activation of MAPKs promoted proinflammatory cytokine synthesis (Fang et al., 2004). Considering these reports, the role of NED in the expression of MAPKs (p38, ERK$^{1/2}$, and JNK) in LPS-stimulated RAW macrophages were studied. Enhanced phosphorylation of all MAP kinases upon stimulation by LPS was significantly inhibited by NED co-treatment in a dose-dependent manner. Thus, NED was found to inhibit a major intracellular signaling pathway involved in the inflammatory response and proved its efficacy in both *in vivo* and *in vitro* models. The inhibition of MAPK/NF-κB signaling could well be the mechanism behind the decreased proinflammatory cytokines and mediator response associated with NED co-treatment in both DSS colitis model and in LPS stimulated RAW macrophages.

Increased intestinal permeability is major factor associated with pathogenesis of UC. Since tight junction proteins are responsible for maintaining the intestinal barrier integrity, further investigations were planned to investigate the mechanism of NED's role in maintaining the intestinal epithelial barrier integrity and on the TJ proteins.

4.4 Study 4: Role of NED in maintaining the intestinal epithelial barrier integrity in *in vivo* and in *in vitro* models of colon inflammation

Intestinal barrier is a dynamic structure that interacts and responds to external stimuli. A disrupted intestinal epithelial barrier leading to increased colonic permeability is the event that precedes the development of intestinal inflammation (Eichele & Kharbanda, 2017). To date, no such investigation has been carried out involving NED interaction on TJ physiology. Therefore, NED's role was investigated in maintaining the epithelial barrier integrity and its effect on various TJ proteins expression using both *in vivo* and *in vitro* models of colon inflammation.

Results:

4.4.1 Effect of NED on colonic permeability

The permeation of FITC-Dextran through the paracellular pathways of intestinal epithelial cells is a marker of intestinal permeability. In the present study, DSS-induced damage in colonic epithelium significantly increased its permeability to FITC-dextran ($p < 0.0001$), indicating a severe intestinal barrier dysfunction. NED significantly prevented the epithelial damage and significantly restored barrier dysfunction induced by DSS colitis, at both doses studied ($p < 0.001$ at NED 100 and 150 mg/kg dose) (Figure 15).

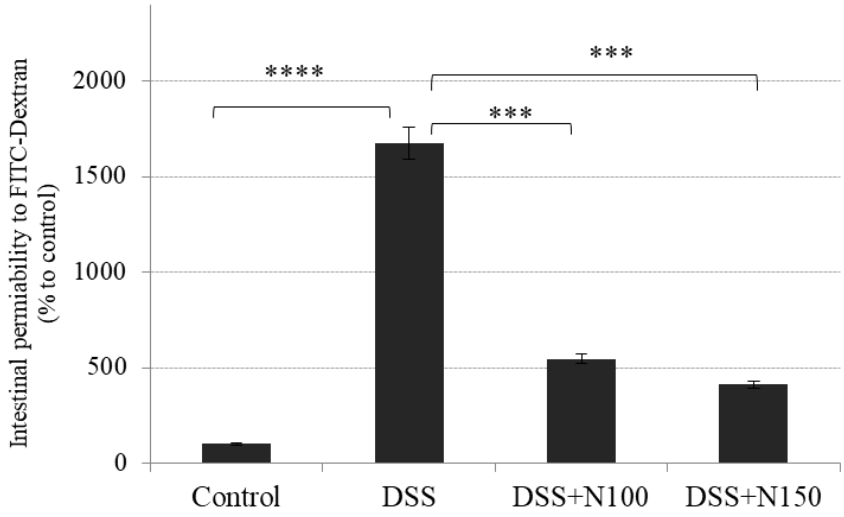


Figure 15: Effect of NED on *in vivo* intestinal permeability using FITC dextran.

NED treatment at 100 and 150 mg/kg dose significantly inhibited the DSS induced increased permeability of FD-4 in mice. Data obtained from $n = 5$ animals and results expressed as mean ± SEM. **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.4.2 Effect of NED on TJ protein expression in the colon of DSS administered mice

TJ proteins present in the intercellular junctions of intestinal epithelial cells play the key role in maintaining intestinal barrier integrity. Western blot analysis showed that the expression of the TJ proteins namely, claudin-1, -3, -7 and occludin were significantly decreased in the mice colon homogenate treated with DSS ($p < 0.01$ for claudin-1, claudin-3, claudin-7 and occluding) (Figure 16a, 16b, 16c, 16d) when compared to control. NED treatment prevented this DSS-induced decrease of TJ proteins ($p < 0.05$ for claudin-1 at NED 100 and 150 mg/kg dose, $p < 0.05$ for claudin-3 at NED 100 mg/kg and $p < 0.01$ at 150 mg/kg dose, $p < 0.05$ for claudin-7 at NED 100 and 150 mg/kg dose, $p < 0.05$ for occludin at NED 100 mg/kg and $p < 0.01$ at 150 mg/kg dose) (Figure 16a, 16b, 16c, 16d).

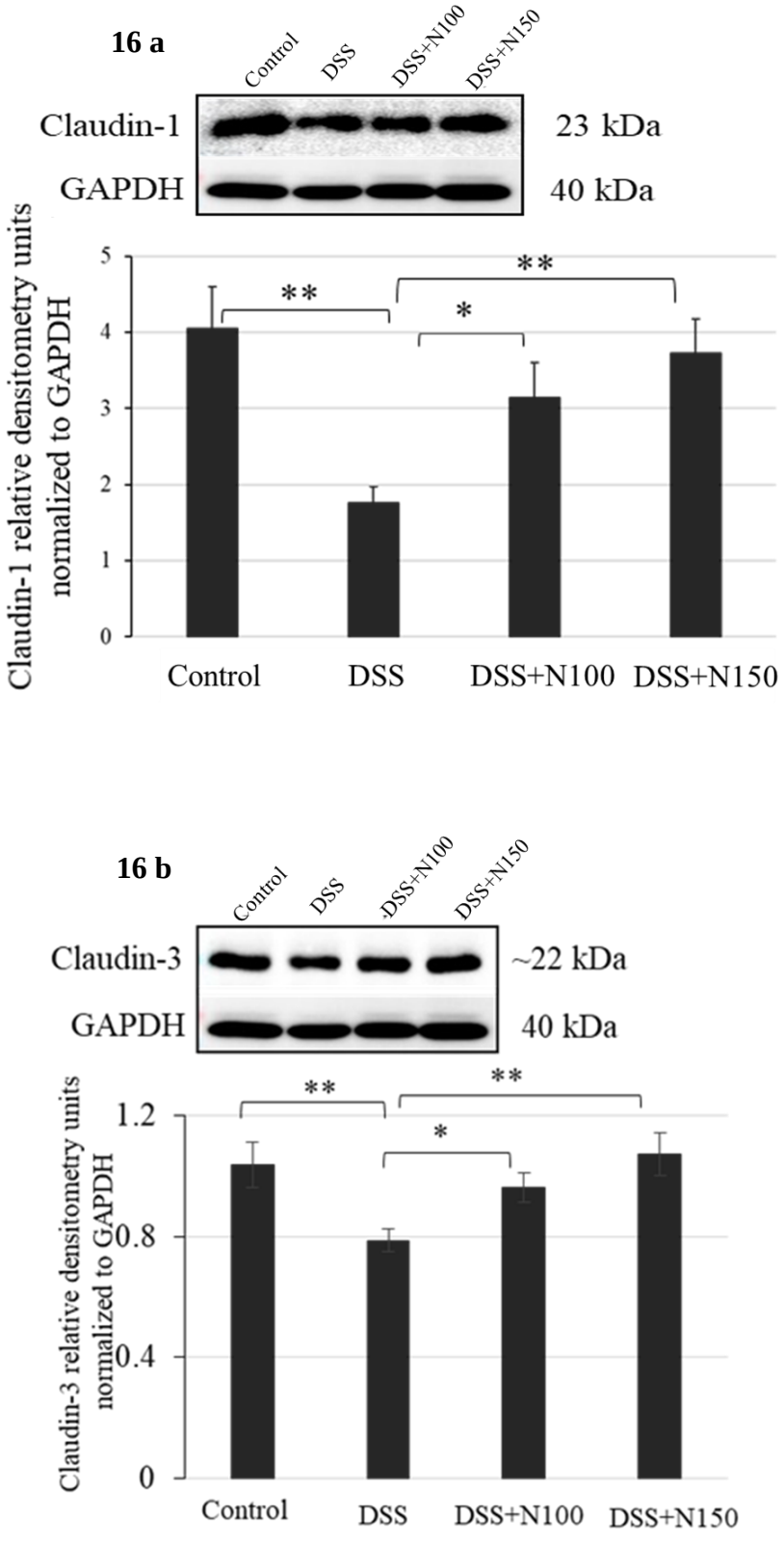


Figure 16: Effect of NED in maintaining the TJ protein expression.

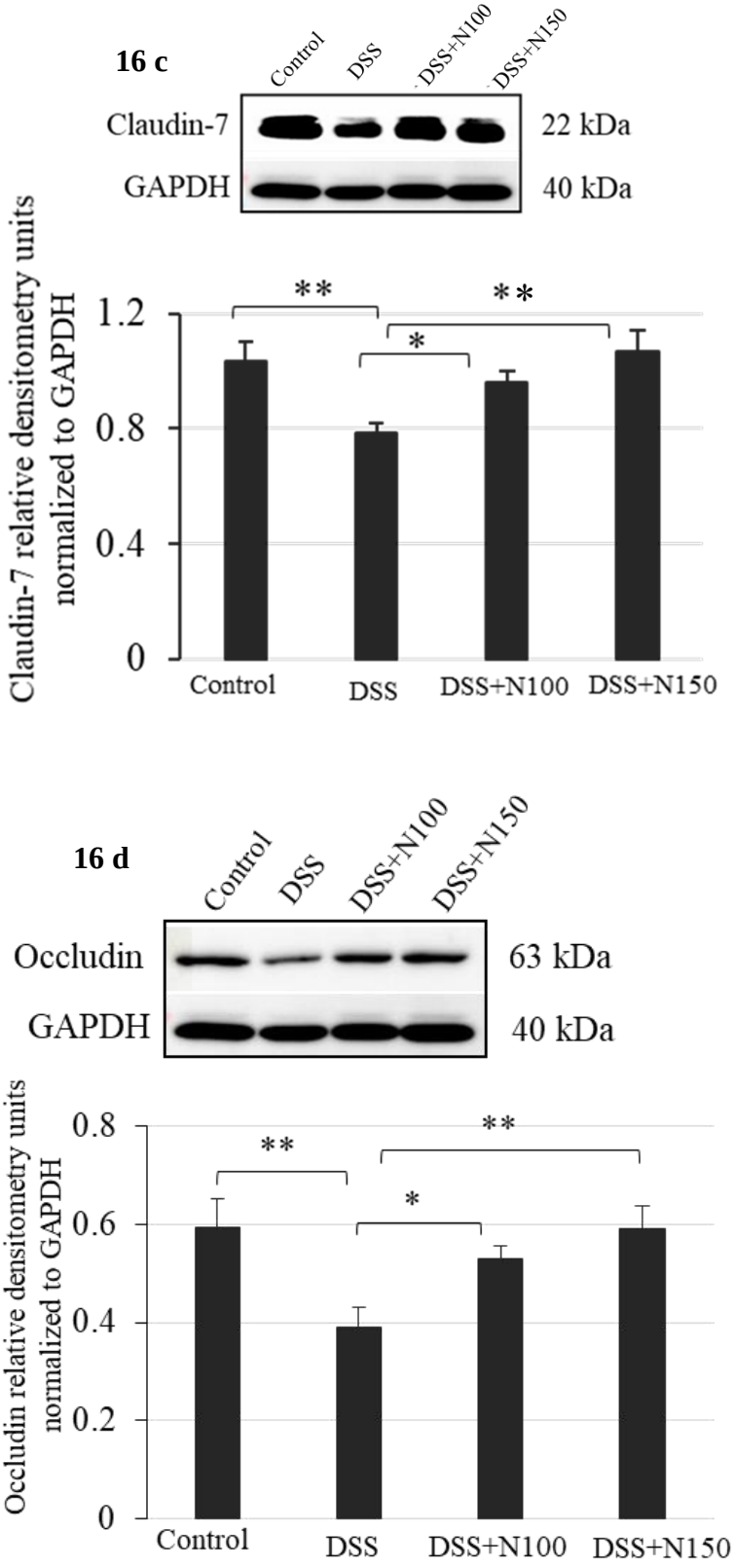


Figure 16: Effect of NED in maintaining the TJ protein expression (continued).

In the animal model DSS significantly downregulated the protein expression of claudin-1, -3, -7 and occludin. The treatment with NED at 100 and 150 mg/kg dose significantly improved the expression of these proteins suggesting the role of NED in protecting the intestinal barrier integrity (Figure 16a, b, c and d). Data obtained from $n = 4$ animals and results expressed as mean ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.4.3 Effect of NED on cell viability, proinflammatory chemokine mRNA expression in Caco-2 cells stimulated with LPS

The effect of NED on mRNA expression of proinflammatory chemokines were evaluated using Caco-2 cell line. To optimize the dose range to be studied, cells were treated with different concentrations of NED ranging from 0 µM to 200 µM for 24 hrs and 48 hrs. NED did not induce cytotoxic effect on the viability of Caco-2 cells. NED was observed to be safe till 100 µM dose (Figure 17a and Figure17b). Based on these observations, 30 and 60 µM doses of NED were selected for subsequent experiments. Stimulating Caco-2 cells with LPS resulted in a significant increase in the mRNA expression of proinflammatory chemokines; CXCL-1 and IL-8; $p \leq 0.0001$ for CXCL-1 and $p \leq 0.001$ for IL-8 (Figure 17c and 17d). All these proinflammatory gene markers were significantly downregulated upon NED treatment in LPS-stimulated Caco-2 cells in a dose-dependent manner, $p \leq 0.01$ and $p \leq 0.001$ for CXCL-1 at NED 30 and 60 µM respectively; $p \leq 0.01$ for IL-8 at NED 30 and 60 µM (Figure 17c and 17d). However, NED alone at 60 µM concentration did not show any significant effect when compared to control. Hence this group was removed from the subsequent experiments.

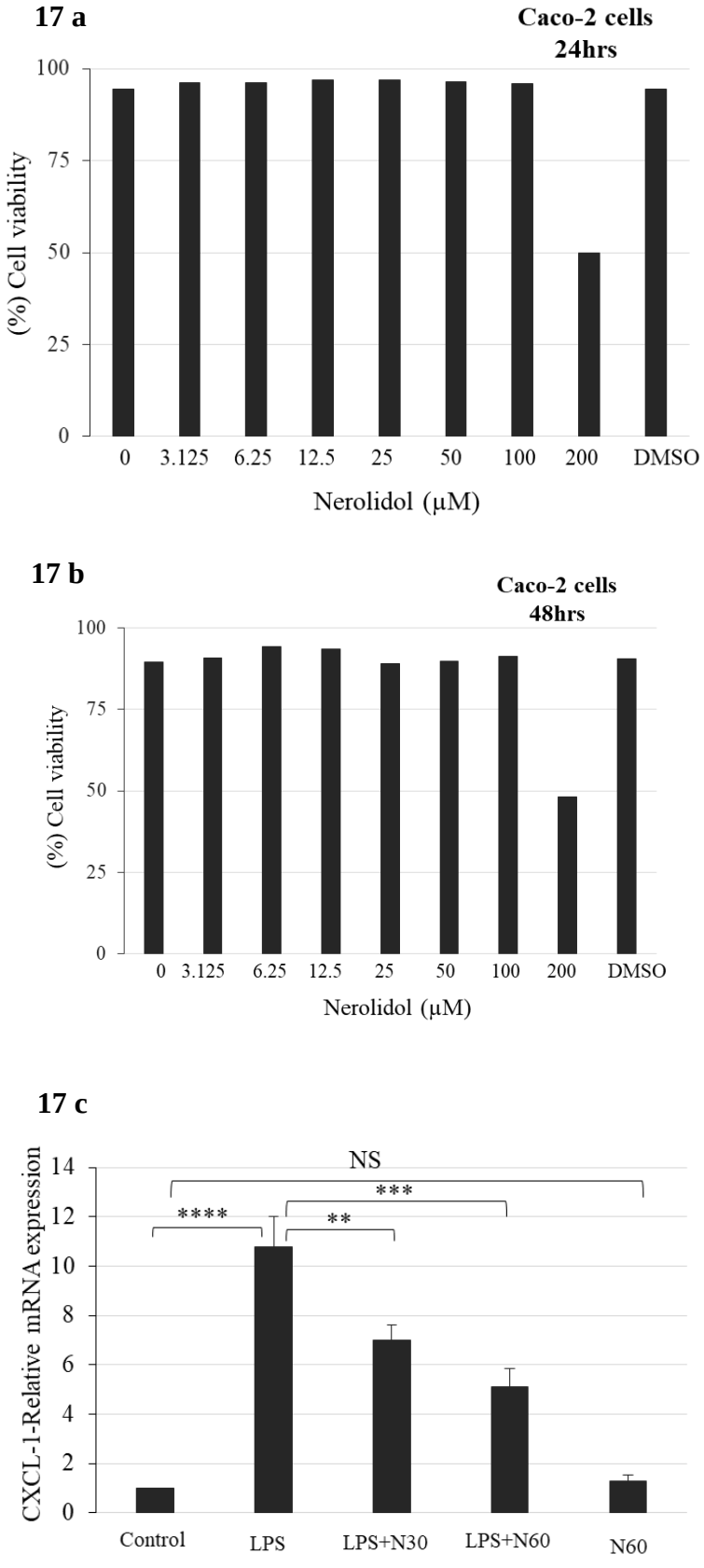


Figure 17: Effect of NED on cell viability and proinflammatory chemokine mRNA expression in LPS treated Caco-2 cells.

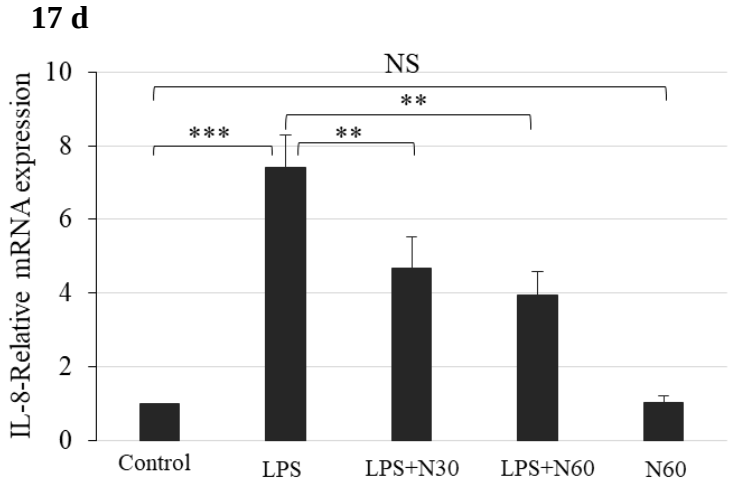


Figure 17: Effect of NED on cell viability and proinflammatory chemokine mRNA expression in LPS treated Caco-2 cells (continued).

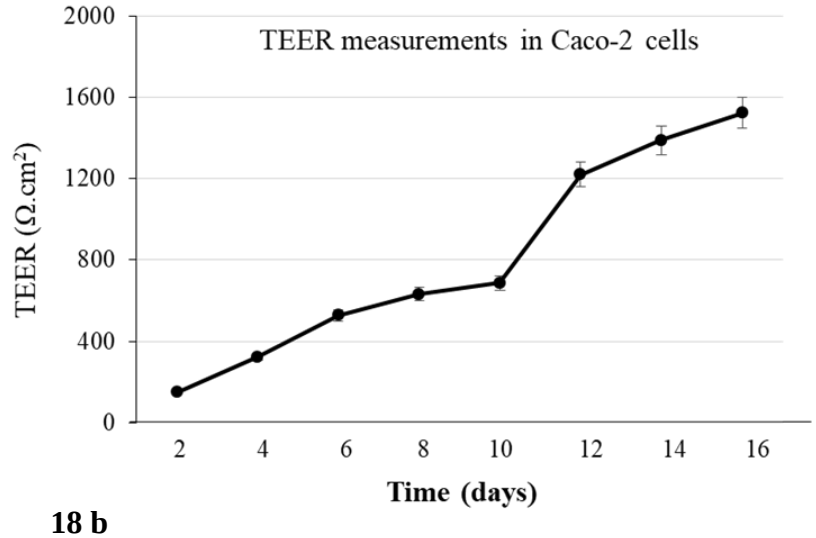
The concentration-dependent cytotoxic effects of NED were investigated on treatment of Caco-2 cells with different concentrations of NED for 24 hrs (Figure 17a) and 48 hrs (Figure 17b). Data expressed as mean ± SEM (n = 6). **** $p \leq$ 0.0001, *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq$ 0.05 considered statistically significant and NS indicate not significant.

4.4.4 Effect of NED on TEER measurements of LPS challenged Caco-2 monolayer

The epithelial barrier integrity of Caco-2 monolayers was evaluated by measuring TEER values daily. TEER showed a steady increase on daily basis, until reached a plateau over a period of 14-15 days (Figure 18a). Caco-2 monolayers showing 1000-1200 $\Omega.cm^2$ were pre-incubated with NED at 30 and 60 µM concentrations. These monolayers were challenged with LPS after 24 hrs. TEER values after 24 hrs showed a 50% decrease compared to the control ($p \leq 0.001$) (Figure 18b). These results indicate that epithelial barrier integrity is compromised by LPS challenge. However, LPS-treated cells exhibited significant decrease in TEER values. However, NED pretreatment in LPS-stimulated Caco-2 monolayer prevented the decrease in TEER ($p \leq 0.05$ at NED 30 µM and $p \leq 0.01$ at NED 60 µM dose) (Figure

18b). Therefore, NED prevented the deleterious effects of LPS on Caco-2 monolayers to a significant level and restored the epithelial barrier integrity in a significant manner.

18 a



18 b

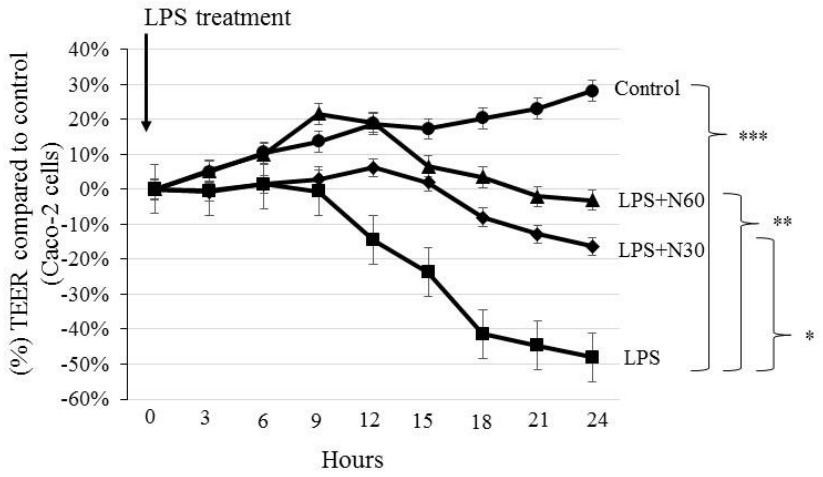


Figure 18: Effect of NED on TEER measurements. TEER values of the Caco-2 monolayer were recorded daily to assess the barrier integrity.

LPS significantly reduced the TEER of Caco-2 monolayers compared to the control. Incubation with NED significantly inhibited the decrease in TEER caused by LPS. Results expressed as mean ± SEM. *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.4.5 Effect of NED on the expression of TJ proteins in Caco-2 monolayers

The effect of NED on the TJ proteins expression was evaluated using western blot experiments. The protein expression of occludin, claudin-1, -3 and -7 were significantly decreased with LPS treatment ($p < 0.05$ for claudin-3) when compared to control, ($p < 0.01$ for claudin-1, claudin-7 and occludin) (Figure 19a, 19b, 19c and 19d). The decreased expression of TJ proteins observed in LPS-treated Caco-2 monolayers indicated the disruption of epithelial barrier. The expressions of the TJ proteins were significantly restored in NED treated Caco-2 monolayers ($p < 0.05$ for occludin and claudin-3 at NED 60 µM dose) (Figure 19a and 19c). However, NED at 30 µM did not induce any significant effect on occludin and claudin-3. For claudin-1 and -7 ($p < 0.05$ at NED 30 µM and $p < 0.01$ at 60 µM dose) (Figure19b and 19d). These results proved that NED was effective in protecting the epithelial barrier integrity.

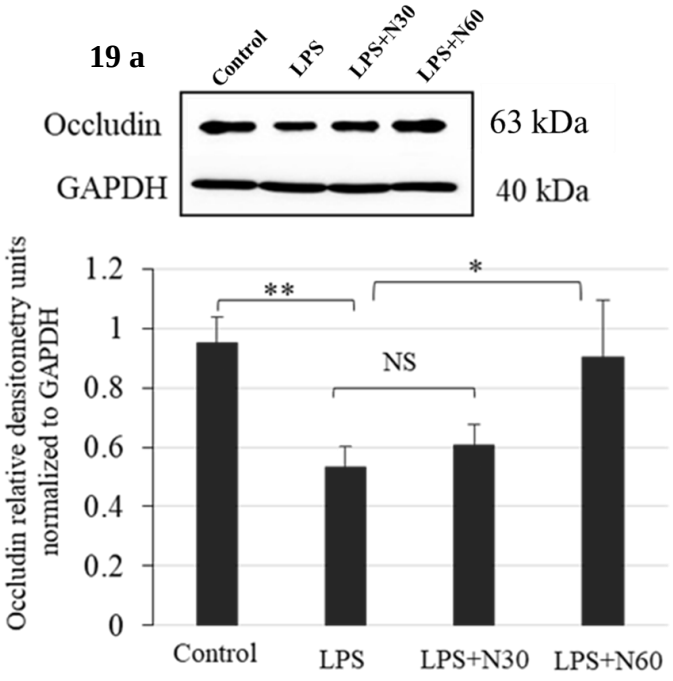


Figure 19: Role of NED in maintaining the TJ protein expression in LPS stimulated Caco-2 cells.

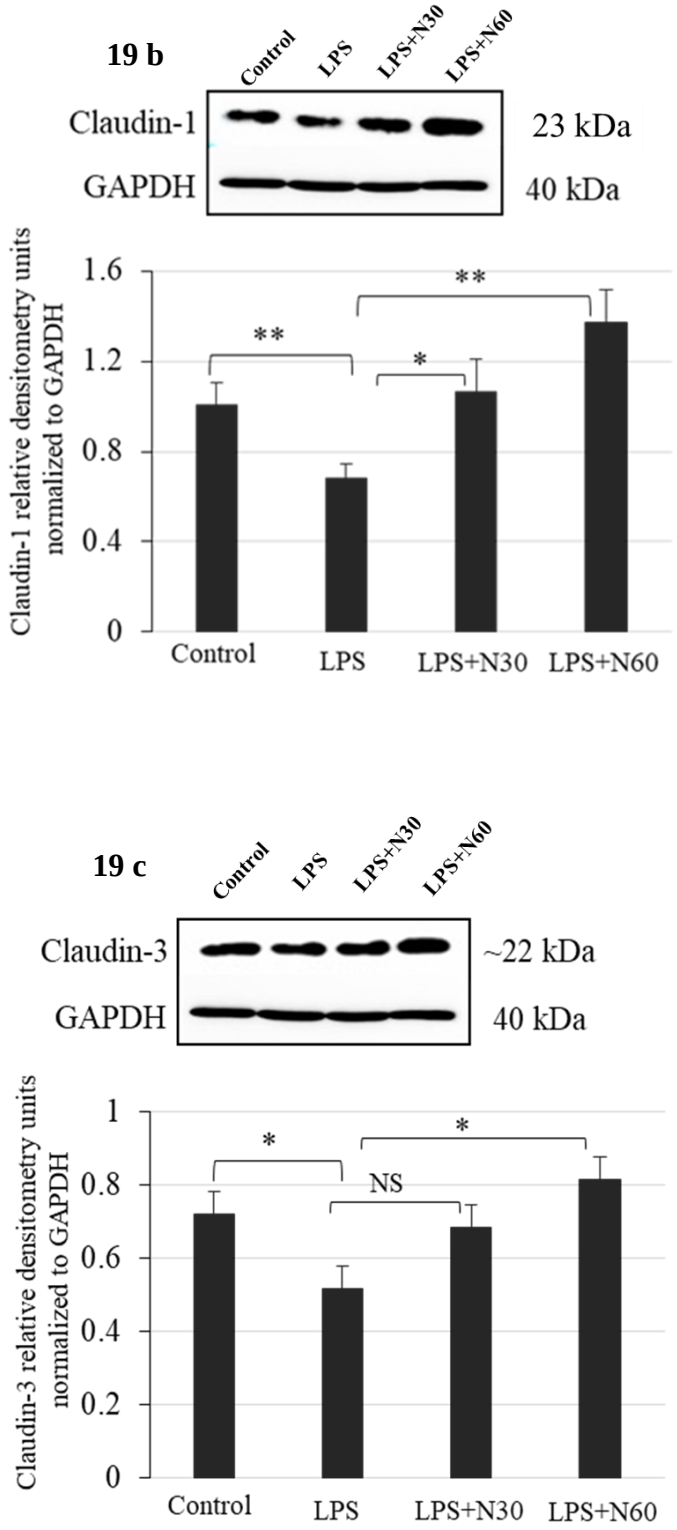


Figure 19: Role of NED in maintaining the TJ protein expression in LPS stimulated Caco-2 cells (continued).

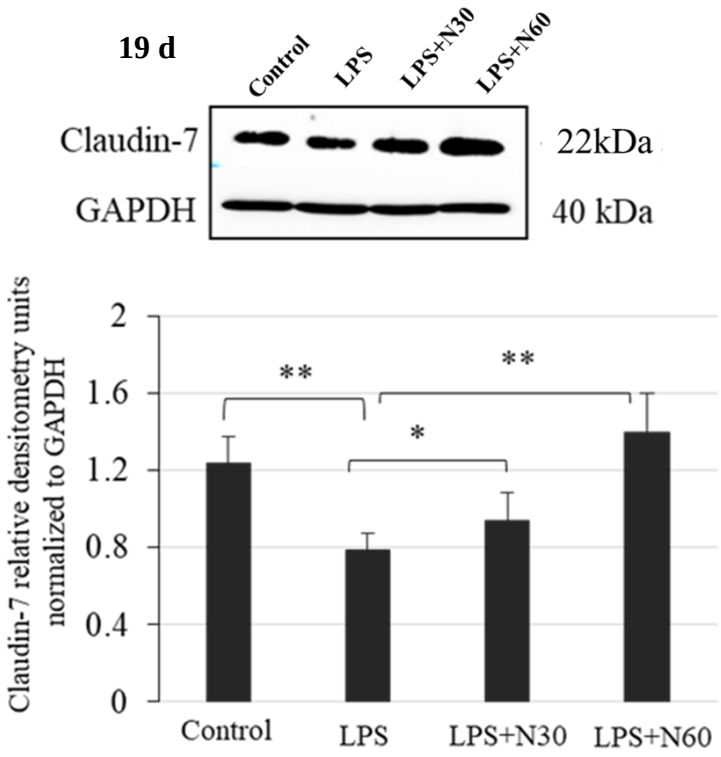


Figure 19: Role of NED in maintaining the TJ protein expression in LPS stimulated Caco-2 cells (continued).

A decrease in TJ protein expression was seen in Caco-2 cells stimulated with LPS. NED treatment at 30 and 60 µM significantly prevented the decrease in the expression of occludin, claudin- 1, -3 and -7 (Figure 19a, 19b, 19c and 19d). *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$, p value obtained by one-way ANOVA followed by Tukey's multiple comparison test using SPSS software and $p \leq 0.05$ considered statistically significant.

4.4.6 Discussion

Intestine mucosal epithelium shows a regularly aligned layer of epithelial cells with an underlying lamina propria and muscularis mucosa. Immune cells, including macrophages reside in the lamina propria. The intestinal epithelium cells help in absorption of water, electrolytes and nutrients along with the maintenance of a physical barrier (Brandtzaeg, 2011). The inflammatory responses seen in UC and CD disrupt the intestine epithelial barrier integrity that contributes the development of a leaky gut.

During normal physiological condition, mucosal epithelium separates the luminal contents, including microbes and other dietary antigens, from entering the underlying intestinal tissue. This is the first and foremost function of intestinal epithelia, and it is termed as the 'barrier function'. However, mucosal epithelium allows the transport of nutrients and ions across the epithelium and thus acts as a selectively permeable barrier (Turner, 2009). Inflammation is the critical event that affects intestine barrier integrity and allows the entry of luminal antigens into the lamina propria via the paracellular pathway due to its compromised barrier (Azuma, Shigeshiro, Kodama, Tanabe, & Suzuki, 2013). The apical junctional complex comprises tight junction (TJ) proteins and helps to regulate the paracellular permeability between two adjacent epithelial cells. The adherence junction located below/along with TJ is involved in regulating cell-cell signaling and desmosomes stabilizes the epithelia (Bischoff et al., 2014).

Two important pathways, namely 1) transcellular and 2) paracellular, are involved in transporting various substances across the intestinal epithelium. Numerous transporters and ion channels located at both apical and basolateral surfaces of the epithelial cells contributes to transcellular transport pathway. The tight junction proteins located at the apical side of intestinal epithelium that regulate the transport of various substances contribute to the paracellular transport pathway (Hung & Suzuki, 2016; Turner, 2009). Two sets of functional proteins guard the TJs: transmembrane proteins, present between adjacent epithelial cells and peripheral membrane or the plaque proteins. As explained before, the major tight junction proteins are the occludin, claudin family, tricellulin, junctional adhesion molecules (JAMs), and zonula occludins (ZO). Occludin, claudins, tricellulin, and JAMs are transmembrane proteins. The intracellular domains of transmembrane proteins are anchored on the F-actin and myosin-II by the membrane/plaque protein - ZOs. This interaction makes TJ proteins

highly dynamic and guarding the epithelial barrier integrity as intact as possible (Mitic & Anderson, 1998).

The phosphorylation, distribution, and expression of these TJ proteins regulate intestinal homeostasis by maintaining the barrier integrity (Lee, Moon, & Kim, 2018). Therefore, any change in the tight junction proteins expression is implicated in barrier disruption in IBD (Galvez, Rodriguez-Cabezas, & Zarzuelo, 2005; Hering & Schulzke, 2009; Oshima, Miwa, & Joh, 2008; Zeissig et al., 2007). Occludin is the first reported TJ protein (Furuse et al., 1993). Claudins are another class of transmembrane proteins in that TJ strand, and approximately 24 different types of claudins are identified in mammals (Van Itallie & Anderson, 2006). Under normal circumstances, the intestinal epithelium is subjected to a renewal process approximately every five days via the differentiation of pluripotent stem cells localized in the crypts. Increased shedding of cells can compromise the barrier integrity of the intestine, but the dynamic nature of TJ causes rapid redistribution of TJ proteins and seals the paracellular space and maintains the barrier integrity (Eisenhoffer et al., 2012; Marchiando et al., 2011). Intestinal epithelial barrier integrity is compromised by ulceration and apoptosis of epithelium and elevated proinflammatory cytokines are also involved in the derangement /or destruction of TJ proteins (Arrieta, Madsen, Doyle, & Meddings, 2009; Heller et al., 2005; Zolotarevsky et al., 2002). Scientific reports have documented that UC colitis patients showed destruction or rearrangement of tight junction proteins like occludin, claudin-1, tricellulin, and JAMs in colon biopsy analysis (Furuse et al., 1998; Oshima et al., 2008; Tsukita & Furuse, 2000). The bacterial component LPS and the elevated levels of proinflammatory cytokines like TNF-α were elevated in IBD patients and associated with an increased intestinal permeability (Duffy et al., 1997; Noerr, 2003; Piechota-Polanczyk & Fichna, 2014).

UC patients showed a significantly elevated expression of tight junction proteins such as claudin-2 and reduced expression of proteins claudin-1, -3, -4 and -7 (Oshima et al., 2008; Prasad et al., 2005). Overall, altered expression of TJ proteins was correlated with the disease severity of UC patients.

In UC pathogenesis, increased epithelial permeability with destruction or rearrangement of tight junction proteins could be an event that precedes luminal microbes' entry into the intestinal mucosa. Elevated levels of proinflammatory cytokines were shown to increase the cell shedding rate and some-times more cells were shedded from the same area, so that redistribution of TJ may not be fast enough to seal the space created by shedded cells. These events increased the paracellular permeability and allowed the entry of luminal microbes, and recruit inflammatory cells to the lamina propria, resulting in further elevated cytokine production (Bischoff et al., 2014).

DSS-colitis mouse model has shared many similarities with human UC, in terms of symptoms, morphological and pathophysiological features. The toxic effect of sulfate moiety of DSS on the epithelial cells disrupt the barrier integrity and allowed the entry of luminal antigens into the mucosa, followed by the innate immune response activation through the recruitment of inflammatory cells (neutrophils and macrophages) (Carlsson et al., 2013). DSS was reported to modulate the expression ofclaudins-1, -3, -7, -8, and occludin in the distal colon and negatively regulated the intestinal permeability. However, there are contradictory reports on the effect of DSS on claudin expression. In rat model of UC, claudin-1 expression was increased up on exposure to DSS, but in the mouse model, DSS caused a decrease of claudin-1 expression (Iwaya et al., 2012; Tsukita & Furuse, 1999; Yuan et al., 2015). DSS effect

on the tight junction proteins increased the intestinal permeability, thereby worsening the mucosal epithelial injury. Besides, DSS exerted significant effect on the plaque protein ZOs expression. One day of DSS exposure was enough to cause significant alterations in the ZO-1 expression (Poritz et al., 2007). A rearrangement of occludin and ZO-1 was also reported in DSS administration for 4 days (Samak et al., 2015). Hence, the DSS-induced colitis model was proved to compromise the intestinal barrier integrity and its effect on various TJ proteins.

Based on the above observations, NED's role was investigated in the regulation of TJ protein expression and on DSS-induced loss of barrier integrity. The intestinal epithelial barrier integrity or intestinal permeability can be evaluated by administering macromolecules that cannot be digestible and can permeate more through compromised colon epithelial barrier, considered the most reliable model for *in vivo* intestinal permeability analysis (Li et al., 2018). The term "intestinal permeability" was first proposed by the electrophysiologists, who demonstrated epithelial permeability experiments in Ussing chambers with animal or tissue explants (Clarke, 2009; Hering, Fromm, & Schulzke, 2012). The biomarkers used in determining intestine permeability experiments include radioisotopes, polyethylene glycols, and sugars (Wang et al., 2015). FITC-dextran (4 kDa molecular weight) was used as a marker to assess the barrier integrity in the present study. Under physiological conditions, FITC-dextran does not cross the intestinal barrier due to its macro size, but can diffuse through the barrier, detected in the blood when barrier integrity is compromised. In this study, FITC-dextran was used to assess the barrier integrity as described in the method section. FITC-dextran takes 3 hrs to reach the colon after passing through GI tract (Li et al., 2018). Blood samples collected from experimental groups after 4 hrs of FITC administration, and the serum concentration of FITC-

dextran indicate the extent of intestinal barrier permeability. Results indicated that DSS administration significantly increased FITC-dextran levels in serum compared to control animals. However, the NED treatment significantly prevented the increase in serum FITC-dextran concentration.

Furthermore, the role of NED modulating the barrier integrity was assessed in an *in vitro* model of colon inflammation using Caco-2, human colorectal adenocarcinoma cells. Human colon epithelial cell line Caco-2 is a well-established model for *in vitro* assessment of intestinal epithelial barrier integrity (Ponce de Leon-Rodriguez et al., 2019). NED doses were selected based on the cell viability assay and showed no cellular cytotoxicity up to 100 µM NED concentration. NED also significantly decreased the LPS-induced chemokines mRNA expression in Caco-2 cells.

To assess the barrier integrity in *in vitro* model, Caco-2 cells were cultured on transwell filter supports and allowed to form a monolayer as described in the method section. As these cells grew and formed a monolayer, the TEER values increased progressively, suggesting a well-formed colonic epithelial barrier. This technique is widely accepted to assess the barrier integrity (Srinivasan et al., 2015; Trevisi et al., 2009). A significant drop was found in the TEER values over 24 hrs post-stimulation with LPS. This TEER decrease was significantly prevented by NED pre-incubation, suggesting NED's role in maintaining the barrier integrity. These results were comparable with other studies, which reported LPS-stimulated TEER reduction modulated by alternative medicines (Chen et al., 2019; Ling, Linglong, Weixia, & Hong, 2016). Considering these results from *in vivo* and *in vitro* experiments on

intestinal barrier integrity, anti-inflammatory properties exhibited by NED could be attributed for its protective effect on the intestinal epithelium.

TJ proteins expression is reported to play a significant role in maintaining the barrier integrity. The NED efficacy was analyzed on protein expression in both *in vivo* and *in vitro* models. Several reports suggest that the DSS administration affects TJ proteins expression and its signaling pathways leading to dysregulation of intestinal epithelial barrier - (Azuma et al., 2013; Mayangsari & Suzuki, 2018). In *in vivo* experiments, western blot analysis was shown that DSS treatment induced a significant reduction in the TJ proteins expression such as, claudin-1, -3, -7, and occludin. Phytochemicals have shown to downregulate the TJ proteins expression in LPS-treated Caco-2 cells and *in vivo* model as well (Chen et al., 2019; Ling et al., 2016). In addition, quercetin, naringenin, and resveratrol have also shown to positively influence the intestinal epithelium barrier integrity by enhancing/rearrangement of the TJ proteins (Azuma et al., 2013; Mayangsari & Suzuki, 2018; Noda, Tanabe, & Suzuki, 2014). Therefore, NED was shown in positively modulating the intestine tight junctions to prevent 'leaky gut' associated with colon inflammation.

It was reported that epithelial growth factor protected the intestinal barrier integrity from hydrogen peroxide-induced disruption of occludin mediated through ERK signaling (Basuroy, Seth, Elias, Naren, & Rao, 2006). Further, glutamine deprivation was found to decrease claudin-1 and ZO-1 expression on Caco-2 monolayers, whereas glutamine treatment leads to ERK/JNK activation and modulation of TJ proteins (Li, Lewis, Samuelson, Liboni, & Neu, 2004; Rhoads et al., 1997). MLC phosphorylation by MLCK regulating the contractions of actomyosin ring, is a key step that regulates barrier integrity by opening of paracellular pathways

(Turner et al., 1997). TNF-α was found to enhance MLCK phosphorylation and resulted in increased paracellular permeability. Moreover, MLCK inhibition was shown to improve barrier function in TNF-α stimulated intestinal epithelial cells (Zolotarevsky et al., 2002). It was reported that p38 MAPK inhibition caused a reduction in TNF-α production in colon biopsies of IBD patientsand shown to be actively involved in barrier impairment (Waetzig et al., 2002). Studies have also reported that TNF-α suppresses occludin promoter activity and caused a rearrangement of ZO-1 and claudin-1 (Mankertz et al., 2000; Wang et al., 2005). In another study, JNK inhibitor SP600125 was shown to decrease the DSS-induced JNK phosphorylation and prevented intestinal barrier disruption by enhanced ZO-1 and occludin protein expression (Samak et al., 2015). It was also reported that curcumin prevented the gut-derived bacterial LPS translocation into the circulation by protecting the intestinal epithelial barrier integrity and its effect attributed to its inhibition of IL-1β signaling. IL-1β was reported to increase p38 MAPK phosphorylation, subsequently enhanced the MLCK expression, resulted in increased paracellular permeability (Wang, Ghosh, & Ghosh, 2017). Hence it may be possible that suppression of MAPK signaling by NED could have resulted in maintaining the epithelial barrier integrity.

Therefore, these results indicated that the anti-inflammatory property of NED helped regulate MAPK/NF-κB signaling and maintain colon epithelial barrier integrity.

Chapter 5: Concluding Remarks

- NED treatment in LPS-stimulated RAW 264.7 macrophages significantly inhibited proinflammatory cytokines (TNF-α, IL-6, IL-1β) protein and mRNA expression. NED treatment also decreased the proinflammatory cytokine mediators COX-2 and iNOS protein and mRNA expression.

- NED treatment in DSS-induced colitis mice prevented both disease progression and severity demonstrated by the DAI score, colon length, MPO activity, and histopathological changes. It was notable that the effects of NED were as efficacious as those of sulfasalazine, a drug commonly used in clinical practice to treat ulcerative colitis. NED treatment in DSS-induced colitis also significantly decreased proinflammatory cytokines (TNF-α, IL-6, IL-1β), and mediators (COX-2 and iNOS) levels. NED treatment in DSS-induced colitis stimulated the nuclear translocation of Nrf-2 transcription factor and enhanced cellular antioxidant response.

- NED treatment in TNF-α stimulated HT-29 cells used as an *in vitro* model colon inflammation significantly inhibited the gene expression of proinflammatory chemokines and mediators.

- NED treatment in DSS-induced colitis and LPS-stimulated murine macrophages significantly prevented the phosphorylation and activation of MAP kinase signaling pathway proteins (p38, JNK and $ERK^{1/2}$), and NF-κB transcription factor.

- NED treatment significantly decreased FITC-dextran colon permeability in DSS-induced colitis and prevented the transepithelial electrical resistance (TEER) reduction in LPS-stimulated Caco-2 (human colon carcinoma cells) monolayer. NED significantly enhanced colonic epithelial tight junction proteins (Occludin, claudin-1, -3, & -7) expression in both DSS-induced colitis and LPS-stimulated Caco-2 monolayers.

- These results indicate that NED, a naturally occurring aliphatic sesquiterpene mitigates colon inflammation and maintains colon integrity by inhibiting the colonic inflammatory response and protecting epithelial tight junction integrity and barrier function.

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1. Raj, V., Ojha, S., Howarth, F. C., Belur, P. D., & Subramanya, S. B. (2018). Therapeutic potential of benfotiamine and its molecular targets. Eur Rev Med Pharmacol Sci, 22(10), 3261-3273. doi:10.26355/eurrev_201805_15089

2. Raj, V., Venkataraman, B., Almarzooqi, S., Chandran, S., Ojha, S. K., Attoub, S., Adrian, T. E., & Subramanya, S. B. (2020). Nerolidol Mitigates Colonic Inflammation: An Experimental Study Using both In Vivo and In Vitro Models. Nutrients, 12(7). doi:10.3390/nu12072032

3. Subramanya, S. B., Chandran, S., Almarzooqi, S., Raj, V., Al Zahmi, A. S., Al Katheeri, R. A., Al Zadjali, S. A., Collin, P. D., & Adrian, T. E. (2018). Frondanol, a Nutraceutical Extract from Cucumaria frondosa, Attenuates Colonic Inflammation in a DSS-Induced Colitis Model in Mice. Mar Drugs, 16(5). doi:10.3390/md16050148

4. Venkataraman, B., Almarzooqi, S., Raj, V., Alhassani, A. T., Alhassani, A. S., Ahmed, K. J., Subramanian, V. S., Ojha, S. K., Attoub, S., Adrian, T. E., & Subramanya, S. B. (2021). Thymoquinone, a Dietary Bioactive Compound, Exerts Anti-Inflammatory Effects in Colitis by Stimulating Expression of the Colonic Epithelial PPAR-gamma Transcription Factor. Nutrients, 13(4). doi:10.3390/nu13041343

5. Venkataraman, B., Ojha, S., Belur, P. D., Bhongade, B., Raj, V., Collin, P. D., Adrian, T. E., & Subramanya, S. B. (2020). Phytochemical drug candidates for the modulation of peroxisome proliferator-activated receptor gamma in inflammatory bowel diseases. Phytother Res, 34(7), 1530-1549. doi:10.1002/ptr.6625