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PhD Thesis

Phytopharmacy Research in the Context of Saudi Arabian Healthcare – the Example of *Nigella sativa* L. Efficacy on Asthma Inflammation and Outcomes

By

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This report is submitted in partial fulfilment for the PhD degree

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Declaration of Originality

This is to certify that this work is entirely my own and not of any other person unless explicitly acknowledged (including citation of published and unpublished sources). This work has not previously been submitted in any form to the UCL School of Pharmacy or any other institution for assessment or any other purpose.

Signed

Date 08/12/2017

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Abstract

Background

Clinical research using herbal medicines requires specific considerations such as production method, which leads to chemical and pharmacological variability. Asthma is a leading chronic respiratory disease, remains sub-optimally controlled despite conventional costly treatments. *Nigella sativa* L. (NS) is a traditional herbal treatment for asthma, but lacking well-established scientific evidence.

Objectives

To develop an integrated research strategy incorporating preclinical and clinical research focusing on NS use in asthma by employing a chemically and pharmacologically well-characterised NS preparation in a well-designed clinical trial.

Methods

Distinct NS preparations were chemically characterised for thymoquinone (main active compound) concentration by High Performance Liquid Chromatography. Human T-lymphocyte, monocyte and A549 epithelial cells were utilised to assess the in-vitro anti-inflammation/immunomodulatory activity of NS preparations. The most potent and suitable NS preparation was clinically evaluated for efficacy as add-on treatment for asthmatics in a phase-II randomised double-blind placebo-controlled clinical trial (RDBPCT). Asthma Control Test (ACT) was the primary outcome. Pulmonary function, blood eosinophils and serum inflammatory markers were secondary outcomes.

Results and Discussion

Ten different NS preparations were obtained, showing variability in thymoquinone concentration and *in-vitro* anti-inflammatory/immunomodulatory activities. Two thymoquinone-rich oily preparations (a super critical fluid extract and a commercial product registered in Saudi Arabia) showed the best *in-vitro* activity via inhibiting inflammatory cytokines in human cellular models. In the RDBPCT including 80 asthmatics, the commercial product capsules showed significant improvements in ACT, eosinophilia and some serum cytokines without serious side effects.

Conclusion

This project addressed important requirements to optimise clinical research using herbal medicines particularly for NS in asthma. Preclinical research on the chemistry and activity of the investigational NS product were the basis for the clinical trial. The RDBPCT revealed a higher level of evidence for the add-on NS treatment in asthma within the Saudi healthcare. This strategy is suggested for future clinical phytotherapeutic research.

Project Flow Chart

1

INTRODUCTION: Review of the literature for *Nigella sativa* (NS) and its related activity in asthma inflammation.

2

EXTRACTION & PHYTOCHEMICAL ANALYSIS: The literature (Chapter 1) showed that NS used in various forms of preparations. Therefore, different types of NS preparations were obtained and chemically characterised (Chapter 2)

3

***IN VITRO* SCREENING:** After the chemical characterisation of NS preparations (Chapter 2), various preclinical *in vitro* models were used to compare the anti-inflammatory/immunomodulatory activity of these preparations to find the most potent NS preparation (Chapter 3).

4

CLINICAL TRIAL: Based on the results of Chapter 2 & 3, the most suitable active NS preparation was selected for the clinical trial. A Phase-II randomised double-blind placebo-controlled clinical trial was conducted to explore the efficacy of the selected NS preparation in asthma (Chapter 4).

Research Impact Statement

Phytotherapy is one of the common approaches of integrative medicine that is practised by different cultures based on traditional knowledge. In the era of evidence-based medicine, it is essential to investigate the specific clinical safety and efficacy information of herbal medicines based on scientific experimental and clinical approaches. In this project, we conducted a clinical trial in asthma using a chemically and pharmacologically characterised *Nigella sativa* (NS) preparation. The method used in this project could be employed as a resource guide for future research in the context of clinical trials with herbal medicines. The method comprised of specific criteria for selecting a suitable active extract with highlighting essential needs for conducting clinical trials on herbal medicines.

Regarding outside academia benefits, this project provided an evidence-based assessment of NS as a complementary treatment for asthma. This may have a positive impact on the quality of life and overall healthcare for patients with asthma by enhancing their disease control and minimising costs and side effects of the current conventional asthma medications.

Also, this project highlighted some important quality characteristics of NS that could be used by herbal medicine industries and medicines agencies to develop a high-quality effective product of NS.

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List of Abbreviations

^{1}H NMR: Proton Nuclear Magnetic Resonance

ACQ: Asthma Control Questionnaire

ACT: Asthma Control Test

AE: Adverse Event

AI: Anti-inflammatory / Immunomodulatory

CNS: Central Nervous System

DMSO: Dimethyl sulfoxide

Dulbecco`s Phosphate Buffered Saline (DPBS)

FEF 25-75%: Forced Expiratory Flow between 25-75%

FeNO: Fractional exhaled Nitric Oxide

FEV1: Forced Expiratory Volume in one second

GAPP: The Global Asthma Physician and Patient

GINA: Global Initiative for Asthma

GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor

HPLC: High Performance Liquid Chromatography

ICAM: Intercellular Adhesion Molecule

IgE: Immunoglobulin E

IL: Interleukin

INF: Interferon

IP: Interferon Gamma-Induced Protein

IQR: Interquartile Range

KAUH: King Abdulaziz University Hospital

L: Linnaeus, Carl (author of NS)

LD50: Lethal Dose

LPS: Lipopolysaccharides from *Salmonella enterica* serotype typhimurium

LTE4: Leukotriene E4

MAPK: Mitogen-Activated Protein Kinase

MCP: Monocyte Chemoattractant Protein

MIP: Macrophage Inflammatory Protein

NS: *Nigella sativa* L.

PEF: Peak expiratory flow

PFT: Pulmonary Function Test

PGE2: Prostaglandin E2

RCT: Randomised Controlled Trial

RDBCT: Randomised Double-Blinded Clinical Trial

RDBPCT: Randomised Double-Blinded Placebo-Controlled Clinical Trial

REALISE: Recognise Asthma and Link to Symptoms and Experience

RSBPCT: Randomised Single-Blinded Placebo-Controlled Clinical Trial

Saudi FDA: Saudi Food and Drug Authority

SD: Standard Deviation

SEB: Staphylococcal enterotoxin B

SINA: Saudi Initiative for Asthma

Th: T helper cells

TLC: thin layer chromatography

TNF: Tumour Necrosis Factor

TQ: Thymoquinone

UCL: University College London

1 Chapter 1. Introduction*

1.1 Introduction

Asthma is considered as an epidemic respiratory disease probably affecting about 334 million people worldwide and becoming a global health priority (Global Asthma Network, 2014). In the United Kingdom, asthma affects one in every eleven people (Asthma UK, 2010). In Saudi Arabia (with a population of 28 million), the prevalence of asthma is increasing and affects more than 2 million Saudis (Al-Moamary, 2012).

Patients with asthma may tend to use complementary and alternative therapies such as herbal medicines as a holistic approach to asthma management due to limitations of conventional asthma medications (Kohn and Paudyal, 2017). In Saudi Arabia, a survey of two hundred patients with asthma found that 34.5% used unconventional therapies for asthma. For example, the seeds of the medicinal plant *Nigella sativa* L. (NS) was one of the most common such therapies (Al Moamary, 2008). However, these therapies often have insufficient evidence for its clinical use in asthma (Kohn and Paudyal, 2017). Therefore, this project explored and assessed the current scientific evidence for the use of NS in asthma, followed by conducting a high-quality clinical trial for investigating the clinical efficacy of NS in patients with asthma.

In this chapter, asthma disease and management were introduced. Also, the traditional medical applications, safety and chemical composition of NS were reviewed as well as the evidence of NS use in asthma was assessed.

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1.2 Asthma definition and pathophysiology

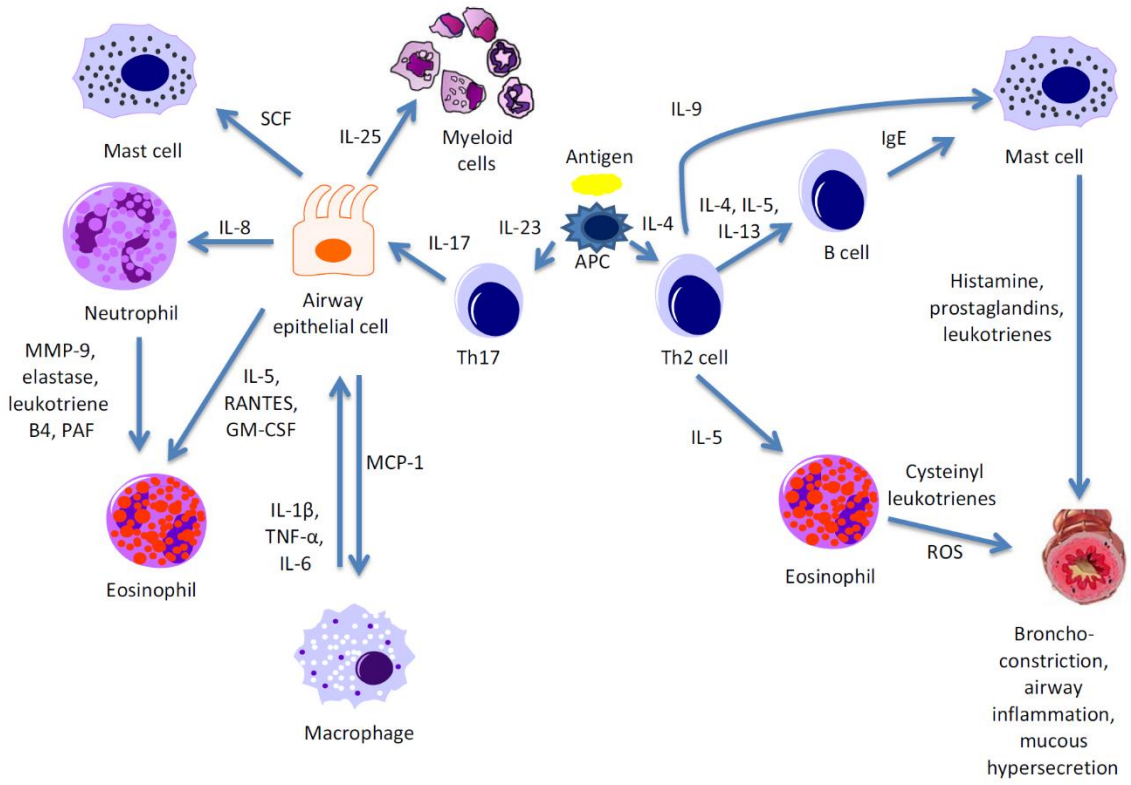
The Global Initiative for Asthma (GINA) guidelines 2017 defined asthma as "a heterogeneous disease, usually characterised by chronic airway inflammation which is identified by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and in intensity, together with variable expiratory airflow limitation". Usually, it is accompanied by lung hyper-responsiveness and inflammation. It is often induced by certain factors such as exercise, allergen or irritant exposure, weather variation, or viral infection (Global Initiative for Asthma, 2017).

Asthma is initiated by multiple interactions between inflammatory cells and mediators. The most important cells involved in asthma inflammation are mast cells, eosinophils, T lymphocytes, neutrophils, and epithelial cells. After exposure to a triggering factor, inflammatory mediators are released from mast cells, macrophages, T-lymphocyte cells and epithelial cells. This causes attraction of other inflammatory cells mainly eosinophils into the pulmonary tissues which produce chemical compounds such as major basic protein (MBP) and eosinophil cationic protein (ECP). This leads to lung injury, mucus hypersecretion and smooth muscle hyperactivity. In fact, there are at least 27 cytokines and 18 chemokines that play a role in asthma pathophysiology (Koda-Kimble, 2009).

Type 2 T helper (Th2) cells cytokines [interleukin IL-4, IL-5, and IL-13] and Type 1 T helper (Th1) cells cytokine interferon-gamma are considered to be the main cytokines in asthma (Ngoc *et al.*, 2005). Although Th2-related eosinophilia is considered to be the classical pathway of inflammation in asthma, Th17-related neutrophilia have been associated with more severe type steroid-resistant asthma (Figure 1) (Trevor and Deshane 2014).

Asthma is classified according to demographic, clinical and pathophysiological characteristics which referred as asthma phenotypes. These phenotypes of asthma include allergic asthma, non-allergic asthma, late-onset asthma, asthma with fixed airflow limitation and asthma with obesity. Allergic asthma is the most easily recognised phenotype that is associated with eosinophilic airway inflammation. The cellular inflammatory profile of non-allergic asthma may be neutrophilic, eosinophilic or contain only a few other inflammatory cells (Global Initiative for Asthma, 2017).

Figure 1. Type 2 T helper cells and Type 17 T helper cells mediated inflammatory pathway in asthma (Trevor and Deshane, 2014).



1.3 Asthma management and assessment of disease control

The key goals for asthma management are to achieve a good control of symptoms and minimise the future risk of exacerbations, airflow limitation and treatment adverse events. Assessment of asthma control level is done by

evaluating the frequency of asthma symptoms (days per week), limitation of activity or night waking due to asthma, and the frequency of reliever medications use. This can be done by using several tools including simple, categorical or numerical tools. Numerical tools provide scores that can be used to assess the progress of control and commonly used in clinical trials. For example, Asthma Control Test (ACT) is a validated numerical tool to assess asthma control characterised by a score range from 5 to 25 (high number means better control). On the other hand, future risk can be assessed by pulmonary function testing, particularly the forced expiratory volume in 1 second (FEV1) (Global Initiative for Asthma, 2017).

Asthma control is achieved by both non-pharmacological and pharmacological therapies. Medications for asthma are categorised into three main classes. First, **controller medications** to decrease lung inflammation, future risks and increase control of symptoms. This includes inhaled corticosteroids, leukotriene receptor antagonists and long-acting β2-agonist. Second, **reliever medications** which are used when required for relief and prevent emergent symptoms such as short-acting β2-agonist. Third, **add-on therapies** for severe asthma that is difficult to control such as anti-IgE (Global Initiative for Asthma, 2017).

Traditionally, the strategy for asthma therapy focused on bronchodilation and decreasing airway inflammation. This can be achieved by inhaled medications. However, inhaled medications have shortcomings such as poor penetration into the peripheral airways and inadequately treat the systemic component of asthma. Oral systemic therapies can reach the small airways and treat the systemic component of asthma (Bjermer, 2001, 2007).

Therefore, the current and future research trend of asthma management is focusing on phenotype-guided add-on **systemic medications,** through targeting disease-specific inflammatory mediators and biomarkers such as anti-IL5 (Global Initiative for Asthma, 2017; Pavord *et al.*, 2017).

1.4 Asthma control and limitations of asthma medications

Currently, asthma control is considered to be suboptimal regardless of the availability of conventional treatments (Demoly *et al.*, 2012; Price, Fletcher and van der Molen, 2014). Data from the Recognise Asthma and Link to Symptoms and Experience (REALISE) survey in 2014 for 8000 European patients revealed that the level of asthma control remains low (Price, Fletcher and van der Molen, 2014).

One of the reasons for low asthma control is poor adherence to asthma medications (Horne *et al.*, 2007; Haughney *et al.*, 2008). Common medication-related reasons for non-adherence include difficulties with inhaler techniques, the complex course of therapy, adverse events, and cost of medications (Bateman *et al.*, 2008; Dima *et al.*, 2015).

The Global Asthma Physician and Patient (GAPP) Survey reported that 39% of asthma patients exchanged or stopped their asthma medication due to adverse events (GAPP, 2005). 76% of patients and 81% of physicians consider that new treatment options are required (GAPP, 2005).

1.5 Phytotherapy in Asthma

In general, asthma patients may tend to use complementary and alternative therapies such as herbal medicines as a holistic approach to disease

management due to limitations of asthma medications (Kohn and Paudyal, 2017). The introduction of novel treatment strategies such as “add-on” treatments is recommended for better asthma control (Lommatzsch and Stoll, 2016). Herbal medicine may be considered as a possible add-on treatment option. For example, butterbur (*Petasites hybridus* L.) was studied as add-on therapy in a small randomised double-blind placebo-controlled study included 16 asthmatic patients. It improved asthma outcomes including FEV_1, exhaled nitric oxide, serum eosinophil cationic protein and peripheral blood eosinophil count (Lee *et al.*, 2004).

Several herbal medicines were reported in clinical trials of asthma such as: ***Nigella sativa* L. (NS)**, *Ginkgo biloba* L., *Ligusticum wallichii* Franch., *Ephedra sinica* Stapf., *Solanum xanthocarpum* L., *Solanum trilobatum* L., *Boswellia serrata* Roxb., *Tylophora indica* Merr., *Cannabis sativa* L., *Hedera helix* L., *Petasites hybridus* L., Eucalyptus oil, *Sophora flavescens* Aiton, *Glycyrrhiza uralensis* Fisch., *and Aleurites moluccanus* L. However, the clinical efficacy and safety evidence for these herbal medicines is limited and there is a need for further proper investigations in randomised controlled trials to evaluate the use of herbal medicines in asthma (Huntley and Ernst, 2000; Slader *et al.*, 2006; Singh *et al.*, 2007; Clarke, Lundy and McGarvey, 2015; Kohn and Paudyal, 2017). Therefore, phytotherapy remains a commonly used but poorly investigated element in asthma management.

In Saudi Arabia, a questionnaire for 69 patients with asthma found that NS was the most frequently used herb as a non-standard treatment. This was followed by Myrrh (*Commiphora myrrha* (Nees)), Garlic (*Allium sativum* L.), Gum (not specified) and Fenugreek (*Trigonella foenum-graecum* L.) (Al Moamary, 2008).

1.6 Clinical research in phytotherapy

Clinical trials are essential to establish the scientific evidence base use of herbal medicines. Systematic reviews and meta-analysis of randomised controlled trials are considered the highest level of evidence (Edwards *et al.*, 2012). Compared to conventional medicines, few herbal medicines were investigated in a good quality clinical trials (Willcox, 2014). For example, St John's wort (*Hypericum perforatum* L.) was found to be equivalent to selective serotonin uptake inhibitors in managing patients with major depression according to a review of 29 randomised double-blind controlled trials (Edwards *et al.*, 2012). Therefore, there is a need for high-quality clinical research for many other herbal medicines.

Due to the nature of herbal medicines, factors such as the chemical composition and plant part used should be considered before commencing to well-designed clinical trials (Edwards *et al.*, 2012). Challenges of clinical trials in phytotherapy include herbal product standardisation (or characterisation) as well as financial, ethical, regulatory, and trial design factors (Parveen *et al.*, 2015). One of the approaches for overcoming the high cost of clinical trials is by collaborating with regional research institutes and with doctoral students, focusing mainly on validated clinical outcomes rather than expensive laboratory analyses (Graz, Elisabetsky and Falquet, 2007).

1.7 Pre-clinical models of pulmonary inflammation

Pulmonary inflammation is a base for several respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and acute respiratory distress syndrome (ARDS). These diseases are most commonly managed by anti-inflammatory treatments to reduce the pulmonary inflammation.

Inflammation is a cellular response that can occur in the lung by the induction of external or internal factors. While it is considered a normal response in the human body, chronic inflammation could damage the lung. Pulmonary inflammation involves many inflammatory cells, such as macrophages, lymphocytes, neutrophils, and eosinophils. Also, the epithelium, smooth muscle, and vascular and neuronal elements are involved in the pathogenesis of pulmonary inflammation. In general, these cells release inflammatory mediators such as histamine, tumour necrosis factor (TNF-α), interleukins (IL-1β, IL-4, IL-5, and IL-6), prostaglandins, leukotrienes, and nitric oxide. These inflammatory mediators are related to the signals and symptoms of pulmonary diseases such as airway hyperresponsiveness and obstruction, airway oedema, mucus hypersecretion, and lung remodelling (Barnes, 2000; Ware and Matthay, 2000; Murphy and O'Byrne, 2017).

Several animal models were used in the drug discovery for pulmonary disease. For example, histamine and acetylcholine induced bronchoconstriction in Guinea pigs, Egg Albumin Sensitized Guinea Pigs and its effects on Broncho Alveolar Lavage Fluid (BALF), Clonidine-induced Catalepsy in Mice, Clonidine-induced Mast Cell Degranulation in Rats, Milk-Induced Leucocytosis and Eosinophilia in Mice were used as *in vivo* animal models in asthma disease. *In vitro* models involving part of an animal were also used in models for asthma such as vascular and airway responses in isolated perfused rat lung preparation, and the contractions induced by histamine, acetylcholine, serotonin and bradykinin in isolated goat tracheal chain preparation or guinea pig ileum (Patil and Ninave, 2016). However, animal models may explore only a part of many pathways in human disease and have the species specific limitations which need to be

considered when translating results from animal models into human (Holmes, Solari and Holgate, 2011).

In comparison to animal *in vivo* models, *in vitro* studies offer several advantages as it may be more easy, flexible, quick, economical and reproducible. Also, it can reduce the number of animals used in research, avoid animal species variation and provide direct access to investigate cellular components and biomolecules (Adamson *et al.*, 2011).

Human tissue-based *ex vivo* and *in vitro* asthma models can be used in pulmonary inflammation research. They can be ranged from little structural organisation to complex 3-D cultures containing different cell types that are organised into a human structure that retains (*ex vivo* tissue explants) or mimics (tissue engineered *in vitro* models) some human characteristics. Cultured tissue from resected human lung is an example of an *ex vivo* model. However, it is limited by the relatively short-term viability and the barrier properties of the epithelial layer.

Cell lines and primary human cells derived from pulmonary tissue are the simplest human based models. It is commonly used for investigating basic cellular responses and cell signalling pathways of the lung. For example, the A549 bronchial epithelial cell line is being used for lung diseases research. They are readily accessible and easy to manipulate but they don't explore the genetic or epigenetic features of the disease. Another example is the *in vitro* cultured primary human cells such as bronchial epithelial cells from bronchial brushings or biopsies, fibroblasts and smooth muscle cells from bronchial biopsies, and immune cells from bronchoalveolar lavage or peripheral blood. These cell types may reflect genetic or epigenetic changes but a special technique such as

bronchoscopy that may limit obtaining a bronchial biopsy for example. A major limitation of such models is that they investigate responses only from a single isolated cell type. Therefore, co-culture models and 3D tissue were manipulated to include more than one type of cell and extracellular matrix components leading to an attempt to mimic the *in vivo* environment. However, such models may be more complex to grow as it needs optimisation of media compatibility for different types of cells (Blume and Davies, 2013).

1.8 *Nigella sativa* L.

The seeds of the medicinal plant *Nigella sativa* L. (NS) are commonly used as a spice known as black cumin seeds. It has traditional medical applications and considered to be a characteristic traditional herbal medicine for diverse diseases in the Unani, Arabic, Prophetic and Indian traditional medicines (Ahmad *et al.*, 2013). Popular ancient physicians such as Hippocrates (460-370 BC), Dioscorides (40-90 AD), Galen (130-210 AD), and Avicenna (980-1037 AD) reported various traditional therapeutic uses of NS (Botnick *et al.*, 2012). In the Islamic literature, it was metaphorically regarded as a cure for all diseases except death (Muhammad al-Bukhari, 854). It is recognised as the curative black cumin in the Bible and mentioned in the Hebrew word “ketzah” in the book of Isaiah (28:27) (Duke, Duke and DuCellier, 2008).

The plant is native to North Africa, Southern Europe, and Southwest Asia. However, it is cultivated in many countries such as India, Pakistan, Syria, Turkey, Saudi Arabia and other Middle Eastern, Mediterranean and South European countries (Khare, 2004). It has several non-scientific names in different languages including common names in English such as black cumin, black seed,

black caraway, fennel flower, nutmeg flower, or Roman coriander. *Nigella sativa* was first described by Carl Linnaeus (L.); 1707-1778 (Kew, 2017).

1.9 Traditional medical uses of *Nigella sativa*

For hundreds of years, NS was an important natural remedy for many diseases. Popular ancient physicians reported its various therapeutic uses in traditional medicine. For instance, Avicenna reported its benefit for shortness of breath (انتصاب النفس) and for stopping phlegm (مقطع البلغم) (Avicenna, 1593). Dioscorides reported its medical use for cleaning rough skin and psoriasis (Osbaldeston, 2000). Moreover, Hippocrates used it for treating hepatic and digestive conditions (Salama, 2010). The Muslim scholar Imam Ibn Qayyim Al-Jawziyya (1292–1350 AD), the author of the Prophetic Medicine, reported that NS alleviate gasping and hard breathing (Abdullah, 2003).

In Arabia, NS is a traditional remedy for asthma, cough, stomach ache, abdominal pain, colic, general fatigue, rheumatism, and skin diseases. Also, it is thought to be good for stimulating digestive, kidney and brain functions. It is traditionally believed to strengthen women after giving birth, stimulate menstruation and support beauty treatments for hair and skin (Lebling and Pepperdine, 2006). In Saudi Arabia, NS was a common non-standard treatment used by patients with asthma (Al Moamary, 2008).

1.10 Chemical composition of *Nigella sativa*

The chemical composition of NS has been studied in considerable detail in several studies. Mainly, it contains fixed oil (24.76 to 40.35%), volatile oil (0.5 to 1.6%), alkaloids, saponins, and other compounds in trace amounts (Table 1) (Ahmad *et al*., 2013; Botnick *et al*., 2012; Liu *et al*., 2011). Because these

compounds have different hydrophilic/hydrophobic properties, the extraction method influences the chemical composition and, consequently, the pharmacological activity of an NS preparation. Therefore, we investigated the pharmacological activity of different NS preparations to select a suitable active preparation of NS as explained in chapter 3.

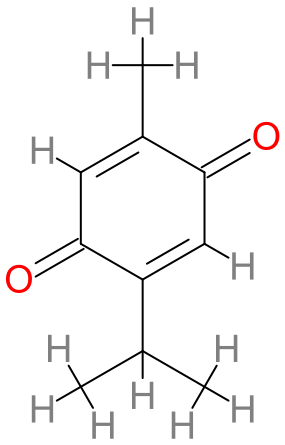
The compounds responsible for NS's activities are still not well-established. It is likely that several compounds are responsible or co-responsible for NS activity and that one must consider a specific extract as the active ingredient rather than a single compound. Thymoquinone (TQ) (Figure 2) is the main component of NS's volatile oil that is considered to be an active compound in several studies (Ahmad et al., 2013). TQ was first isolated from NS oil in 1963 (El–Dakhakhny, 1963). TQ has very low stability profile in aqueous solutions (Salmani et al., 2014). Other compounds such as dithymoquinone, thymohydroquinone, thymol, carvacrol and α-hederin were also reported to produce relevant pharmacological effects in preclinical models (Marsik *et al.*, 2005; Landa *et al.*, 2009; Fallahi *et al.*, 2016).

Table 1. Identified metabolites in *Nigella sativa*.

Class	**Compounds**
Alkaloids	- **Dolabellane-type diterpene alkaloids:** Nigellamines A1, A2, A3, A4, A5, B1, B2 and C. - **Indazole alkaloids:** Nigellidine, nigellicine and nigellidine-4-O-sulfite. - **Isoquinoline alkaloids:** Nigellimine and nigellimine N-oxide.
Fixed oils (lipids)	- **Diacylglycerols, fat-soluble vitamins, lipases, monoacylglycerols, phospholipids, triacylglycerols.**

(24.76-40.35%)	- **Saturated fatty acids:** Arachidic, behenic, lauric, lignoceric, smyristic, palmitic and stearic acid. - **Sterols:** Campestrol, cholesterol, stigmasterol, β-sitostanol, β-sitosterol, Δ^5-avenasterol, Δ^7-avenasterol, Δ^7-stigmasterol and lophenol. - **Unsaturated fatty acids:** Dihomolinoleic, eicosadienoic, erucic, linoleic (52.6% - 55.6%), linolenic, margaroleic, oleic (23.4% - 23.5%) and palmitoleic acids.
Saponins	- α-hederin and hederagenin.
Volatile oils (0.5–1.6%)	- **Aldehydes:** 2E,4Z-decadienal, 2E,4E-decadienal. - **Monoterpene alcohols:** Carvacrol, terpinene-4-ol and thymohydroquinone. - **Monoterpene ester:** bornyl acetate. - **Monoterpene ethers:** cis-4-methoxythujane, trans-4-methoxythujane, 4,5-epoxy-1-isopropyl-4-methyl-1-cyclohexene. - **Monoterpene hydrocarbons:** Limonene, myrcene, p-cymene, sabinene, terpinolene, α-pinene, α-terpinene, α-thujene, β-pinene and γ-terpinene. - **Monoterpene ketones:** Carvone, thymoquinone and nigellone. - **Sesquiterpenes:** Longifolene, longipinene, trans-caryophyllene and zonarene.
Other	Vitamins, minerals, proteins, resins and tannins.

Figure 2. The chemical structure of thymoquinone (PubChem database).



1.11 Safety of *Nigella sativa*

Generally, NS considered a food substance, and for centuries has been used by many communities. The toxicity of NS has been investigated in various animal and clinical studies. The acute and chronic toxicity of NS **oil** was studied as shown in the following animals. In mice, the administration of 28.8 ml/kg oral and 2.06 ml/kg intraperitoneal single doses of NS oil resulted in high lethal doses (LD_{50}) of 28.8±1 ml/kg for oral, and 2.06 ± 0,1 ml/kg for intraperitoneal doses. In rats, oral dose of 2 ml/kg for 12 weeks showed no changes in the levels of liver enzymes or the histopathology of the heart, liver, kidneys and pancreas as well as decreased serum levels of cholesterol, triglycerides and glucose (Zaoui *et al.*, 2002).

Additionally, the acute and subacute toxicity of the **aqueous, methanol and chloroform extracts** of NS were shown to be low (Vahdati-Mashhadian, Rakhshandeh and Omidi, 2005). After oral administration of four different single doses of each extract (6, 9, 14 and 21 g/kg) in mice, no mortality occurred with all doses. Also, prolonged administration of these extracts for 14 days showed no significant effects on weight, plasma levels of liver enzymes and overall health (Vahdati-Mashhadian, Rakhshandeh and Omidi, 2005).

Powdered NS in the dose of up to 1 g/kg/day for 28 days had no changes in liver enzymes level and did not cause any toxic effects on the liver function in rats (Dollah *et al.*, 2013). A dose of 2g/day for 6 weeks of powdered NS did not show any adverse effects on liver or kidney function tests in a clinical trial including 64 patients (Qidwai *et al.*, 2009). Furthermore, high doses of powdered NS (3g/day) did not show any toxic effects in clinical trials (Bamosa, 2014). The relative safety of the major active compound **TQ** was evident due to high LD_{50}

values which were 870.9 mg/kg (647.1–1094.8) for oral and 104.7 mg/kg (89.7–119.7) for intraperitoneal administration of TQ in experimental animals (Al-Ali *et al.*, 2008).

1.12 Pharmacokinetics of thymoquinone

The clinical bioavailability and pharmacokinetic parameters for thymoquinone are not well established (AbuKhader, 2013). However, in an animal rabbit study, the oral bioavailability of 20 mg/kg of TQ was about 58% with slow absorption (T ½ 217 min) and rapid elimination (T ½ 274.61 ± 8.48 min) (Alkharfy *et al.*, 2015).

1.13 Evidence for *Nigella sativa's* efficacy in asthma

Between the years 1990 and 2016, a literature search for scientific studies published in electronic databases (PubMed, Science Direct, Scopus, and Google Scholar) was done using the terms *Nigella sativa*, black seed, thymoquinone and asthma. At least nineteen **pre-clinical studies** and six **clinical studies** reported the effects of NS in asthma.

1.13.1 Pre-clinical studies of *Nigella sativa* in cellular and animal models of asthma

NS and its active compounds TQ, nigellone and α-hederin have been investigated in eighteen whole or cellular animal models and one human cellular model related to asthma. NS oil, TQ or α-hederin showed anti-inflammatory and immunomodulatory effects in seven studies (Mansour and Tornhamre, 2004; Abbas *et al.*, 2005; El Gazzar *et al.*, 2006; Shahzad *et al.*, 2009; Balaha *et al.*, 2012; Saleh, ElDenshary and Mahran, 2012; Saadat *et al.*, 2015). NS extracts,

TQ or α-hederin demonstrated a bronchodilatory or relaxant effect in six studies (Al-Majed *et al.*, 2001; Gilani *et al.*, 2001; Boskabady, Keyhanmanesh and Saadatloo, 2008; Abd El Aziz, El Sayed and Mahran, 2011; Keyhanmanesh *et al.*, 2013; Saadat *et al.*, 2015). The anti-histaminic effect was shown in four studies used NS oil/aqueous extract, nigellone or α-hederin (Chakravarty, 1993; Abd El Aziz, El Sayed and Mahran, 2011; Saleh, ElDenshary and Mahran, 2012; Saadat *et al.*, 2015). Pathological improvements were shown using TQ or NS oil in five studies (Boskabady and Sheiravi, 2002; El Gazzar *et al.*, 2006; Shahzad *et al.*, 2009; Kalemci *et al.*, 2013; Arabzadeh, Mirdar and Moradiani, 2016). The summary of findings of these studies is shown in Table 2.

Generally, these studies used animal models sensitised with ovalbumin or isolated guinea pig trachea. Some studies used cellular models such as human granulocytes or animal mast cells. However, these studies had limitations such as the variability of NS preparations used between most them, and absence of control group in some studies (Table 2).

Table 2. Pre-clinical studies of *Nigella sativa* in asthma.

Studies	Study material	Minimal active dose	Model	Negative Control	Positive control	Effects							limitations
						Serum Immunoglobulins	Inflammatory mediators	Inflammatory cells	Histamine release	Block H1 receptors	Relaxation of SM	Other	
(Chakravarty, 1993)	Nigellone	11µg/ml *in vitro*	Peritoneal cells of egg albumin induced Wistar rats	N/A	N/A				↓				No control group (without TQ)
(Gilani *et al.*, 2001)	NS (70% aqueous-methanol extract)	0.1 - 3.0 mg/ml *in vitro*	Guinea pig trachea induced by carbachol, histamine or K+	N/A	N/A						+		No control group (without TQ)
(Al-Majed *et al.*, 2001)	TQ	50µM *in vitro*	Guinea-pig trachea induced by carbachol	N/A	N/A					+	+		No control group (without TQ)
(Boskabady and Sheiravi, 2002)	NS (aqueous extracts)	0.3ml *in vitro*	Guinea pig trachea induced by histamine	Saline	CHL					+			
(Mansour and Tornhamre, 2004)	TQ	3 and 10 µM *in vitro*	Human granulocytes induced by A23187, LTA4, and AA	Untreated human granulocytes	N/A		↓ leukotrienes						No positive control
(Abbas *et al.*, 2005)	NS fixed oil	5 ml/kg/day ip for 17 days	Conalbumin sensitised albino mice	Untreated mice	DEX	↓ IgG	↓ serum IL-2 & IL-12	↓ eosinophils in blood					
(Büyüköztürk *et al.*, 2005)	NS fixed oil	0.3ml/day for 1 month	OVA sensitised BALB/c mice	Saline			No change in IL-4, IL-10 and IFN- γ in splenic mononuclear cells						No positive control

(El Gazzar *et al.*, 2006)	TQ + 10% DMSO	3 mg/kg TQ in 10% DMSO ip for 5 days	OVA sensitised BALB/c mice and lung cells	Saline + 10% DMSO	OVA + 10% DMSO	↓ OVA IgE & IgG1	↓ IL-4, IL-5, IL-13 ↑ IFN-γ in BALF	↓ eosinophils in the lung & BALF				↓ goblet cells hyperplasia	
(Boskabady, Keyhanmanesh and Saadatloo, 2008)	NS (methanol and dichlorom-ethane extracts)	-0.8 g% of methanol extract -1.2 g% of dichloromethane extracts	Guinea pig trachea	Saline	THEO						+		
(Shahzad *et al.*, 2009)	NS fixed oil	4 ml/kg/day ip for 7 days	OVA sensitised E3 rats	Saline	N/A	↓ IgE ↓ IgG1 ↓ OVA IgG1	↓ mRNA expression of IL-4, IL-5, IL-6 and TGF-β1 from lung cells ↓ nitric oxide in BALF	↓ eosinophils, macrophages & lymphocytes in the lung & BALF				↓ bronchial and alveolar epithelial hyperplasia ↓goblet cells and collagen fibres	No positive control
(Abd El Aziz, El Sayed and Mahran, 2011)	TQ	-3 mg/kg ip for 5 days in guinea pig -8 mg/kg ip for 21 days in rats	OVA sensitised guinea pig trachea Mast cells of egg-albumin sensitised rats	Saline	N/A				↓		+		No positive control
(Balaha *et al.*, 2012)	NS fixed oil	Oral NS oil 4 ml/kg/day for 31 days	OVA sensitised BALB/c mice	Saline	N/A	↓ IgE ↓ OVA IgE & IgG1	↑ BALF Th1 cytokines ↓ BALF Th2 cytokines	↓ leukocytes, macrophages & eosinophils in BALF				↓ Airway hyperresponsivness	No positive control
(Saleh, ElDenshary and Mahran, 2012)	NS fixed oil	Oral NS oil 2.5 ml/kg/day for 3 weeks	OVA sensitised guinea pig Isolated rat peritoneal mast cells	Saline	N/A		↑ PGE2 in lung tissue ↓ leukotrienes		↓				No positive control
(Keyhanmanesh *et al.*, 2013)	NS (fractions of 20% methanolic extract)	(50, 100, 150, 200 mg/L)	Guinea pig trachea	Saline	THEO						+		
(Kalemci *et al.*, 2013)	TQ	3 mg/kg/day ip for 5 days	OVA sensitized BALB/c mice	Saline	DEX							↓ Sub-epithelial and epithelial hyperplasia ↓ Number of mast and goblet cells	

(Keyhanmanesh *et al.*, 2014)	TQ	0.3 mg/kg ip	OVA sensitized guinea pig	Saline	N/A		↑ Blood IFN-γ	↓ Eosinophil ↓ Basophils in BALF				↓ Tracheal responsiveness ↓ Airway membrane hyperplasia ↓ Respiratory epithelial denudation ↓ Cellular infiltration ↓ Emphysema	No positive control
(Saadat *et al.*, 2015)	α-hederin	0.3 mg/kg ip	OVA sensitized guinea pig	Saline	TQ			↓ Total WBC ↓ Eosinophils ↓ Basophils in BALF			+	↓ Tracheal contractile response to histamine	
(Fallahi *et al.*, 2016)	α-hederin	0.2 mg/kg ip	OVA sensitized Wistar rats	Saline	TQ		↓ IL-13 mRNA ↓ miRNA-126					↓ Pneumocyte and fibroblastic hypertrophy and hyperplasia ↓ Hyperemia ↓ Haemorrhage ↓ Edematous and exudative changes	
(Arabzadeh, Mirdar and Moradiani, 2016)	NS (ethanolic extract)	Oral 500 mg/kg/day for 3 weeks	exercise-induced Wistar rats	exercise-induced Wistar rats without NS	N/A							↓ thickness of epithelial bronchi, tunica media (muscle) bronchi, and tunica adventitia bronchi ↓ number of goblet cells	No positive control

Ip; intraperitoneal. OVA; ovalbumin. BALF; Bronchoalveolar lavage fluid. N/A; data not included in the original study. TQ; Thymoquinone. DEX; Dexamethasone. CHL; Chlorpheniramine. THEO; Theophylline. A23187; Calcium Ionophore to investigate LTC4 and LTB4 formation. LTA4; Leukotriene A4 to investigate LTA4 hydrolase and LTC4 synthase activity by the formation of LTC4 and LTB4 formation. AA; Arachidonic Acid to investigate 5-lipoxygenase activity by formation of 5-Hydroxyicosatetraenoic acid.

1.13.2 Clinical studies of *Nigella sativa* in patients with asthma

Six clinical studies showed a potential efficacy of NS on asthma outcomes and biomarkers. Two Randomised Double-Blinded Placebo-Controlled Clinical Trials (RDBPCT) and Two Randomised Single-Blinded Placebo-Controlled Clinical Trials (RSBPCT) using NS crushed seeds powder or oil/aqueous extract, showed an improvement in clinical symptoms and pulmonary function test in adult asthmatics (Kalus *et al.*, 2003; Boskabady *et al.*, 2007; Kardani *et al.*, 2013; Salem *et al.*, 2017). A nonsignificant reduction of blood eosinophilia and total serum IgE was found within the treatment group only in an RDBPCT (Kalus *et al.*, 2003). A significant reduction of fractional exhaled nitric oxide and increase of serum interferon gamma were found in a RSBPCT (Salem *et al.*, 2017). A Randomised Double-Blinded Clinical Trial (RDBCT) showed a short bronchodilatory effect in patients with asthma after administration of a single dose of NS (Boskabady, Mohsenpoor and Takaloo, 2010). Two studies used NS in combination with other treatments showed an improvement in either clinical symptoms or pulmonary function (Al Ameen *et al.*, 2011; Kardani *et al.*, 2013) (Table 3).

However, these clinical trials had some important limitations as mentioned in Table 3. The quality of trials was not high as two studies only were RDBPCT. There was a variability in the NS preparation type between the trials and the phytochemical characterisation of the investigational NS product was not shown in many of these trials. In most studies, the sample size was comparatively small and the outcomes were generally limited to symptoms or pulmonary function. A meta-analysis is suggested for these clinical trials. However, due to the variability

of outcomes measured between trials and limited sample size, it was not suitable to perform a meta-analysis for the outcomes of these trials.

Therefore, there is a need for a longer, larger and high quality multicentre clinical trial with chemically well-characterised NS product. Also, to use validated asthma control measurement tool with consideration of additional asthma outcomes and biomarkers such as FeNO, Sputum eosinophils, total blood eosinophils, total serum Immunoglobulin E (IgE), allergen-specific IgE and urinary leukotriene E4 LTE4 (Szefler *et al.*, 2012). Additionally, measuring serum inflammatory cytokines may be worth considering, since asthma is regulated by multiple inflammatory cytokines and asthma control might be predicted with some cytokines. For example, high serum level of the anti-inflammatory cytokine Interleukin-10 (IL-10) was associated with reduced risks of deteriorating asthma control, asthma attacks and low IgE levels (Akiki *et al.*, 2017).

Table 3. Clinical studies of *Nigella sativa* in patients with asthma.

Study reference	Study material	Study design	Control	NS dose	Duration	Sample	Outcomes			Advantages (+) & Limitations (-)
							Symptoms	Pulmonary function	Other	
(Kalus *et al.*, 2003)	NS fixed oil	RDBPCT	Placebo	40–80 mg/kg/day Three times daily	3 weeks	63 allergic adults: - 31 allergic rhinitis - 3 bronchial asthma - 6 atopic eczema	Improved subjective severity of symptoms		↓ eosinophils (not significant) ↓ serum IgE (not significant)	- Sample of mixed allergic patients - No pulmonary function measurement - Blood biomarkers were not compared between groups - limited NS characterisation - invalidated symptoms scoring system
(Boskabady *et al.*, 2007)	Aqueous extract of NS	RDBPCT	Placebo	15 mL/kg of 0.1 g%	3 months	29 adult asthmatics 15 active 14 control	Improved asthma symptoms	↑ FVC ↑ FEV1 ↑ PEF ↑ MMEF		+ NS was chemically characterised + High standard study design - Small sample size - Limited outcomes to symptoms and pulmonary function - Invalidated symptoms scoring system
(Boskabady, Mohsenpoor and Takaloo, 2010)	Aqueous extract of NS	RDBCT crossover	THEO	50 mg/kg	150 min	15 adult asthmatics		↑ FEV1 ↑ MMEF ↑ PEF		+ NS was chemically characterised - Very small sample size - Not placebo controlled + limited outcomes to pulmonary function
(Al Ameen *et al.*, 2011)	Whole NS seeds + bee honey	Non RCT open-label	N/A	2g of NS seeds + 1 tsp honey	3 months	5 adult asthmatics		↑ FVC in asthmatics		- Very small sample size - NS was not chemically characterised and was used in combination - Low standard study design - Outcomes were limited and statistically compared within the same group only, and not between groups. - No symptoms measurement
(Kardani *et al.*, 2013)	NS powder + IM (House dust mite)	RSBPCT	IM + placebo	15 mg / kg / day	14 weeks	31 child asthmatics G1: 8 IM + placebo G2: 8 IM + NS G3: 8 IM + probiotic G4: 7 IM + NS + probiotic	↑ ACT		No change in Th17 cells	- Small sample size + NS not chemically characterised and was used in combination - Single-blinded - Outcomes limited to symptoms only
(Salem *et al.*, 2017)	NS Powder	RSBPCT	Placebo	1 & 2 g/day	3 months	76 adult asthmatics -24 placebo -26 (1 g NS) -26 (2 g NS)	↑ ACT	↑ FEV1 ↑ FEF25-75% ↑ PEF	↓ serum IgE ↑ serum IFNγ ↓ FeNO	+ Large sample size but still comparatively small + Longer duration - Single-Blinded - NS was not chemically characterised

RDBPCT; Randomised Double-Blind Placebo-Controlled Trial. RSBPCT; Randomised Single-Blind Placebo-Controlled Trial. RDBCT: Randomised Double-Blind Controlled Trial. ACT; Asthma control test. FEV1; forced expiratory volume in 1 second. FVC; forced vital capacity. MMEF; maximal mid expiratory flow. PEF; peak expiratory flow. Tsp; tea spoonful. FeNO; fractional exhaled nitric oxide. FEF25-75%; mid expiratory flow. IM; Immunotherapy. THEO; Theophylline. G; Group.

Table 3.1 Asthma symptoms outcomes in the clinical studies of *Nigella sativa* in patients with asthma.

<table>
<tr><th>Study reference</th><th>Asthma symptoms</th></tr>
<tr><td>(Boskabady et al., 2007)</td><td>
<table>
<tr><td></td><td colspan="3">Baseline</td><td colspan="3">Post-treatment</td></tr>
<tr><td></td><td>Control (Placebo)</td><td>Study</td><td>p</td><td>Control (Placebo)</td><td>Study</td><td>p</td></tr>
<tr><td>Asthma severity score</td><td>3.20 ± 0.19</td><td>3.27 ± 0.18</td><td>Not significant</td><td>5.36 ± 3.87</td><td>49.45 ± 3.50</td><td>< 0.001</td></tr>
</table>
Data are presented as mean ± SEM
</td></tr>
<tr><td>(Kardani et al., 2013)</td><td>
<table>
<tr><td></td><td colspan="3">Control (IM + Placebo)</td><td colspan="3">Treatment (IM + NS)</td></tr>
<tr><td></td><td>Baseline</td><td>Post-treatment</td><td>p</td><td>Baseline</td><td>Post-treatment</td><td>p</td></tr>
<tr><td>Asthma Control Test</td><td>15.86 (5.15)</td><td>18.00 (7.42)</td><td>0.062</td><td>16.00 (2.73)</td><td>20.75 (3.37)</td><td>0.001</td></tr>
</table>
Data are presented as mean (SD)
</td></tr>
<tr><td>(Salem et al., 2017)</td><td>Actual scores not reported</td></tr>
</table>

Table 3.2 Pulmonary function outcomes in the clinical studies of *Nigella sativa* in patients with asthma.

<table>
<tr><th>Study reference</th><th>Pulmonary function</th></tr>
<tr><td>(Boskabady et al., 2007)</td><td>
<table>
<tr><td></td><td colspan="3">Baseline</td><td colspan="3">End of treatment</td></tr>
<tr><td></td><td>Control (Placebo)</td><td>Study</td><td>p</td><td>Control (Placebo)</td><td>Study</td><td>p</td></tr>
<tr><td>FEV1</td><td>52.1 ± 4.50</td><td>58.8 ± 5.00</td><td>Not significant</td><td>3.30 ± 6.50</td><td>29.47 ± 5.04</td><td>< 0.005</td></tr>
<tr><td>PEF</td><td>38.8 ± 3.70</td><td>53 ± 5.21</td><td>< 0.05</td><td>-0.66 ± 7.20</td><td>31.18 ± 3.80</td><td>< 0.001</td></tr>
</table>
Data are presented as mean ± SEM
</td></tr>
<tr><td>(Boskabady, Mohsenpoor and Takaloo, 2010)</td><td>
<table>
<tr><td></td><td colspan="3">Control (Theophylline)</td><td colspan="3">Treatment</td></tr>
<tr><td></td><td>Baseline</td><td>Post-treatment</td><td>p</td><td>Baseline</td><td>Post-treatment</td><td>p</td></tr>
<tr><td>FEV1</td><td>2.32 ± 0.35</td><td>↑ by 8.58 ± 1.43 %</td><td>< 0.01</td><td>2.14 ± 0.21</td><td>↑ 3.85 ± 0.54 %</td><td>< 0.001</td></tr>
<tr><td>PEF</td><td>3.46 ± 0.55</td><td>↑ by 6.15 ± 1.51 %</td><td>Not significant</td><td>3.18 ± 0.39</td><td>↑ 11.57 ± 3.00 %</td><td>< 0.05</td></tr>
</table>
Data are presented as mean ± SEM
</td></tr>
<tr><td>(Salem et al., 2017)</td><td>
<table>
<tr><td></td><td colspan="3">Control (Placebo)</td><td colspan="3">Treatment</td></tr>
<tr><td></td><td>Baseline</td><td>Post-treatment</td><td>p</td><td>Baseline</td><td>Post-treatment</td><td>p</td></tr>
<tr><td>FEV1</td><td>2.3 (0.6)</td><td>2.3 (0.6)</td><td>Not significant</td><td>2.3 (0.7)</td><td>2.4 (0.7)</td><td>Not significant</td></tr>
<tr><td>FEV1 (% predicted)</td><td>81.1 (19.1)</td><td>80.8 (20.6)</td><td>Not significant</td><td>78.1 (21.4)</td><td>85.5 (22.9)</td><td>< 0.05</td></tr>
<tr><td>PEF</td><td>76.6 (7.3)</td><td>78.5 (8.8)</td><td>Not significant</td><td>73.7 (11.2)</td><td>81.4 (8.7)</td><td>< 0.01</td></tr>
</table>
Data are presented as mean (SD)
</td></tr>
</table>

Table 3.3 Serum IgE outcomes in the clinical studies of *Nigella sativa* in patients with asthma

<table>
<tr><th>Study reference</th><th>Pulmonary function</th></tr>
<tr><td>(Kalus et al., 2003)</td><td>
<table>
<tr><td></td><td colspan="3">Control (Placebo)</td><td colspan="3">Treatment</td></tr>
<tr><td></td><td>Baseline</td><td>Post-treatment</td><td>p</td><td>Baseline</td><td>Post-treatment</td><td>p</td></tr>
<tr><td>Serum IgE</td><td>387.6 (429)</td><td>337.9 (374)</td><td>0.02</td><td>291.9 (650)</td><td>269.6 (550)</td><td>0.14</td></tr>
</table>
Data are presented as mean (SD)
</td></tr>
<tr><td>(Salem et al., 2017)</td><td>
<table>
<tr><td></td><td colspan="3">Control (Placebo)</td><td colspan="3">Treatment</td></tr>
<tr><td></td><td>Baseline</td><td>Post-treatment</td><td>p</td><td>Baseline</td><td>Post-treatment</td><td>p</td></tr>
<tr><td>Serum IgE</td><td>623.8 (802.5)</td><td>595.9 (752.5) 391.5</td><td>Not significant</td><td>393.8 (470.1)</td><td>322.0 (373.2)</td><td>0.01</td></tr>
</table>
Data are presented as mean (SD)
</td></tr>
</table>

1.14 Conclusion and rationale for the PhD work

NS is a medicinal plant with a long history of use in asthma. The therapeutic benefits of NS were reported in different systems of traditional medicine as well as in modern scientific studies (pre-clinical and clinical studies). Pre-clinical studies showed a preliminary evidence for various preparations of NS (oil or extracts of aqueous/organic solvents) and TQ in asthma. Further clinical studies showed a promising activity of different NS preparations in patients with asthma but with variable NS dosage forms (oil, aqueous extract and whole crushed seeds).

These pre-clinical and clinical studies had important limitations in the quality of design, sample size, and investigated outcomes. Also, the variability of the investigational NS preparations and its poor chemical characterisation were major drawbacks in these studies.

Therefore, a specific method of pre-clinical characterisation of NS (chemical and pharmacological characterisation) was carried to find the best suitable active NS preparation that can be used in a clinical trial (Chapter 2 & 3). Followingly, NS was introduced as a complementary (add-on) treatment and assessed its efficacy in patients with asthma using a high-quality clinical trial (Chapter 4).

1.15 General aims and objectives of the dissertation

1. Review the scientific literature for the current evidence for the medical use of *Nigella sativa* in asthma (Chapter 1).
2. Obtaining a series of different *Nigella sativa* preparations, and chemically characterise them using phytochemical analytical techniques (Chapter 2).
3. Pharmacological characterisation of the obtained *Nigella sativa* preparations using different pre-clinical *in vitro* models and compare the anti-inflammatory/immunomodulatory (A/I) activity of these *Nigella sativa* preparations. Ultimately, to find the most suitable active extract that can be used for the clinical trial (Chapter 3).
4. Investigate the clinical efficacy of the selected *Nigella sativa* preparation on asthma inflammation and outcomes in a high-quality clinical trial (Chapter 4).

2 Chapter 2. Phytochemical analysis: method development for extraction and analysis of different *Nigella sativa* preparations

2.1 Introduction

NS was used in various form of preparations in traditional medicine as well as pre-clinical and clinical studies. Different companies are producing NS products based on various extraction and processing methods. Also, many studies investigated the pharmacological activity of NS with poor chemical standardisation or characterisation. Ultimately, this can lead to a variability in the pharmacological activity of NS. Therefore, we intended to compare the phytochemical composition (Chapter 2) and the pharmacological activity of different NS preparations (Chapter 3); either prepared in our laboratory or imported as a commercial ready-made product from different companies.

There is no official monograph defining the standard quality or composition of NS. Biological studies attribute the activity of NS mainly to TQ (Darakhshan *et al.*, 2015). Therefore, TQ was selected as an indicator of NS quality, and NS preparations were characterised for the TQ level by High Performance Liquid Chromatography (HPLC). Additionally, Proton Nuclear Magnetic Resonance (^{1}H NMR) was used to overview the variability in the phytochemical composition of the different NS extracts.

2.2 Specific objectives

- Obtain various types of NS preparations by either local laboratory extraction or importation of ready-made products of NS.
- Quantification of the main active compound TQ among the NS preparations.

- Compare the TQ concentration level and ^{1}H NMR fingerprints of the NS extracts.

2.3 Materials and methods

2.3.1 Solvents, reagents and chemicals

- Ethanol absolute batch no. 11E160502, VWR.
- Dimethyl sulfoxide-d6 NMR solvent, Sigma-Aldrich.
- Thymoquinone (purity >98%), Cayman Chemicals. Catalogue number: CAY15039.

2.3.2 Apparatus and instrumentation

- HPLC instrument: WATERS 2695 separations module, attached to Waters 996. Photodiode Array Detector.
- Ependorff Minispin plus centrifuge, model 5453.
- Fisher ultrasound bath, model FB 11020.
- Rotamixer, rotary mixer, serial no. 8011.
- HPLC 1.5ml amber screw vials, VWR. Cat no. 548-1367.
- VWR NMR sample tubes, 5mm economy, 7” Length, 100MHz.
- Bruker Avance 500MHz nuclear magnetic resonance (NMR) spectrometer (BrukerAnalytik, Rheinstetten, Germany).
- Wet and Dry Grinder, Andrew James, model B00I0SF1N0.
- Millex HA filter unit 0.45mm.
- HPLC Column: phenomenex® P/NO 00F-4252-E0, Desc Luna 5u C18(2) 100 Å, size 150x4.6mm 5 micron, S/NO 312985-48.

2.3.3 Software

- Topspin software version 1.3 was used for NMR spectra observation and utilisation.
- Microsoft Office Word and Excel 2010.

2.3.4 *Nigella sativa* extracts collection and preparation

Two main types of NS preparations were obtained in this study. First, the locally prepared extracts from crude NS seeds in our laboratory at the UCL School of Pharmacy. Second, a series of ready-made NS oil products from different companies were included.

The crude seeds of NS were obtained from the spice market of Jeddah city in Saudi Arabia produced by Bafart company (Wadi Nimar, Al-Balad, Jeddah 22236 - www.bafarat.sa). This product was selected because it has a high concentration of volatile oil content using Thin Layer Chromatography (TLC), according to my previous work for an analysis of 23 different NS samples from various origins as part of an MSc dissertation (Koshak, 2013). Apparently, this was probably due to the proper packaging and storage conditions of the product.

Due to the discrepancies found in the literature about the type of NS preparation used in biological and clinical studies (such as aqueous extract, organic solvent extract, oil and powder), an approach of using different ratios of extraction solvents was used to obtain different combinations of water/alcohol soluble compounds. The crude seeds were used to prepare six different extracts (1-6) via different ratios of ethanol:water extraction solvents. Ten grammes of crushed NS were filled into six different 100 ml glass bottles. Then, different solvent ratios were used to produce the extracts by means of maceration for extracts 1-5 with frequent agitation at room temperature for three days, and by

means of decoction for extract 6 as following; Extract no.1 (100:0), 2 (80:20), 3 (60:40), 4 (30:70), 5 (0:100), 6 (0:100 with continuous boiling for 30 minutes). After filtration, the ethanol was evaporated using a rotavap until complete dryness, while water removed by freeze-drying until complete dryness. On the other hand, commercial ready-made NS oil products were obtained from different producers (extracts 7-10) as shown in Table 4.

Table 4. The list of obtained *Nigella sativa* preparations.

NS preparation	Extraction method or origin
Extract 1	100% Ethanol
Extract 2	80% Ethanol: 20% Water
Extract 3	60% Ethanol: 40% Water
Extract 4	30% Ethanol: 70% Water
Extract 5	100% Water
Extract 6	100% boiled water (30min boiling)
Extract 7	Super Critical Fluid (SCF) extract by Sami Labs Ltd., India
Extract 8	Cold pressed NS oil capsules by The Blessed Seeds, UK
Extract 9	Cold pressed NS oil capsules by Sanct Bernhard, Germany
Extract 10	Cold pressed NS oil capsules by Marnys, Spain. (A licenced herbal product in Saudi Arabia)

Extract 1-6; Laboratory prepared extracts from crude NS seeds using different ratios of combined extraction solvents. Extract 7-10; Commercial ready-made *Nigella sativa* oil extracts.

2.3.5 High Performance Liquid Chromatography method for analysis of thymoquinone in *Nigella sativa* preparations

The High Performance Liquid Chromatography (HPLC) analysis method was developed using the approach of Hadad *et al*. (2012). The analysis was performed using a WATERS 2695 HPLC instrument. 20µl sample injected using gradient methanol-water (20-80, v/v) mobile phase over 10min and injection run time of 12min at flow rate of 1.5ml/min.

The NS preparations were dissolved in 1 ml methanol inside 1.5 ml Eppendorf tubes separately, vortexed for 10 seconds and centrifuged for 2 min at 10000 rpm to separate any remaining of solid extract. Afterwards, the supernatant was transferred to an HPLC amber vial via 0.45mm filter for analysis with HPLC. The remaining solid extract was also analysed with HPLC to ensure complete extraction of TQ.

After setting up the method on the HPLC instrument software, the samples were submitted in triplet, and the average reading results were used. Next, a calibration curve was made based on the area under curve readings of each sample peak. Microsoft Excel software was used to draw the calibration curve, and the level of TQ was determined accordingly. The individual HPLC chromatogram for each sample is provided in the appendix (Page 152).

2.3.6 Proton Nuclear Magnetic Resonance method for the analysis of *Nigella sativa* preparations

A Bruker Avance 500MHz nuclear magnetic resonance (NMR) spectrometer (BrukerAnalytik, Rheinstetten, Germany) equipped with a multi-nuclear probe head with z-gradient was utilised to produce proton NMR analytical

spectra. 50mg of each NS extract (Extract 1-10) and 5mg of standard TQ was transferred into a 1.5ml Eppendorf tube. Then, samples were dissolved in 1.0ml of DMSO-d6, vortexed for 15 seconds, and ultrasonicated for 30 minutes, followed by further mixing on a rotary mixer. Afterwards, centrifugation was carried out for 5 minutes at 10,000 rpm. Next, 0.7 ml of the supernatant was transferred to a 5mm diameter NMR tube. These were submitted for NMR analysis. The NMR submission settings were adjusted to scan type: proton NMR, number of spins: 256 for optimum resolution, solvent: DMSO.

2.4 Results and discussion

2.4.1 Extraction yield of *Nigella sativa* preparations from different extraction methods

The extraction yields of 10 grammes of crude NS seeds using different solvent systems (Extract 1-6) are shown in Table 5. Apparently, Extract 1, in which 100% ethanol was used as a solvent, showed the highest yield among other preparations because of the relative high fatty acid content of NS which is more soluble in ethanol than water.

Table 5. Extraction yield of the locally prepared *Nigella sativa* extracts 1-6.

Preparations	Extraction yield from 10 g crude NS	Percentage yield
Extract 1 (100% ethanol)	3.25 g	32.5 %
Extract 2 (80:20)	1.23 g	12.3 %
Extract 3 (60:40)	1 g	10 %
Extract 4 (30:70)	1.21 g	12.1%
Extract 5 (100% water)	0.89 g	8.9 %
Extract 6 (100% boiled water)	1.57 g	15.7 %

2.4.2 High Performance Liquid Chromatography analysis of thymoquinone in *Nigella sativa* preparations

This experiment was conducted to determine the concentration of the main active compound thymoquinone (TQ) among all NS preparations. The method used to identify and quantify TQ in HPLC revealed the TQ peak at around 10.3 min (Figure 3). Different serial dilutions of known concentrations of standard TQ solutions were prepared and analysed by HPLC (Table 6) to establish a calibration curve by Microsoft Excel (Figure 4). This curve was utilised to establish TQ levels among NS preparations (Table 7).

The NS preparations had a variability in the TQ concentrations. This is attributed to the methods of extraction and preparation as well as origins of NS seeds. Extract 7 (prepared by supercritical fluid extraction method) appeared to have the highest concentration of TQ, followed by extract 10. Among the locally prepared extracts (extract 1-6), the 100% ethanolic extract 1 showed the highest level of TQ in comparison to the rest of the extracts. This is due to the solubility characteristics of TQ which is highly soluble in organic solvents. On the other hand, extracts 2-6 had very low or undetectable level of TQ, and this was confirmed by the absence of TQ peaks in the NMR analysis.

Figure 3. The standard TQ (0.2mg/ml) peak at 10.3 min in HPLC analysis.

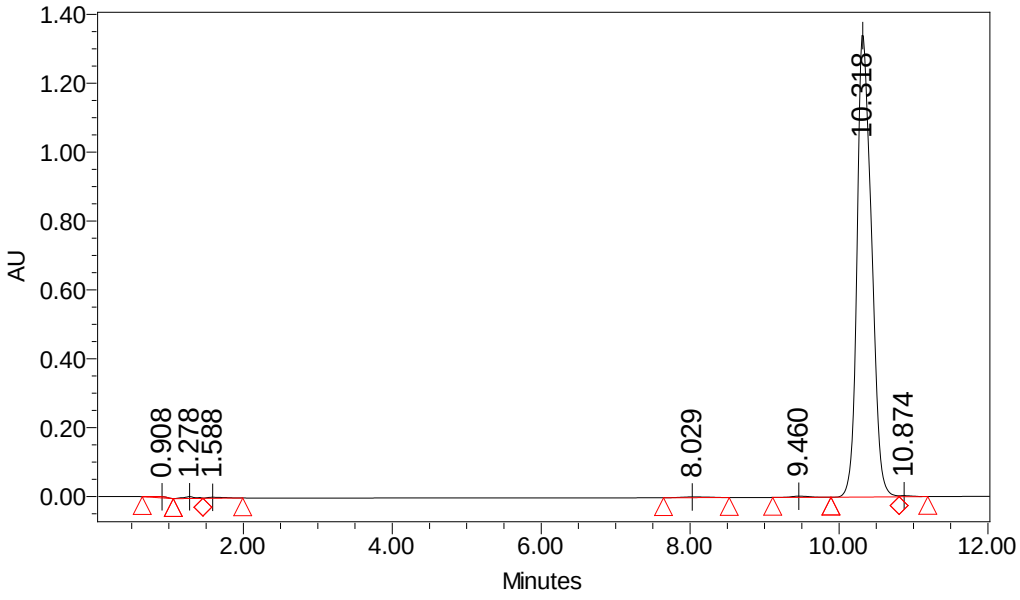


Table 6. High Performance Liquid Chromatography peak areas of six known standard thymoquinone concentrations.

TQ Concentration (mg/ml)	Peak area (area under curve)
0.005	341,676
0.050	3,868,814
0.100	8,138,286
0.200	18,148,300
0.400	33,733,063
0.600	42,756,647

Figure 4. Calibration curve produced from the peak area results of six known concentrations (mg/ml) of standard thymoquinone.

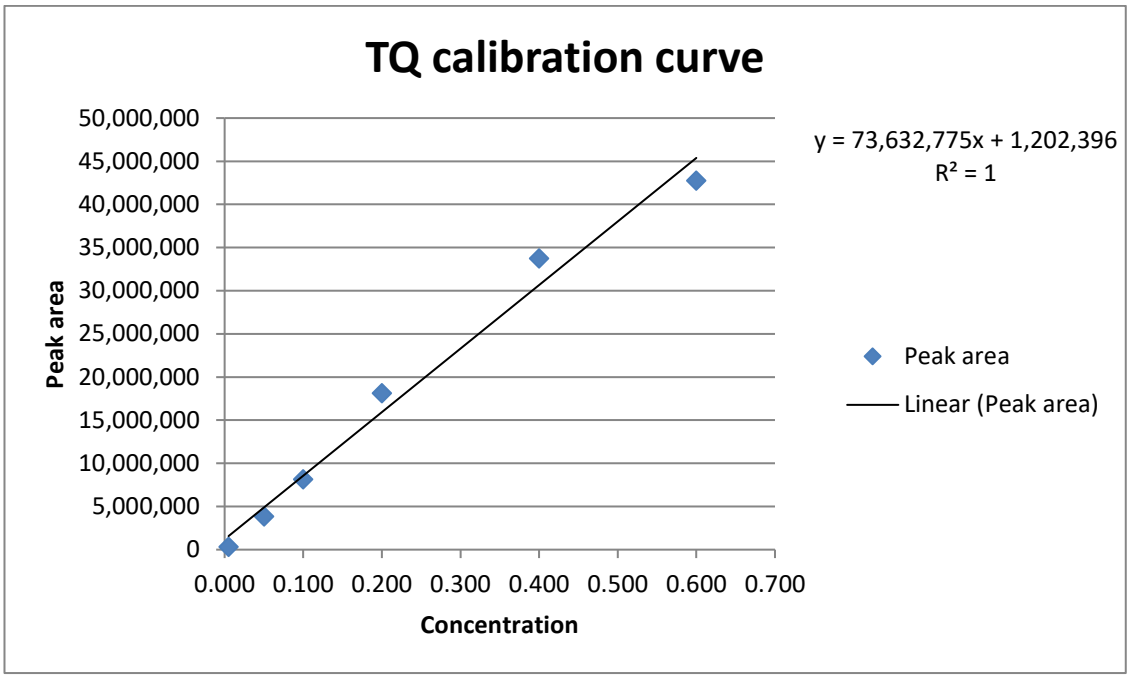


Table 7. The established levels of TQ among *Nigella sativa* preparations

Sample	Prepared concentration (mg/ml)	Peak area	TQ concentration (mg/ml)	TQ concentration %(wt/wt)
Extract 1	84	28,577,583	0.371	0.4%
Extract 2	36	1,522,845	0.004	0.01%
Extract 3	80	3,996,096	0.037	0.04%
Extract 4	109	486,202	~0.005	~0.005%
Extract 5	138	124,386	< 0.005	< 0.005%
Extract 6	125	N/A	N/A	N/A
Extract 7	27	49,727,558	0.659	2.4%
Extract 8	64	29,926,597	0.391	0.6%
Extract 9	110	19,561,802	0.249	0.2%
Extract 10	65	34,914,364	0.457	0.7%

2.4.3 Proton nuclear magnetic resonance analysis of *Nigella sativa* preparations

The NS preparations and standard TQ were analysed by proton nuclear magnetic resonance (^{1}H NMR). The typical ^{1}H NMR peaks of TQ are shown in Figure 5. A comparison of all generated ^{1}H NMR spectra is shown in Figure 6. Also, the presence of characteristic TQ peaks was assessed in NS preparations. As a result, extracts 1, 7, 8, and 10 appeared to have the characteristic peaks of TQ such as the 2 singlet peaks at 6.6 and 6.7ppm (Figure 6). However, the rest of extracts barely showed any presence of TQ peaks by matching with the reference standard TQ. These ^{1}H NMR figures provided information on the presence/absence of TQ as well as a general overview of the variability in chemical compositions of the NS preparations.

Figure 5. Proton nuclear magnetic resonance spectrum of thymoquinone using the nuclear magnetic resonance predictor online tool at nmrdb.org.

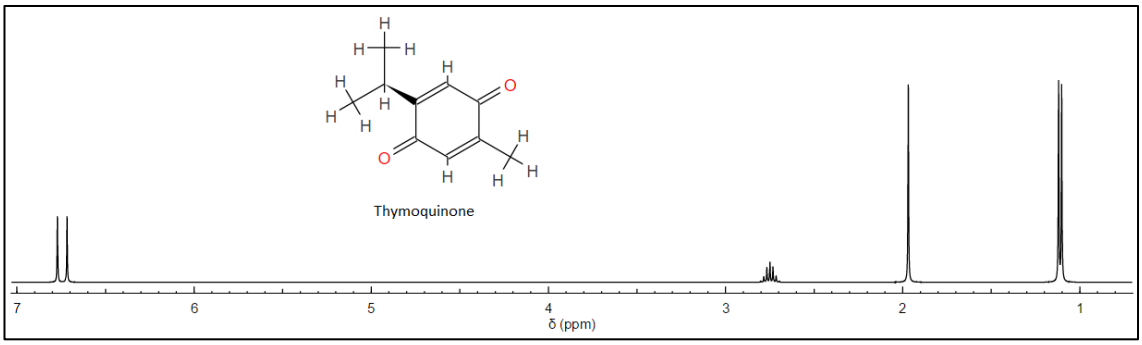
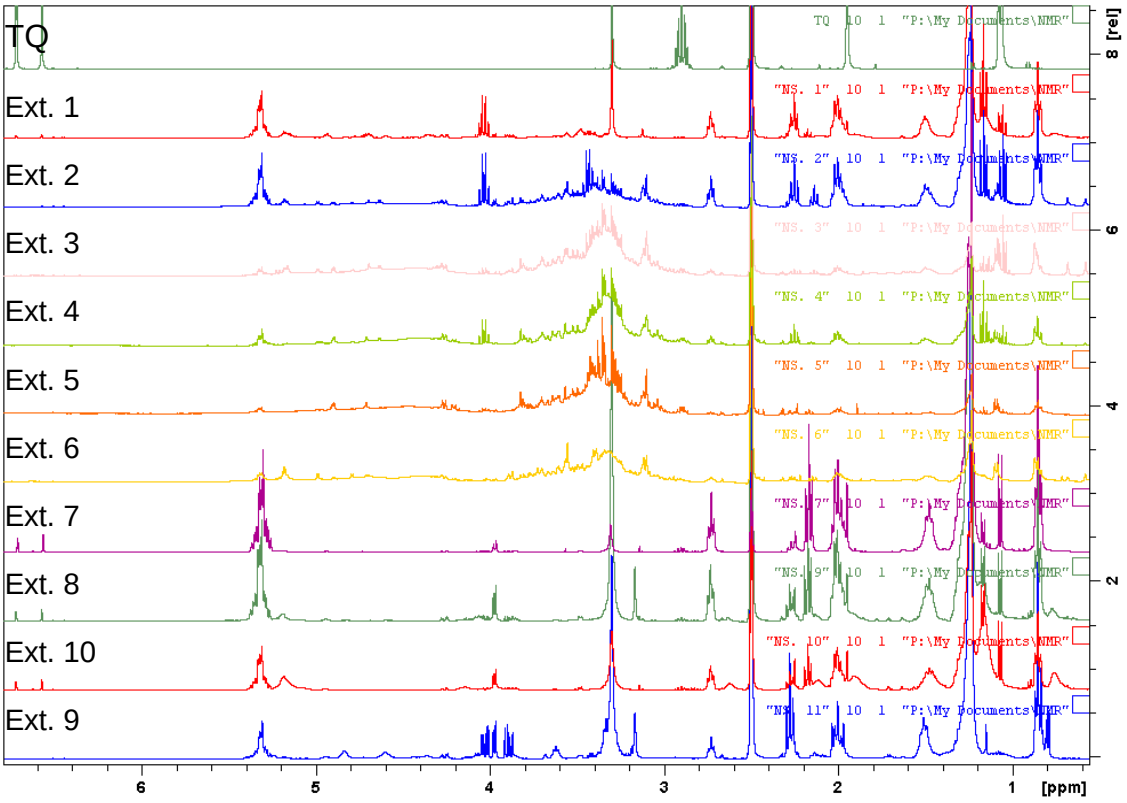


Figure 6. Comparison of proton nuclear magnetic resonance spectra of *Nigella sativa* preparations (Extracts 1-10) and thymoquinone.



2.5 Conclusion

Ten different preparations of NS were obtained via local preparation (at our laboratory) using different ratios of combined extraction solvents (water:methanol) along with the inclusion of commercial ready-made products. Using HPLC, high level of TQ was observed in the oily preparations due to the lipophilic nature of TQ. It was highly concentrated in the supercritical fluid extract (no. 7) followed by the commercial product of Marnys company (no. 10). ^{1}H-NMR fingerprints showed variability in the phytochemical content between the extracts and confirmed the presence of TQ in the oily preparations. This variability in the chemical composition among NS preparations was linked to the biological activity (Chapter 3), and a the best suitable and biologically active preparation was used in the clinical trial (Chapter 4).

3 Chapter 3: Pre-clinical (*in vitro*) screening

3.1 Introduction

The work of this chapter was done in collaboration with Dr Bernd Fiebich at the University Medical Center Freiburg in Germany. The goal of this work was to have a comparative screening for the general anti-inflammatory/immunomodulatory (A/I) activity of the obtained NS preparations (Extracts 1-10, Chapter 2) in various pre-clinical *in vitro* models. There are several inflammatory mediators and signalling pathways from different human cells are involved in asthma inflammation as discussed in Chapter 1. Due to the capabilities of Dr Fiebich's laboratory, certain targets were used for screening the activity of NS preparations on the expression of the inflammatory mediators IL-2, IL-6 and PGE2, as well as the inflammatory signalling protein phospho-p38. This was investigated in different laboratory-induced human cells including peripheral blood mononuclear cells (T-lymphocyte and monocytes cells), and A549 human lung epithelial cell line.

The relevance of the selected targets was discussed in section 3.2. The preclinical *in vitro* activity (A/I) of NS preparations was investigated in this chapter. Consequently, the most active extracts that demonstrated potent A/I activity were selected for the clinical trial of NS in patients with asthma (Chapter 4).

3.2 The relevance of the selected targets to asthma

Interleukin-2 (IL-2) is an inflammatory cytokine mainly released by activated T cells (Hoyer *et al.*, 2008). It is linked to asthma because it was found to be increased in the broncho-alveolar lavage fluid (BALF) of patients with

asthma (Virchow *et al.*, 1996). Moreover, patients treated with inhaled IL-2 had asthma-like sign and symptoms (Loppow *et al.*, 2007).

Interleukin-6 (IL-6) is a small glycoprotein released from different cells of the immune system such as macrophages, dendritic, mast cells, B cells, some T cells, and non-leukocytes (such as epithelial cells, endothelial cells and fibroblasts) during an inflammatory or immune reaction. It is found to be a major pro-inflammatory mediator and a relevant target for several diseases (Hirano, 1998). In patients with asthma, IL-6 was found at a high level in serum as well as in BALF (Yokoyama *et al.*, 1995; Tillie-Leblond *et al.*, 1999). IL-6 was elevated in induced sputum of allergic asthma patients (Neveu *et al.*, 2010). There was an increase expression of IL-6 in bronchial epithelial cells of patients with asthma (Marini *et al.*, 1992). Out of 170 adult patients with asthma, elevated serum IL-6 was found in 54% of patients and that was associated with the use of high-dose inhaled corticosteroids (Ilmarinen *et al.*, 2016).

Prostaglandin E2 (PGE2) is one of the common prostanoids synthesised in humans and has an important role in the inflammatory cascade. Prostaglandins are produced by cyclooxygenase enzymes (COX-1 and COX-2). COX-1 is produced by most cell types in the body. COX-2 is expressed in response to stimuli mainly in antigen-presenting cells such as macrophages (Kaur *et al.*, 1999). PGE2 is considered a pleiotropic prostaglandin with stimulating or inhibiting properties. In general, PGE2 is commonly assumed as a proinflammatory mediator in several inflammatory diseases (Sastre and Del Pozo, 2012). PGE2 can induce Th2 cell development and Th2 cytokines profile secretion. Also, PGE2 can enhance the production of Immunoglobulin E (Kaur *et al.*, 1999). However, PGE2 is also important because of the multiplicity of its effects on immune response in respiratory diseases. It is considered a major

mediator in the lower respiratory tract released mainly by epithelium and airway smooth muscle. It has a beneficial role in asthma via protection from lung smooth muscle proliferation and some anti-inflammatory effects (Sastre and Del Pozo, 2012). PGE2 has an ability to reduce mast cells activity and relax smooth muscle (Torres, Picado and de Mora, 2015). In a clinical trial, inhaled PGE2 therapy had a protective role against the delayed response to allergen-induced asthma (Pavord *et al.*, 1993). In patients with asthma, PGE2 showed bronchodilatory effects via inhibition of allergen-induced bronchoconstriction and some inflammatory mediators (Chung, 2005).

The inflammatory cell signalling p38 protein kinases is part of mitogen-activated protein kinase (MAPK) family that plays a role in an inflammatory reaction and involved in some pro-inflammatory cytokines production (Hommes, Peppelenbosch and van Deventer, 2003). Inhibition of p38 may provide a wide range of anti-inflammatory effects which can be useful in several inflammatory diseases such as asthma (Newton and Holden 2003). Targeting p38 is a potential treatment option for asthma because it showed several beneficial effects such as inhibition of allergen-induced pulmonary eosinophilia, mucus overproduction, and lung hypersensitivity (Chung, 2011). Also, p38 inhibition may reduce corticosteroid insensitivity in severe asthma (Bhavsar *et al.*, 2010). Detection of phospho-p38 stain was obvious in the epithelial cells of severe asthma patients (Liu *et al.*, 2008).

3.3 Specific objectives

- Determination and comparison of the A/I activity of the prepared NS extracts (Chapter 2) using different *in vitro* cellular models.

- Selection of a suitable active extract that shows a potent A/I activity and can be used for the clinical trial of NS in asthma (Chapter 4).

3.4 Material and methods

3.4.1 NS preparations

- Extracts 1-10 as mentioned in Chapter 2 (Table 4).

3.4.2 Experimental materials and apparatus

- (HRP)-coupled rabbit anti-goat IgG secondary antibodies, Santa Cruz.
- 500ml of Roswell Park Memorial Institute medium (RPMI) 1640 Media, GE Healthcare.
- CELLSTAR® 50 ml Centrifuge Tubes, VWR.
- Dimethyl sulfoxide (DMSO) solvent, Merck Millipore, Germany.
- Dulbecco`s Phosphate Buffered Saline (DPBS), GIBCO®, Life Technologies.
- Eppendorf Minispin plus centrifuge, model 5453.
- Falcon® 24 Well Clear Flat Bottom TC-Treated Multiwell Cell Culture Plate (Product #353047), Corning B.V. Life Sciences.
- Fisher ultrasound bath, model FB 11020.
- Fresh human blood from healthy donors, University of Freiburg health centre.
- Human IL-2 ELISA kit, R&D Systems.
- Human IL-6 ELISA ready-SET-GO kit, eBioscience.
- Human lung epithelial cell line (A549), Sigma-Adrich.
- IMT-2 microscope, Olympus optical CO. LTD.
- Interleukin-1β, human (hIL-1β) Product No. 11457756001, Roche Life Science.
- LPS: L9516 Lipopolysaccharides (rough strains) from Salmonella enterica serotype typhimurium SL1181, Sigma-Aldrich.

- Lymphocyte Separation Medium (LSM 1077), The Cell Culture Company, PAA Laboratories GmbH.
- Magellan Biosciences microplate reader, Dynex.
- p.38 primary antibodies, Cell Signaling Co.
- PGE2 Enzyme Immunoassay kit, Biotrend.
- Pierce™ BCA Protein Assay Kit, Thermo Scientific™ [BCA Reagent A, BCA Reagent B, Albumin Standard Ampules 2mg/mL].
- Rotary mixer, Rotamixer serial no. 8011.
- SEB: Staphylococcal enterotoxin B, Sigma-Aldrich.
- Thermo Scientific™ Megafuge™ 3.0 RS.
- Thermomixer basic, Cell Media Co.
- Thermomixer compact, Eppendorf.
- Thymoquinone (purity >98%), Cayman Chemicals. Catalogue number: CAY15039.

3.4.3 Preparation of *Nigella sativa* extracts for *in vitro* testing

Initially, a preparation step was carried out on all NS preparations for the *in vitro* screening. A 20mg of each NS preparation was dissolved in 1ml Dimethyl sulfoxide (DMSO) solvent inside 1.5ml Eppendorf tube with mixing on a rotamixer for 10 seconds. This is followed by centrifugation for 5 minutes at 1300rpm for separation of insoluble substances. Then, the clear supernatant was re-diluted in DMSO to produce three different dilutions as in the following concentrations; 10µg/ml, 100µg/ml, and 500µg/ml as required for *in vitro* testing. Also, the standard TQ was prepared by using DMSO solvent to produce concentrations of 0.1µM, 1µM, 5µM, and 10µM as required. As the molecular weight of TQ is 164 g/mol, 1µM of TQ equals to 0.164µg/ml.

3.4.4 Isolation of human peripheral monocytes and T-lymphocyte cells for *in vitro* testing

Human blood from three different healthy donors at the University Medical Centre Freiburg was obtained in 450 ml blood plastic bags. The isolation of human monocytes and T-lymphocyte cells were carried out according to a standardised protocol described by (Noble and Cutts, 1968; English and Andersen, 1974). In six CELLSTAR® 50ml Centrifuge tubes, 25 ml of Lymphocyte Separation Medium (LSM 1077) were added to each tube. Then, 20 ml of human blood were carefully added into each tube. These tubes were applied to centrifugation with Thermo Scientific™ Megafuge™ instrument at 1800 rpm and acceleration 1 for 1 hour at room temperature. After centrifugation, the buffy coat was transferred to 50 ml CELLSTAR® tube prefilled with a 10ml of Dulbecco`s Phosphate Buffered Saline (DPBS) solution. This was completed to 50 ml with the addition of more DPBS solution. Then, centrifugation for 10min at 1600rpm, normal acceleration and room temperature. The supernatant was discarded, and DPBS was added on top the buffy coat layer to fill until 50 ml. Afterwards, centrifugation at 1200rpm for 10mi; supernatant discarded, and 20ml of DPBS was added with proper mixing. Additional DPBS was added to fill the tube until 50ml. The last step was repeated, and the supernatant was discarded, and the cells remained in the tube. Finally, the cells were re-suspended in 50 ml RPMI-1640 fortified with 10% human serum. Cells were counted using Olympus microscopy with the addition of 180 µL of Microscopy Solution (Trypan Blue 0.4%) and 20µl of sample cells solution. Further dilution with RPMI-1640 solution to maintain a proper cell density of 150-200 cells per ml.

3.4.5 Stimulation of monocytes and T-lymphocyte cells

Sixteen of 24-well tissue culture plate were obtained and seeded with 1ml of cells solution in each well. This was incubated in 37c for 1 hour. Then the medium was changed with (RPMI 1640 + 10% human serum) and further incubated for another 1 hour at 37c. After that, a 5 µL of NS preparations (in concentrations 10, 100, 500µL) and standard TQ (10 µM) were added to their assigned well. In each plate, two wells were left without any extract to serve as positive (Cells+stimulant+DMSO) and negative (Cells+DMSO) reference. After 30min incubation, 10µl of the stimulant LPS (Lipopolysaccharides, 10ng/ml) was added to each well (except –ve reference) of eight plates to stimulate monocytes. In another eight plates, 10 µl of the stimulant SEB (Staphylococcal enterotoxin B, 100ng/ml) was added to each well (except –ve reference) to stimulate T-lymphocyte cells. These plates were incubated at 37c overnight to allow stimulation of both monocytes and T-lymphocyte cells to release inflammatory mediators. In the next day, the supernatant was collected from each well into separate Eppendorf tubes and centrifuged to filter out any remaining cells. This step was further repeated, and the supernatant was stored at -80c for further analysis of the inflammatory mediators.

3.4.6 Stimulation of human lung epithelial cells (A549)

In four of 6-well plates containing A549 cells, 50 µl of each NS preparation (in both concentrations of 100 & 500 µg/ml) and standard TQ (10 µM) were added to plate wells. In each plate, two wells were left without any extract to serve as a positive control (Cells + stimulant + DMSO) and negative control (Cells + DMSO). After 30 min incubation, 20 µl of the stimulant IL-1β (10 µl /ml) was added to each well (except negative reference) to stimulate the A549 cells. These plates were

incubated at 37°c overnight to allow stimulation and production of inflammatory mediators. After incubation, the media was discarded and the cells were washed with DPBS for complete removal of media (cells are precipitated and attached to the bottom of wells). This is followed by a cell lysis step to break the cells and release the inflammatory mediators and cells proteins without protein degradation. This was done by the addition of 140 µl of a mixture of [2 ml lysis buffer + 2 µl protease inhibitor + 20 µl phosphatase inhibitor] in each well. Followed by proper mixing and scratching over a box of ice for 2 min. Then, the supernatant in each well was transferred into separate Eppendorf tubes and heated to 90°c on a thermomixer at 300rpm for 5 min. Afterwards, the protein estimation step was carried (3.3.7).

3.4.7 Protein estimation in A549 cells

This step was carried as preliminary step for quantification of phospho-p38 in the following immunoblotting step (3.4.8). The protein content was measured according to the manufacturer instructions (Pierce™). A solution of a mixture of BCA Reagent A and BCA Reagent B (ratio 50:1) was prepared in the required amount that is 200 µl for each well. In a 96-well plate, a 200 µl of this mixture was added to all test wells. BCA Albumin Standard was added in different amounts (1, 2, 4, 8, 10, 20 µl) to be used as a standard curve for estimation of protein from samples. All samples were diluted with 1 ml of lysis buffer, and 10 µl of each sample was added to its corresponding well. The plate was placed on a thermomixer for 30min at 95c and 300rpm. Accordingly, the protein amount was detected by extrapolating the wavelengths readings of samples to the standard BCA Albumin readings, using DYNEX Magellan Biosciences microplate reader. This information was used in immunoblotting.

3.4.8 Immunoblotting (Western Plot) for phospho-p38 inflammatory signalling proteins

This was carried on the A549 cells exudates to quantify the expression of phospho-p38 inflammatory signalling proteins. This was done to explore a possible mechanism of the anti-inflammatory action of NS according to the methodology of (Olajide *et al.*, 2013). In phospho-p38 signalling protein detection, rabbit anti-phospho p38 MAPK poly-clonal antibody (Cell Signalling, Schwalbach, Germany) used as primary antibodies and goat anti-total p38 MAPK (Santa Cruz, Heidelberg, Germany) as secondary antibodies. Quantification of the proteins was done by ImageJ software after normalisation with total protein. This was done as a single experiment for screening NS preparations. Then, the same experiment was replicated on selected NS preparations using new A549 cell line in each experiment.

3.4.9 Enzyme-linked immunosorbent assay for interleukin-6, interleukin-2 and Prostaglandin E2 determination

Measurements of the levels of the pro-inflammatory mediators: IL-6, IL-2, and PGE2 were performed by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Human IL-6 ELISA ready-SET-GO kit by eBioscience; Human IL-2 ELISA kit by R&D Systems; PGE2 Enzyme Immunoassay kit by Biotrend). All experiments were replicated at least three times with different new blood donor or cell line.

3.4.10 Statistical analysis

The results were statistically analysed using one-way ANOVA with post-hoc Dunnett's test to investigate significant effects of each NS preparation against the

control group. For each experiment, P value <0.05 was considered statistically significant.

3.5 Results and discussion

3.5.1 Effect of *Nigella sativa* preparations on human T-lymphocyte cells inflammatory mediators

The effect of NS preparations on the release of the inflammatory mediators IL-2, IL-6 and PGE2 was investigated in SEB-induced human T-lymphocyte cells. Initially, three different concentrations of NS preparations were used in this experiment (10, 100 and 500 µg/ml). The optimum concentrations were found to be 100 µg/ml for most NS preparations. This was due to the poor effect appeared by using the concentration of 10µg/ml (no significant inhibition) and the very high effect shown by the concentration of 500µg/ml (complete inhibition of cytokines release) by most NS preparations.

There was a suppression of IL-2 release from T-lymphocyte cells by all NS preparations, which was noteworthy (by more than 50%) with extracts 1, 2, 3, 5, 7, 8, and 10 (Figure 7). This suppressive effect of NS on IL-2 release from human T-lymphocyte cells is not reported in the literature. However, in an animal study, there was a suppression of serum IL-2 in mice by NS fixed oil (Abbas *et al.*, 2005). This suppressive effect of IL-2 release from T lymphocyte cells may be beneficial in the context of asthma as it is considered a pro-inflammatory mediator and found in a high level in BALF of patients with asthma.

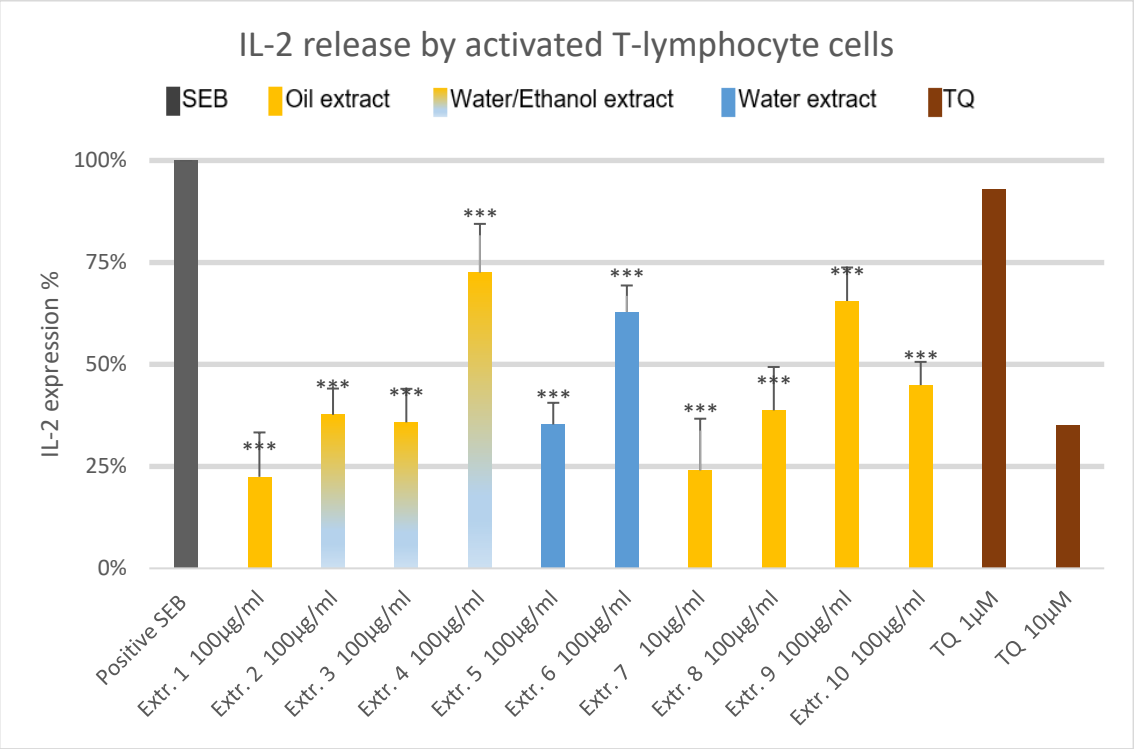
IL-6 release from T-lymphocyte cells was markedly reduced (by more than 50%) with the oily extracts 1, 7, 10. (Figure 8). Conversely, IL-6 release from T-

lymphocyte cells was increased with water or mixed (water/oil) extracts 3, 4, 5, and 6. This effect of NS on IL-6 release from human T-lymphocyte cells is not reported in the literature. This suppressive effect of IL-6 release from T lymphocyte cells may be beneficial in the context of asthma as it is considered a pro-inflammatory mediator and found in a high serum level among patients with asthma.

PGE2 release from T-lymphocyte cells was inhibited markedly (by more than 50%) only with the oily extracts 7 and 10 (Figure 9). Conversely, there was a marked stimulation of PGE2 release (by more than 150%) with water/ethanol extracts, and particularly with pure water extracts (by up to 10 times). Interestingly, there was a positive relationship between the NS extracts prepared by water (low TQ level) and the release of the PGE2 from T-lymphocyte cells. This suppressive effect of NS on PGE2 release from human T-lymphocyte cells is not reported in the literature. However, the NS volatile oil administration significantly lowered the plasma level of PGE2 in rats (Salim, 2010). In an *in vitro* cyclooxygenase-1 (COX-1) and -2 (COX-2) assays, TQ caused a general anti-inflammatory activity via inhibition of COX-1 and COX-2 catalysed PGE2 production (Marsik *et al.*, 2005). Carvacrol, a volatile oil constituent of NS, inhibited the release of PGE2 catalysed by COX-2 (Landa *et al.*, 2009). This suppressive effect of PGE2 release from T lymphocyte cells may be considered a favourable effect due to the general pro-inflammatory properties of PGE2 and the enhancement effect of PGE2 on Th2 and IgE responses.

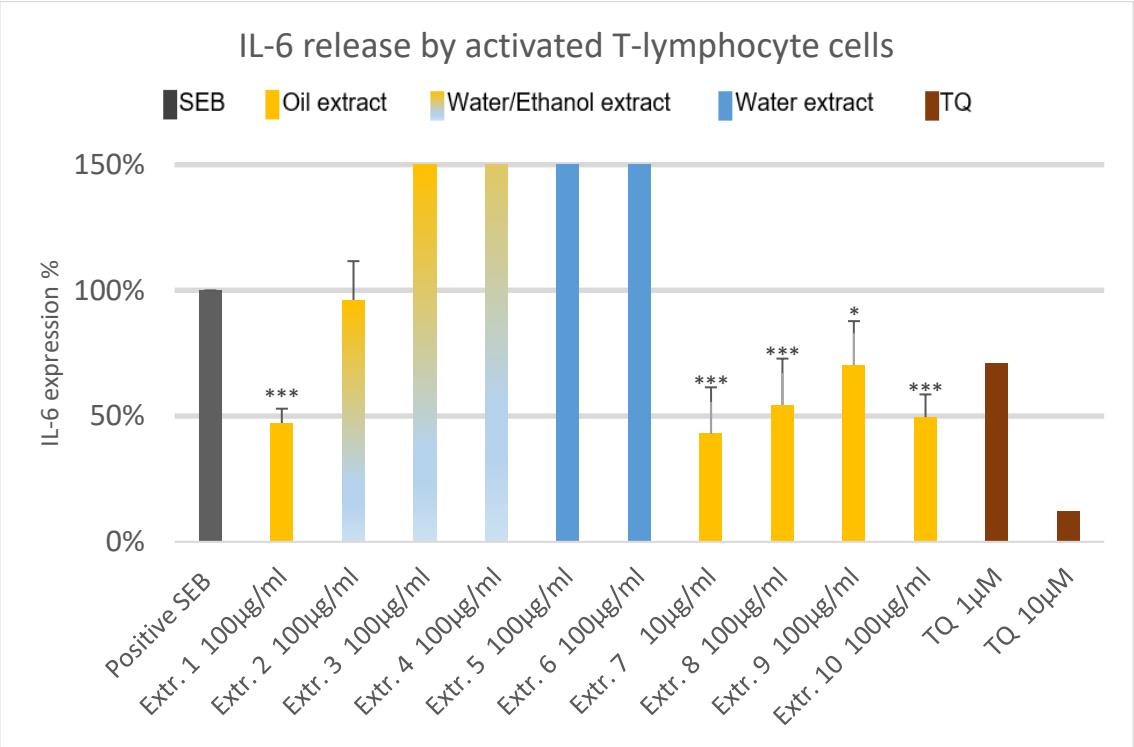
Interestingly, there was a positive relationship between the TQ level of the oily NS preparations and the inhibition of the inflammatory mediators IL-2, IL-6 and PGE2.

Figure 7. The effect of *Nigella sativa* preparations on interleukin-2 production by SEB-induced T-lymphocyte cells (n=3).



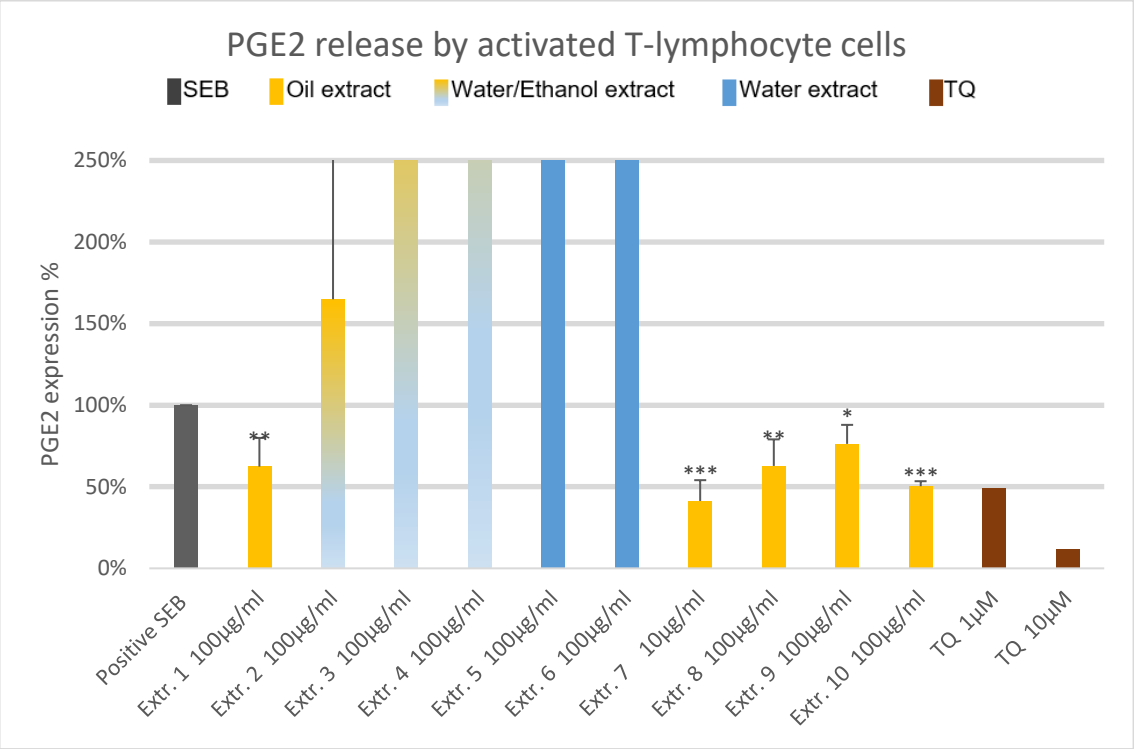
Significant effects are indicated by an asterisk (***P<0.001).

Figure 8. The effect of *Nigella sativa* preparations on interleukin-6 production by SEB-induced T-lymphocyte cells (n=3).



Significant effects are indicated by asterisks (*P<0.05, ***P<0.001).

Figure 9. The effect of *Nigella sativa* preparations on prostaglandin E2 production by SEB-induced T-lymphocyte cells (n=3).



Significant effects are indicated by asterisks (*P<0.05, **P< 0.01, ***P<0.001).

3.5.2 Effect of *Nigella sativa* preparations on Monocytes inflammatory mediators

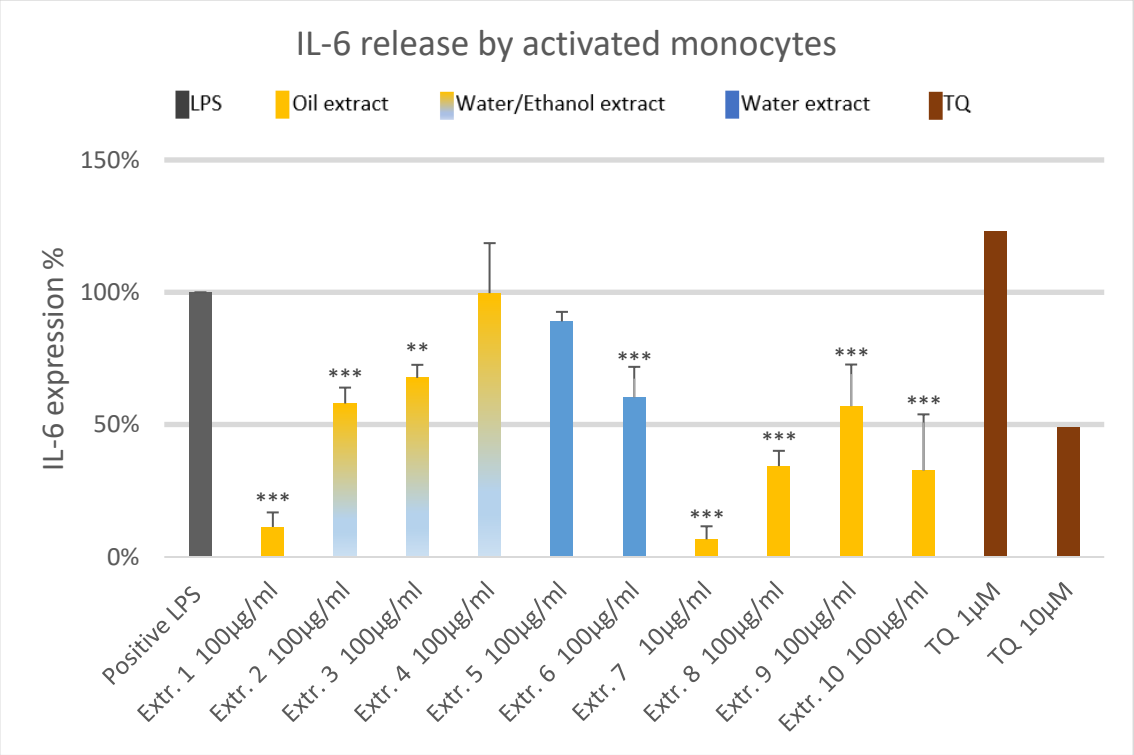
The effect of NS preparations on the release of inflammatory mediators IL-6 and PGE2 was investigated in LPS-induced monocytes. The concentration of 100µg/ml for most NS preparations was used according to the findings of the previous experiment on T-lymphocyte cells.

There was a suppression of IL-6 release from monocytes with most extracts, and a marked inhibition occurred (by more than 50%) with the oily extracts 1, 7, 8 and 10 (Figure 10). This effect by NS is not reported in the literature. However, TQ alone suppressed the expression of IL-6 from *Mycobacterium tuberculosis* infected human monocyte THP-1 cells (Mahmud *et al.*, 2017). TQ inhibited the production of IL-6 in LPS-activated murine macrophage-like RAW264.7 cells (Hossen *et al.*, 2017). This suppressive effect of IL-6 release from monocyte cells may be beneficial in the context of asthma as it is considered a pro-inflammatory mediator and found in a high level in the serum of patients with asthma.

There was a marked suppression of PGE2 release from monocyte cells (by more than 50%) with the oily extracts 1, 7, 8 and 10 (Figure 11). This suppressive effect of NS on PGE2 release from human monocyte cells is not reported in the literature. This suppressive effect of PGE2 release from monocyte cells may be considered a favourable effect due to the general systemic pro-inflammatory properties of PGE2 and the enhancement effect of PGE2 on Th2 and IgE responses.

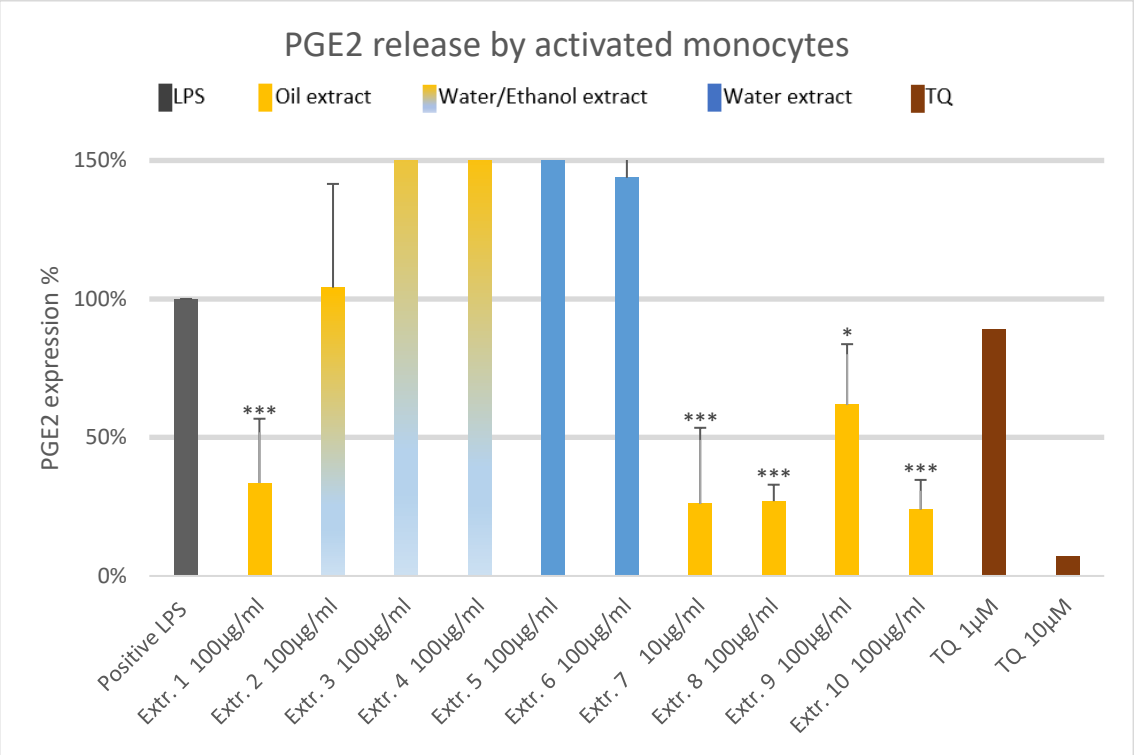
The marked inhibitory activity of the oily extracts may be attributed to the high TQ content particularly in the oily extracts 7 and 10, which showed an inhibitory action at 10 µM of TQ.

Figure 10. The effect of *Nigella sativa* preparations on interleukin-6 production by LPS-induced monocytes (n=3).



Significant effects are indicated by asterisks (**P< 0.01, ***P<0.001).

Figure 11. The effect of *Nigella sativa* preparations on prostaglandin E2 production by LPS-induced monocytes (n=3).



Significant effects are indicated by asterisks (*P<0.05, ***P<0.001).

3.5.3 Effect of *Nigella sativa* preparations on inflammatory mediators from A549 lung epithelial cells

There was no significant inhibition of IL-6 release by all NS preparations in IL-1β-induced A549 lung epithelial cells (Figure 12). The concentration of 100µg/ml for most NS preparations was used according to the findings of the previous experiment on T-lymphocyte cells.

A previous study demonstrated that NS fixed oil significantly suppressed mRNA IL-6 in whole lung tissue of rats (Shahzad *et al.*, 2009). Moreover, TQ suppressed the expression of IL-6 from *M. tuberculosis* infected A549 cells (Mahmud *et al.*, 2017). The inhibitory effects of TQ were also shown on IL-6 expression from Human proximal tubular epithelial cells stimulated with advanced glycation end products (Sayed and Morcos, 2007). Studies have found that

suppression of IL-6 may possess beneficial anti-inflammatory effects in bronchial epithelial cells of asthmatic patients (Mattoli, Marini and Fasoli, 1992). Unlike beta-agonists (a conventional treatment in asthma), which can enhance IL-6 expression in the lung, NS in this experiment had a mild inhibitory effect of on IL-6 release like steroids (Edwards *et al.*, 2007).

Conversely, the release of PGE2 was increased markedly (by more than 150%) with extracts 1, 2, 6, 7, 8, and 10 (Figure 13). This stimulatory effect of NS on PGE2 release from A549 human lung epithelial cells is not reported in the literature. However, such finding of increased PGE2 release was shown in perfused guinea-pig lung preparation used NS oil (Saleh, ElDenshary and Mahran, 2012). Interestingly, the increased PGE2 release by TQ-rich NS preparations may have an encouraging effect in the context of asthma as several studies showed that PGE2 may possess beneficial local protective and bronchodilatory effects in the airways of the lung (3.2).

Figure 12. The effect of *Nigella sativa* preparations on interleukin-6 production by interleukin-1β-induced A549 cells (n=3).

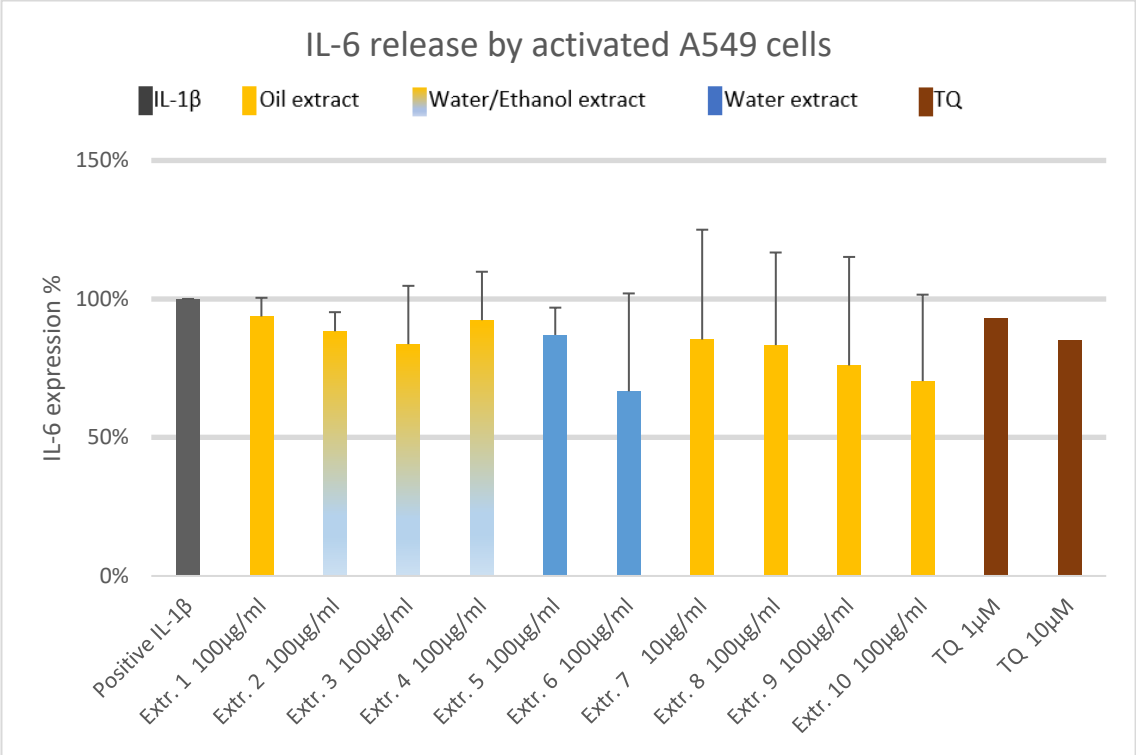
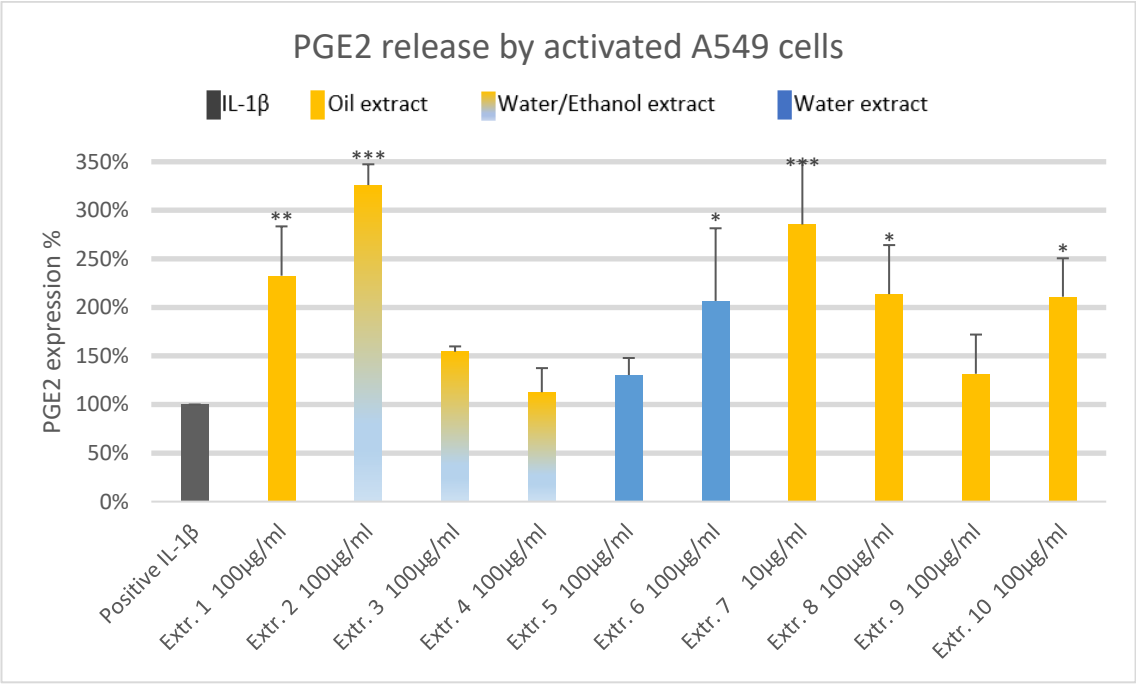


Figure 13. The effect of *Nigella sativa* preparations on prostaglandin E2 production by interleukin-1β-induced A549 cells (n=3).



Significant effects are indicated by asterisks (*P<0.05, **P< 0.01, ***P<0.001).

3.5.4 Summary of the activity of *Nigella sativa* preparations in preclinical *in vitro* models.

The oily extracts 7 (a supercritical fluid extract) and 10 (a commercial oil product by Marnys, Spain), which are characterised by the highest level of TQ, showed the most remarkable effect on suppressing the inflammatory mediators IL-2, IL-6 and PGE2 from human T-lymphocyte cells. In addition, these two extracts showed a marked suppression of IL-6 and PGE2 release from human monocytes. The other oily extracts 1, 2 and 8 had also shown a suppression of inflammatory mediators in T-lymphocyte and monocytes but in a limited number of mediators compared to extracts 7 and 10. On the other hand, the oily extracts 1, 2, 7, 8, 10 had a remarkable enhancement effect on the release of PGE2 from A549 human lung epithelial cells (Table 8).

Therefore, extracts 7 and 10 showed the most potent and favourable effects in the context of asthma, due to their anti-inflammatory actions by limiting the release of inflammatory mediators in human immune cells (T-lymphocyte and monocyte cells) as well as their possible bronchodilatory effect by enhancement of PGE2 release in A549 human lung epithelial cells.

Table 8. Summary of the most active *Nigella sativa* preparations by more than 50% suppression or 150% enhancement of inflammatory mediators in cellular *in vitro* models of inflammation (n=3).

Sample	T-lymphocyte cells			Monocytes		A549
	IL-6	IL-2	PGE2	IL-6	PGE2	PGE2
Extract 1	↓ ≥ 50%	↓ ≥ 50%		↓ ≥ 50%	↓ ≥ 50%	↑ ≥ 150%
Extract 2		↓ ≥ 50%				↑ ≥ 150%
Extract 7	↓ ≥ 50%	↓ ≥ 50%	↓ ≥ 50%	↓ ≥ 50%	↓ ≥ 50%	↑ ≥ 150%
Extract 8		↓ ≥ 50%		↓ ≥ 50%	↓ ≥ 50%	↑ ≥ 150%
Extract 10	↓ ≥ 50%	↓ ≥ 50%	↓ ≥ 50%	↓ ≥ 50%	↓ ≥ 50%	↑ ≥ 150%

3.5.5 Effect of *Nigella sativa* preparations on p38 mitogen-activated protein kinase signalling pathway in A549 cells

Investigation of the NS activity on p38 mitogen-activated protein kinase (MAPK) inflammatory signalling pathway inhibition was done to explore a possible mechanism of NS anti-inflammatory activity. In IL-1β-induced A549 cells, extracts 8, and 10 showed a remarkable inhibition of phospho-p38 protein expression, but this was not correlated with TQ (Figure 14 and 15). The results of phospho-p38 were normalised with total p38 protein for verification (Figure 15). Additional experimental repeats were done with the most active NS preparations (extract 8 and 10) to verify their activity on p38 MAPK signalling pathway inhibition (Figure 16).

This finding of p38 MAPK signalling pathway inhibition in A549 cells was not reported in the literature. However, TQ blocked the induction of p38 MAPK phosphorylation in LPS activated murine macrophage-like RAW264.7 cells and inhibited LPS mediated upregulation of phospho-p38 in mouse peritoneal primary macrophages (Hossen *et al.*, 2017). Also, TQ inhibited TNF-α-induced phospho-p38 expression in human rheumatoid arthritis synovial fibroblasts (Umar *et al.*, 2015). In contrast, TQ caused a dose-dependent increase of phospho-p38 expression in rabbit articular chondrocytes (Goldstein and Gabriel, 2005). The anti-tumour effect of TQ was found to be mediated through induction of p38 in breast cancer cell line (Woo *et al.*, 2013). This contradicting effect of TQ on p38 signalling pathway might be due to differences in cell type.

There was a mild suppressive effect by TQ and a remarkable downregulation by the oily extracts 8 and 10 on the expression of phospho-p38 in A549 cells. This may indicate that other compounds in the TQ-rich extracts 8

and 10 may play an active role in inhibition of p38 MAPK signalling pathway rather than TQ alone. This may indicate that the mechanism of the anti-inflammatory effect of NS is possibly through the inhibition of p38 MAPK signalling pathway. Thus, NS may act as a p38 inhibitor which has possible therapeutic benefits in inflammatory conditions (Goldstein and Gabriel, 2005). In future, further investigations are encouraged to elucidate another possible mechanism of anti-inflammatory action through other pathways.

Figure 14. The preliminary inhibitory activity of *Nigella sativa* preparations on phospho-p38 expression in interleukin-1β-induced A549 cells (n=1).

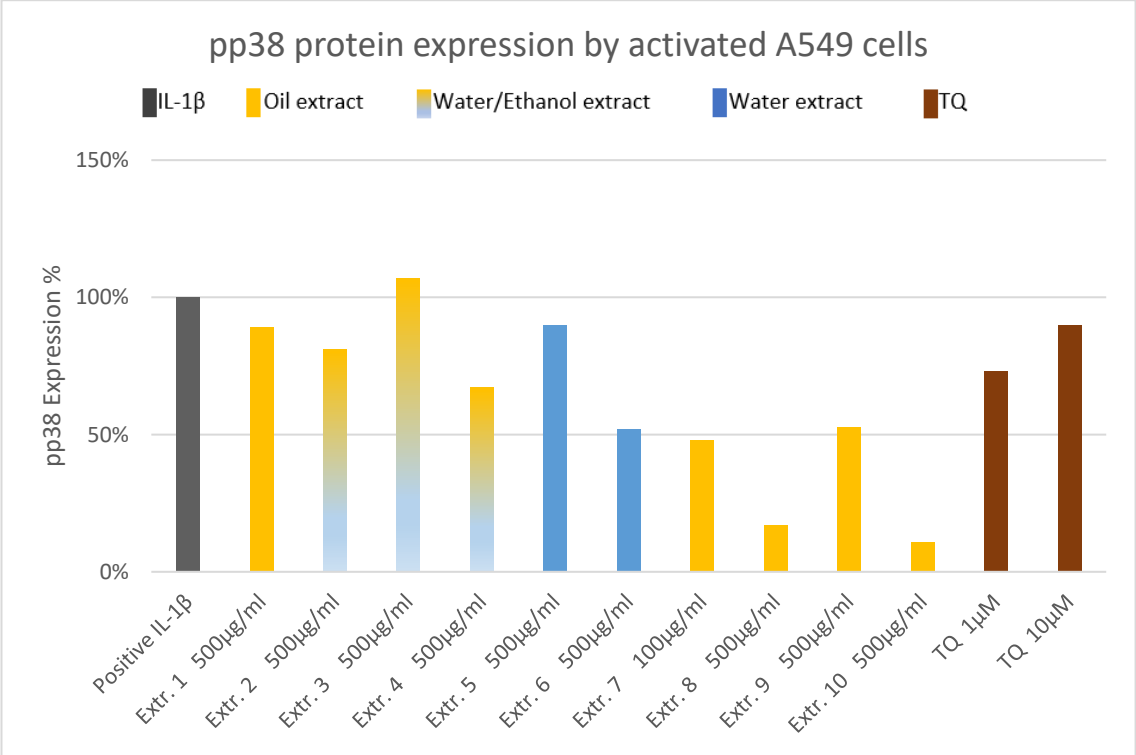


Figure 15. Western blot bands of the effect of *Nigella sativa* preparations on p38 signalling pathway in interleukin-1β-induced A549 cells. The phospho-p38 results was normalised by total p38 results (n=1).

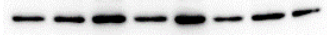
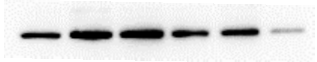
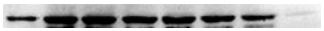
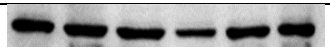
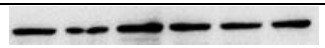
	Extracts 1-3	Extracts 4-5	Extracts 6-8	Extracts 9-10	Extract 11 and TQ
Phospho-p38					
Total p38	 1 2 3 4 5 6 7 8	 9 10 11 12 13 14	 15 16 17 18 19 20 21 22	 23 24 25 26 27 28	 29 30 31 32 33 34 35
Numbers identification	1. -ve: DMSO 2. +ve: IL-1β 10 U/ml 3. Extr. 1 100 µg/ml 4. Extr. 1 500 µg/ml 5. Extr. 2 100 µg/ml 6. Extr. 2 500 µg/ml 7. Extr. 3 100 µg/ml 8. Extr. 3 500 µg/ml	9. -ve: DMSO 10. +ve: IL-1β 10 U/ml 11. Extr. 4 100 µg/ml 12. Extr. 4 500 µg/ml 13. Extr. 5 100 µg/ml 14. Extr. 5 500 µg/ml	15. -ve: DMSO 16. +ve: IL-1β 10 U/ml 17. Extr. 6 100 µg/ml 18. Extr. 6 500 µg/ml 19. Extr. 7 100 µg/ml 20. Extr. 7 500 µg/ml 21. Extr. 8 100 µg/ml 22. Extr. 8 500 µg/ml	23. -ve: DMSO 24. +ve: IL-1β 10 U/ml 25. Extr. 9 100 µg/ml 26. Extr. 9 500 µg/ml 27. Extr. 10 100 µg/ml 28. Extr. 10 500 µg/ml	29. -ve: DMSO 30. +ve: IL-1β 10 U/ml 31. Extr. 11 100 µg/ml 32. Extr. 11 500 µg/ml 33. TQ 1 1 µM 34. TQ 1 5 µM 35. TQ 1 10 µM

Figure 16. The effect of selected *Nigella sativa* preparations on phospho-p38 signalling pathway inhibition in activated A549 cells (n=2).

Significant effects are indicated by an asterisk (***P<0.001).

3.6 Conclusion

The results of this chapter, provided direct *in vitro* evidence that the release of some cytokines with inflammatory properties (IL-2, IL-6, PGE2) from human immune cells (including T-lymphocytes and monocytes), were suppressed by the oily NS preparations. These cytokines are normally upregulated in patients with asthma, and such suppressive effect by NS may mimic corticosteroids in inducing remission of asthmatic symptoms.

However, there was a variability in the anti-inflammatory / immunomodulatory effects of different NS preparations on the expression of inflammatory mediators (IL-6, IL-2, PGE2) in human T-lymphocyte, monocyte and A549 lung epithelial cells. Apparently, the oily preparations (especially extract 7 and 10) appeared to be among the most potent active extracts by inhibiting inflammatory mediators in more than one type of human cells. There was a positive relationship between the level of TQ and inhibition of inflammatory mediators in T-lymphocyte and monocyte cells. Interestingly, the oily preparations (including extracts 7 and 10) showed an increase in PGE2 production in A549 cells, which may possess a favourable effect in the context of asthma. Also, suppression of the inflammatory p38 signalling pathway as a possible mechanism of anti-inflammatory action was discovered in this study, but this inhibition was not correlated with TQ itself but inhibited by TQ-rich extracts (such as extract 10). In the end, extracts 7 and 10 appeared to be the most potent preparations recommended for the clinical trial.

4 Chapter 4. The clinical trial*

4.1 Introduction

Due to the high incidence of asthma, suboptimal control of asthma, and poor adherence to asthma medications, there is a need for novel strategies to improve asthma control (Chapter 1). Empirical evidence suggests potential benefits of NS in pre-clinical asthma models and preliminary clinical trials (Chapter 1). However, the current clinical trials of NS in asthma had limitations such as low standard of trial design, small sample size, limited investigated outcomes, and poor chemical characterisation of the NS preparation used in most trials (Chapter 1).

Therefore, we intended to investigate the effects of NS oil supplementation on asthma outcomes and biomarkers in a phase-II randomised double-blind placebo-controlled clinical trial (RDBPCT) with a higher number of sample size and biomarkers. Exclusively, the investigational product in this trial was chemically and pharmacologically characterised preparation of NS. Based on the results of Chapter 2 and 3, extracts 7 and 10 appeared to be the most active NS preparations, but extract 10 was used in the clinical trial due to the logistical suitability as discussed in this chapter. This trial was important to assess the efficacy and safety of NS oil supplementation for patients with asthma in a well-designed study. The clinical trial design, implementation plan, ethical and logistical considerations, and results were discussed in details in this chapter.

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*Part of the results in this chapter is published;

Koshak, A., Wei, L., Koshak, E., Wali, S., Alamoudi, O., Demerdash, A., Qutub, M., Pushparaj, P. N. and Heinrich, M. (2017) '*Nigella sativa* Supplementation Improves Asthma Control and Biomarkers: A Randomized, Double-Blind, Placebo-Controlled Trial', *Phytotherapy Research*. doi: 10.1002/ptr.5761.

4.2 Specific objectives

- To develop the design and implementation plan and highlight the ethical considerations of the NS clinical trial in patients with asthma.
- To study the efficacy of NS supplementation in patients with asthma, using a properly designed clinical trial.

4.3 Material and Methods

4.3.1 Clinical trial design

The design and outcome choice of the clinical trial (Table 9) was developed according to the guidance on clinical investigation of medicinal products for the treatment of asthma (European Medicines Agency, 2013), Asthma Outcome Measures (Shen, Johnston and Hays, 2011), and Asthma outcomes: Biomarkers (Szefler *et al.*, 2012). The design has been discussed and developed along with three co-investigators at the study site (King Abdulaziz University Hospital “KAUH”, Jeddah, Saudi Arabia) according to the best applicable practice in the context of the planned study; Prof Emad Koshak (consultant in allergy, asthma and immunology), Prof Siraj Wali (consultant in pulmonary and sleep medicine), and Prof Omer Alamoudi (consultant in pulmonary medicine). Also, the secondary supervisor at UCL Dr Li Wei (epidemiology and medical statistics) and the UCL Research Ethics Committee further reviewed the trial design.

Table 9. Summary of the clinical trial design of *Nigella sativa* oil supplementation on asthma inflammation and outcomes

Title	Potential Benefits of *Nigella sativa* Oil Supplementation on Asthma Inflammation and Outcomes
Phase	Phase II clinical trial
UCL Number	UCL REC project ID number 6419/002
Online registration	ClinicalTrials.gov: NCT02407262
Research type	Prospective randomised (1:1) double-blind placebo-controlled parallel-group clinical trial (RDBPCT).
Sample size	80 participants
Age range	18-65 years of age
Study locations	King Abdulaziz University Hospital (KAUH) including Allergy clinic, pulmonology clinic, and family medicine clinic.
Investigational product	Marnys® Cuminmar (*Nigella sativa* oil, 500mg). Dose: 500mg twice daily for 4 weeks.
Placebo	Olive oil (pharmaceutical excipient grade), 500mg capsules produced by Marnys. Dose: 500mg twice daily for 4 weeks.
Treatment duration	4 weeks

Primary outcomes	-Asthma Control Test (ACT)
Secondary outcomes	-Pulmonary function test (PFT) -Absolute blood eosinophil count -Blood biomarkers of airway inflammation
Primary safety endpoint	Occurrence of any severe side effect at any point during the intervention period
Inclusion criteria	-Adult male/female (age 18-65 years) -Asthma diagnosis according to the GINA guidelines (Global Initiative for Asthma, 2017) -Asthma symptoms not fully controlled based on ACT score from 5 to 24 -No severe asthma exacerbation in the last four weeks -Able to obtain consent
Exclusion criteria	-Patients with serious co-morbid conditions such as; cancer, renal, hepatic, cardiovascular, gastrointestinal diseases, mental health conditions and respiratory disorders. -Smoking history -Pregnant women

	-Currently taking any form of NS -Known history of hypersensitivity to NS or olive oil -Taking medications that may interact with NS or olive oil; oral corticosteroids, anticoagulant, antiplatelet, CNS depressants, and immunosuppressant drugs.

4.3.1.1 Sample size estimation

The sample size was estimated based on the expected improvement of an average primary outcome (ACT) from 17 to 20 (Nathan *et al.*, 2004). Using nQuery Advisor software for power calculation, it is estimated to have at least 40 patients in each group of both treatment and placebo based on the following parameters:

- Significance level: 0.05
- Two-sided independent t-test
- Mean1 (average ACT of not well-controlled population): 17
- Mean2 (estimated ACT): 20
- Difference in mean (estimated effect): 3
- Common SD: 4.7
- Effect size: 0.638
- Power: 80 %

4.3.1.2 Interventions and compliance check

In the treatment group, the interventional product was softgel capsules of *Nigella sativa* oil (Figure 17). It was characterised by **0.7% thymoquinone** at our

labs (UCL School of Pharmacy, London, UK) by High Performance Liquid Chromatography (HPLC). It was produced according to pharmaceutical GMP standards by Marnys® (Cartagena, Spain; brand name: CUMINMAR; batch number: L885) and is a licensed product of the Saudi Food and Drug Authority (SFDA) as a herbal medicinal product. The placebo group received similar capsules of virgin olive oil (produced in identical appearance by Marnys®, batch number: M398). Both groups received a dose of one capsule (500mg oil) twice daily for four weeks. Patient compliance with interventions was determined by counting the remaining capsules at the final visit.

Figure 17. The investigational products (*Nigella sativa* oil capsules as the treatment and olive oil capsules as the placebo)

4.3.1.3 Blinding and randomisation

Both the investigator and the patients were blinded to treatment or placebo groups. An external medical scientist created a randomisation list (with a unique code for each trial medication) using Sealed Envelope Limited online tool in random permuted blocks of sizes 2 and 4 (Sealed Envelope Ltd, 2016). These

mixed block sizes were used to reduce the selection bias and achieve a better balance in the allocation of participants. Co-investigators enrolled participants from their clinics and the principal investigator allocated patients to their concealed study medications. The packaging label of study medications contained the randomisation code, study title, expiration date, date of packaging, and contact information.

4.3.1.4 Participant recruitment and obtaining consent

Adult asthmatic patients were invited to the trial from the study clinics of respiratory, allergy, and family medicine or by contacting registered asthmatic patients within the hospital's directory of KAUH. After a patient accepted to participate and was checked for the fulfilment of eligibility criteria, the investigator introduced and discussed the informed consent sheet with the patient. This informed consent included a description of the study procedures, risks and benefits of participation. Also, the patient was informed that participation in this study is voluntary and he/she have the right to withdraw at any time without giving a reason. Afterwards, the patient was given enough time to decide on participation. Once the patient decided to participate, he/she was asked to sign a consent form along with the researcher signature and handled a copy of the consent. The original copy was kept in a safe and locked place.

4.3.1.5 Primary outcome

The primary outcome was the ACT score. This was chosen because of the current asthma guidelines, including the Global Initiative for Asthma, recognised asthma control as a principal component for asthma management (Global Initiative for Asthma, 2017). Traditionally, asthma practice guidelines were

focused on optimising lung function, minimising symptoms and reducing exacerbations. Pulmonary function was regarded as the primary outcome in previous guidelines, until the discovery of a poor correlation between lung function, inflammation and symptoms. Clinical practices and trials have focused increasingly on assessing asthma control (Shen, Johnston and Hays, 2011). The Global Initiative for Asthma guidelines recommends using one of the numerical "asthma control" tools such as the Asthma Control Test (ACT) and the Asthma Control Questionnaire (ACQ) for scoring the level of asthma control and patients' progress (Global Initiative for Asthma, 2017). The ACT is one of the best validated instrument to measure asthma control (Cloutier et al., 2012). It is commonly used by the Saudi Initiative for Asthma (SINA) Group and considered to be a favourable option because it is short and easy to fill by the patient (Reddel *et al.*, 2009). An Arabic validated version of the ACT (Figure 18) was used in this study (Lababidi, Hijaoui and Zarzour, 2008).

The ACT consists of five questions with a total score of 5-25. Each question (Q1-Q5) scaled from 1 to 5 (Nathan et al., 2004). A total score of 25 is regarded as complete control, and a change of 3 points is considered to be clinically important (Global Initiative for Asthma, 2017). The following are the five questions of the ACT:

1. Q1=In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school or home?
2. Q2=During the past 4 weeks, how often have you had shortness of breath?
3. Q3=During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?

4. Q4=During the past 4 weeks, how often have you used your rescue inhaler or nebuliser medication?
5. Q5=How would you rate your asthma control during the past 4 weeks?

Figure 18. The Arabic version of Asthma Control Test used in the clinical trial

اختبار التحكم في الربو

- الاختبار التالي يمكن أن يساعد الأشخاص المصابين بالربو (عمر ١٢ سنة فأكثر) في تقييم تحكمهم في الربو.
- برجاء رسم دائرة حول الدرجة المناسبة لكل سؤال، جميعها خمسة أسئلة.
- يمكنك أن تحسب مجموع درجاتك في اختبار التحكم في الربو بجمع درجاتك عن كل إجابة.
- تأكد من مراجعة نتائجك مع طبيبك.

اعرف درجة الربو عندك

الخطوة الأولى:

ارسم دائرة حول درجتك عن كل سؤال واكتب نفس الرقم في الخانة المحددة على اليسار.

برجاء الإجابة على الأسئلة بأصدق ما يمكن. فإن هذا سيساعدك أنت وطبيبك على وضع تصور حقيقي للربو عندك.

الدرجة

١. خلال ال ٤ أسابيع الماضية، كم من الوقت منعك الربو من إنجاز القدر الكافي في العمل، أو الدراسة، أو المنزل؟

١ كل الوقت ٢ معظم الوقت ٣ بعض الوقت ٤ قليل من الوقت ٥ لم يحدث أبداً

٢. خلال ال ٤ أسابيع الماضية، كم مرة حدث لك ضيق تنفس؟

١ أكثر من مرة واحدة في اليوم ٢ مرة واحدة في اليوم ٣ ٣ إلى ٦ مرات في الأسبوع
٤ مرة أو مرتين في الأسبوع ٥ لم يحدث أبداً

٣. خلال ال ٤ أسابيع الماضية، كم مرة أيقظتك أعراض الربو (الصفير، السعال، ضيق تنفس، ضيق صدر أو ألم في الصدر) أثناء الليل أو في وقت أبكر من العادة في الصباح؟

١ ٤ ليالي أو أكثر في الأسبوع ٢ ٢ إلى ٣ ليالي في الأسبوع ٣ مرة واحدة في الأسبوع
٤ مرة أو مرتين ٥ لم يحدث أبداً

٤. خلال ال ٤ أسابيع الماضية، كم مرة استخدمت الدواء المسعف عن طريق البخاخ أو جهاز الرذاذ (مثل سلبيوتامول)؟

١ ٣ مرات أو أكثر في اليوم ٢ مرة واحدة أو مرتين في اليوم ٣ ٢ أو ٣ مرات في الأسبوع
٤ مرة واحدة في الأسبوع أو أقل ٥ لم يحدث أبداً

٥. ما هو تقييمك للتحكم في الربو عندك خلال ال ٤ أسابيع الماضية؟

١ لايوجد تحكم أبداً ٢ تحكم ضعيف ٣ متحكم إلى حد ما ٤ تحكم جيد ٥ تحكم تام

المجموع

4.3.1.6 Secondary and exploratory outcomes

There are several outcomes can be used in clinical trials of asthma. Based on the feasibility and availability as well as co-investigators recommendations for the choice of other than primary outcomes, it was agreed to use the following:

1- Secondary outcomes:
   a. Pulmonary function (FEV1 as % predicted).
   b. Absolute blood eosinophil count.
2- Exploratory outcomes:
   a. Individual questions of ACT
   b. Pulmonary function (PEF and FEF25-75% as % predicted)
   c. Total serum Immunoglobulin E (IgE)
   d. Serum cytokines

4.3.1.7 Pulmonary Function Test

Pulmonary function test (PFT) was performed using the “Easy on-PC” spirometer (ndd Medical Technologies, Inc., Switzerland) as shown in Figure 19. The pulmonary function for each patient was measured in triplicate, and the best reading was recorded. Spirometry is used for pulmonary function testing since it is considered one of the important tools to measure asthma control (Shen, Johnston and Hays, 2011). It provides an objective measure of airway limitation caused by smooth muscles contraction or structural abnormalities (Shen, Johnston and Hays, 2011). However, due to its poor association with asthma symptoms, it may be used to provide complementary information on asthma control (Shen, Johnston and Hays, 2011).

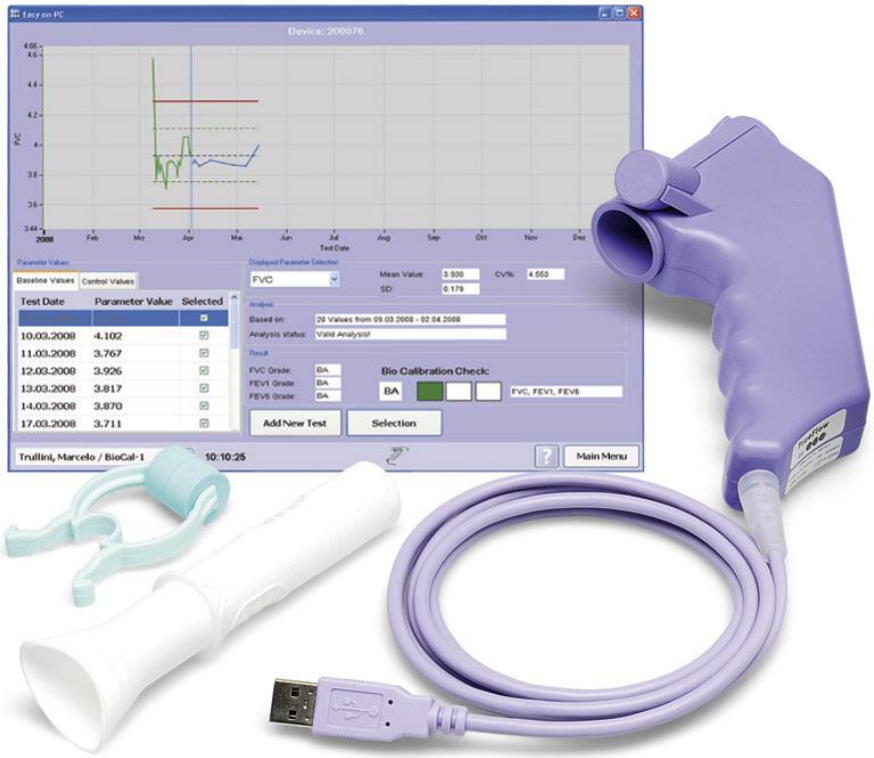
The forced expiratory volume in 1 second (FEV1) is the amount of air that can be forcefully blown out in a second (Thillai and Hattotuwa, 2012). It used to

predict airway obstruction as well as response to the bronchodilatory effect of asthma medications (Thillai and Hattotuwa, 2012). It is considered the most appropriate parameter of spirometry for asthma control monitoring (European Medicines Agency, 2013). Predicted FEV1 is derived from FEV1 when baselines characteristics (gender, race, age and height) is considered. The normal level of predicted FEV1% is >80% (British Thoracic Society/Scottish Intercollegiate Guidelines Network, 2016). FEV1 starts to improve within days in patients taking regular corticosteroids therapy and the minimal important difference for improvement in predicted FEV1% is about 10% (Global Initiative for Asthma, 2017).

PEF (Peak expiratory flow) is a parameter of the maximal flow achieved during the maximally forced expiration initiated at full inspiration (Thillai and Hattotuwa, 2012). It may indicate an airway obstruction, but it is less important than FEV1 (Shen, Johnston and Hays, 2011). PEF has no advantage over FEV1 in reproducibility, has limited reference values for many populations, and may underestimate the level of airway obstruction in patients with airway remodelling (Reddel *et al.*, 2009). The normal level of predicted PEF% is >80% (British Thoracic Society/Scottish Intercollegiate Guidelines Network, 2016).

FEF 25-75% (Forced Expiratory Flow between 25-75% of pulmonary volume) is a value of average flow over the middle portion of the flow-volume curve (Thillai and Hattotuwa, 2012). It provides important information regarding small airway obstruction (Thillai and Hattotuwa, 2012).

Figure 19. Easy on-PC spirometer device for pulmonary function test.



4.3.1.8 Blood biomarkers

Biomarkers of airway inflammation provide additional objective measurements in asthma clinical trials (Shen, Johnston and Hays, 2011). In this study, total blood eosinophil count and total serum IgE were used as secondary outcome measures. Changes in multiple serum inflammatory mediators were also explored in this study.

Eosinophil cells play a major role in asthma inflammation, and blood eosinophil count is considered to be a vital biomarker in asthma trials (Szefler *et al.*, 2012). Several studies found a positive relationship between blood eosinophil count and the risk of exacerbations (Hambleton and Pavord, 2016). Total peripheral eosinophil count was positively correlated with asthma severity level in adult asthmatics (Koshak and Alamoudi, 1999). High blood eosinophil level (≥250 cells/uL) was associated with higher bronchial hyperresponsiveness, poor

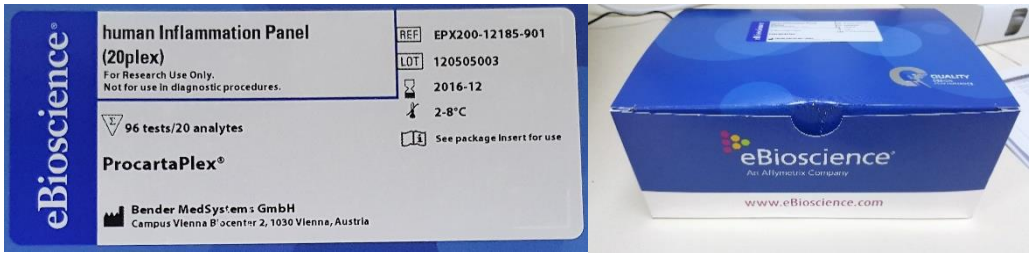
pulmonary function and higher total serum IgE level in the analysis of 474 asthmatics (Nadif *et al.*, 2016). Total serum Immunoglobulin E (IgE) is associated with asthma. It is considered to be a complementary biomarker in allergic asthma and as an outcome in interventional studies (Szefler *et al.*, 2012). Since asthma is regulated by multiple inflammatory cytokines and early studies pointed out its possible association with asthma control, we explored the changes in serum inflammatory cytokines and other mediators (Akiki *et al.*, 2017).

The blood analysis was carried out using the Celldyn-3500 counter (Abbot) at KAUH's haematology laboratory. This flow cytometry counter provided differential white blood cell counts automatically and was used to measure blood eosinophil count. Total serum IgE was measured with the radioimmunofluorescent CAP system (Phadia, Sweden) at the clinical immunology laboratory of KAUH.

4.3.1.9 Serum inflammatory mediators

The serum inflammatory mediators were investigated as an exploratory outcome using the Multiplex system of ProcartaPlex Human Inflammation Panel 20 plex kit (Affymetrix eBioscience, UK) and Luminex® MAGPIX® system reader. The kit was pre-set to measure 20 different inflammatory mediators (including Cytokines, Chemokines, Growth Factors) in the serum of patients. The explored mediators were TNF-α, IFN-α, IFN-γ, IL-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-13, IL-17A/CTLA-8, IP-10, MCP-1, MIP-1α, MIP-1β, P-Selectin, E-Selectin, GM-CSF, ICAM-1. The kits (Figure 20) were purchased in the UK and transferred (temperature controlled) to the study site to analyse inflammatory mediators in patients' serum samples.

Figure 20. ProcartaPlex Human Inflammation Panel 20 plex kit was used for serum inflammatory mediators' analysis.



4.3.1.10 Trial procedure

This study involves two visits. The first visit is for patients' enrolment and assessment of baseline characteristics and outcomes. The second visit is for the assessment of intervention outcomes after 4 weeks.

- **Visit 1:** After identification of eligible patients and signing the consent form, the following was conducted:
    1. **Recording of patients' baseline characteristics and outcomes**: age, body weight, body mass index, pulse, blood pressure, symptoms, medications, concomitant therapies, and scores of ACT and PFT.
    2. **Blood samples collection:** participants were sent to the phlebotomy unit at the hospital to collect blood samples for the analysis of secondary outcomes.
    3. **Randomisation and Allocation:** Concealed medications were dispensed to participants in a random and blind manner according to randomisation table with unique codes recorded on each investigational product package. Both the investigator and the patient are blinded to treatment or placebo groups.

- **Visit 2 (after 4 weeks):**

The same outcomes measured at baseline were assessed after four weeks of treatment to measure the effects of NS oil supplementation. Also, a blood sample was collected to detect the change in blood biomarkers. Medication compliance was evaluated, and the appearance of any adverse event was checked after the first week of treatment as well as at the end of treatment.

4.3.1.11 Blood and serum collection

Venous blood samples were collected twice (at the baseline and follow-up visit) for the assessment of the secondary outcomes (absolute peripheral blood eosinophils count, total serum IgE and multiple serum inflammatory mediators). 4ml fresh blood was collected in ordinary blood plain tube for differential WBC. Also, 6 ml blood was collected in gel tubes (BD vacutainer 367955 tubes with gel, BD Plymouth, UK) and centrifuged (by Eppendorf centrifuge 5810 at 3500rpm for 5min) to yield about 2ml serum which was used for the analysis of total IgE and inflammatory mediators.

4.3.1.12 Statistical analysis

IBM SPSS v.23 statistical software was used for statistical analysis and interpretation of the clinical trial results. The normality of data was assessed visually by Q-Q plot. Normally distributed data were represented as mean (SD), and independent t-test was used to compare the means of the outcomes between two groups. However, non-normal data was represented as median (IQR) and Mann–Whitney U test was used to compare the medians of outcomes between both groups. Intention-to-treat analysis was used for the primary outcome. Multiple regression-substitution was used for imputing primary outcome missing data, considering the baseline values. The level of significance for the primary

and secondary outcomes was $p<0.05$. The level of significance for the exploratory outcomes was $p<0.01$. Subgroup group analysis was planned for patients with abnormal pulmonary function (FEV1 as % predicted < 80%) and patients with high blood eosinophils count (>300 Cells/uL).

4.3.2 Ethical and logistical considerations

4.3.2.1 Trial approvals and registrations

Ethical approval was granted by both the University College London (ref. 6419/002, Figure 21) and the study site's ethical committee of King Abdulaziz University Hospital (ref. 95-15, Figure 22). Also, the study was approved by the national regulatory framework of clinical trials in Saudi Arabia "Saudi FDA" (ref. 15051902). Also, the Saudi Ministry of Health approved the study (ref. 00637).

The trial was registered online at www.clinicaltrials.gov in April 2015 (ref. NCT02407262, Figure 23), the Saudi Clinical Trials Registry (ref. 15051902, Figure 24). Ultimately, the trial became available on the National Health Service website (NHS, Figure 25) and the World Health Organisation website (WHO, Figure 26) via the international clinical trials registry platform. This was done to make the study available to the public and allow publication of the trial results.

Figure 21. Approval letter of University College London' ethics.

UCL RESEARCH ETHICS COMMITTEE
ACADEMIC SERVICES

23 April 2015

Professor Michael Heinrich
School of Pharmacy
UCL

Dear Professor Heinrich

Notification of Ethical Approval
Project ID: 6419/002: Potential benefits of Black Seed Oil Supplementation on Asthma Inflammation and Outcomes

Further to your satisfactory response to the committee's comments, I am pleased to confirm in my capacity as Chair of the UCL Research Ethics Committee (REC) that your study has been approved by the UCL REC for the duration of the project i.e. until April 2016.

Approval is also subject to the following conditions:

1. You must seek Chair's approval for proposed amendments to the research for which this approval has been given. Ethical approval is specific to this project and must not be treated as applicable to research of a similar nature. Each research project is reviewed separately and if there are significant changes to the research protocol you should seek confirmation of continued ethical approval by completing the 'Amendment Approval Request Form':

2. It is your responsibility to report to the Committee any unanticipated problems or adverse events involving risks to participants or others. Both non-serious and serious adverse events must be reported.

   Reporting Non-Serious Adverse Events
   For non-serious adverse events you will need to inform Helen Dougal, Ethics Committee Administrator (ethics@ucl.ac.uk), within ten days of an adverse incident occurring and provide a full written report that should include any amendments to the participant information sheet and study protocol. The Chair or Vice-Chair of the Ethics Committee will confirm that the incident is non-serious and report to the Committee at the next meeting. The final view of the Committee will be communicated to you.

   Reporting Serious Adverse Events
   The Ethics Committee should be notified of all serious adverse events via the Ethics Committee Administrator immediately the incident occurs. Where the adverse incident is unexpected and serious, the Chair or Vice-Chair will decide whether the study should be terminated pending the opinion of an independent expert. The adverse event will be considered at the next Committee meeting and a decision will be made on the need to change the information leaflet and/or study protocol.

On completion of the research you must submit a brief report (a maximum of two sides of A4) of your findings/concluding comments to the Committee, which includes in particular issues relating to the ethical implications of the research.

With best wishes for the research.

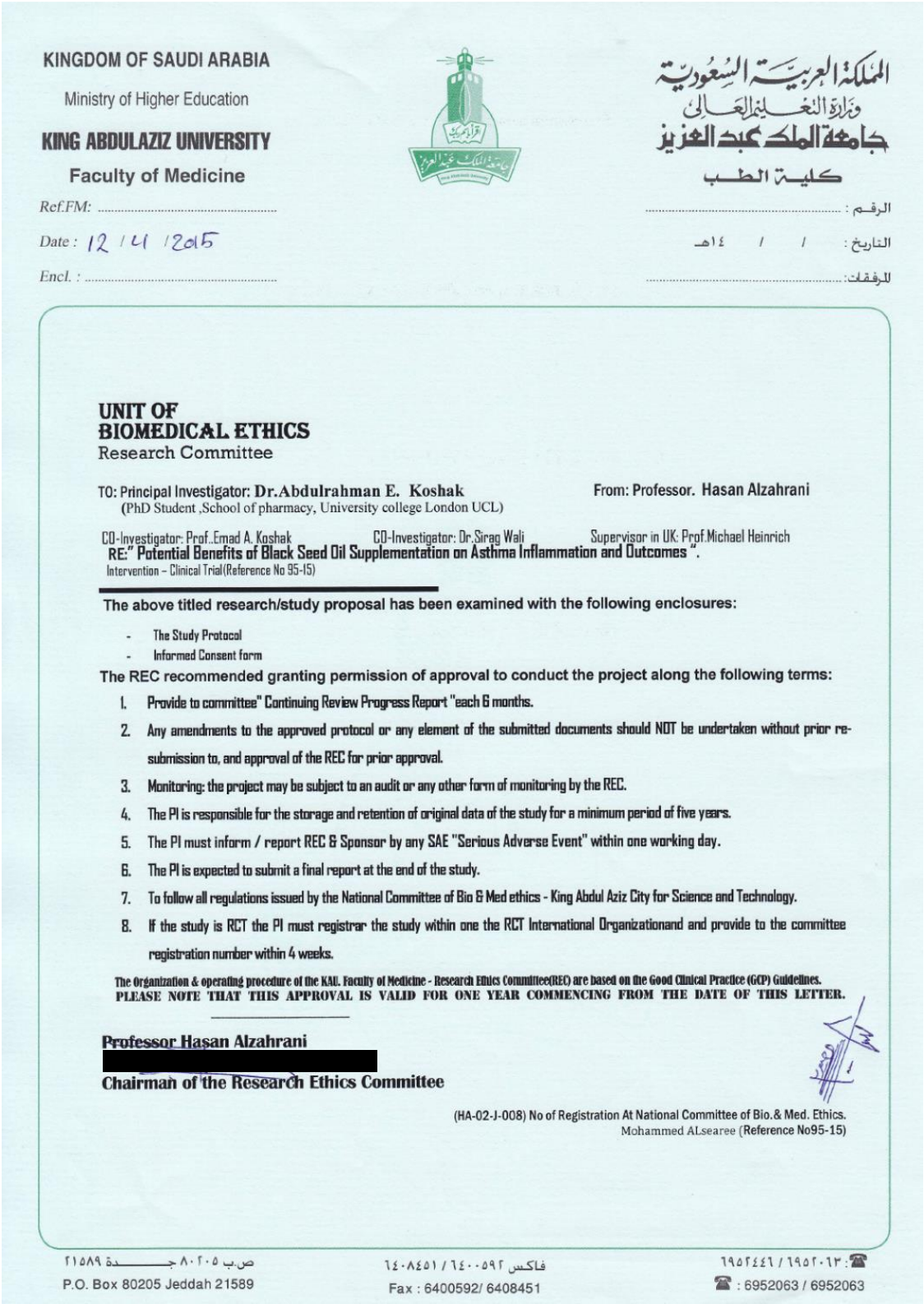
Yours sincerely

[redacted]

Professor John Foreman
Chair of the UCL Research Ethics Committee

Cc: Abdulrahman Koshak, Applicant

Figure 22. Approval letter of King Abdulaziz University's ethics committee.



KINGDOM OF SAUDI ARABIA
Ministry of Higher Education
KING ABDULAZIZ UNIVERSITY
Faculty of Medicine

المملكة العربية السعودية
وزارة التعليم العالي
جامعة الملك عبد العزيز
كلية الطب

Ref.FM: .....
Date : 12 / 4 / 2015
Encl. : .....

الرقم : .....
التاريخ : / / ١٤هـ
المرفقات: .....

UNIT OF
BIOMEDICAL ETHICS
Research Committee

TO: Principal Investigator: **Dr.Abdulrahman E. Koshak** From: Professor. Hasan Alzahrani
(PhD Student ,School of pharmacy, University college London UCL)

CO-Investigator: Prof..Emad A. Koshak CO-Investigator: Dr.Sirag Wali Supervisor in UK: Prof.Michael Heinrich

RE:" Potential Benefits of Black Seed Oil Supplementation on Asthma Inflammation and Outcomes ".
Intervention – Clinical Trial(Reference No 95-15)

The above titled research/study proposal has been examined with the following enclosures:

- The Study Protocol
- Informed Consent form

The REC recommended granting permission of approval to conduct the project along the following terms:

1. Provide to committee" Continuing Review Progress Report "each 6 months.
2. Any amendments to the approved protocol or any element of the submitted documents should NOT be undertaken without prior re-submission to, and approval of the REC for prior approval.
3. Monitoring: the project may be subject to an audit or any other form of monitoring by the REC.
4. The PI is responsible for the storage and retention of original data of the study for a minimum period of five years.
5. The PI must inform / report REC & Sponsor by any SAE "Serious Adverse Event" within one working day.
6. The PI is expected to submit a final report at the end of the study.
7. To follow all regulations issued by the National Committee of Bio & Med ethics - King Abdul Aziz City for Science and Technology.
8. If the study is RCT the PI must registrar the study within one the RCT International Organizationand and provide to the committee registration number within 4 weeks.

The Organization & operating procedure of the KAU. Faculty of Medicine - Research Ethics Committee(REC) are based on the Good Clinical Practice (GCP) Guidelines.
PLEASE NOTE THAT THIS APPROVAL IS VALID FOR ONE YEAR COMMENCING FROM THE DATE OF THIS LETTER.

Professor Hasan Alzahrani
[redacted]
Chairman of the Research Ethics Committee

(HA-02-J-008) No of Registration At National Committee of Bio.& Med. Ethics.
Mohammed ALsearee (Reference No95-15)

ص.ب ٨٠٢٠٥ جـــــــدة ٢١٥٨٩
P.O. Box 80205 Jeddah 21589

فاكس ٦٤٠٠٥٩٢ / ٦٤٠٨٤٥١
Fax : 6400592/ 6408451

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Figure 23. The trial registration within clinicaltrials.gov website.

Figure 24. The trial registration within the Saudi Clinical Trials Registry website.

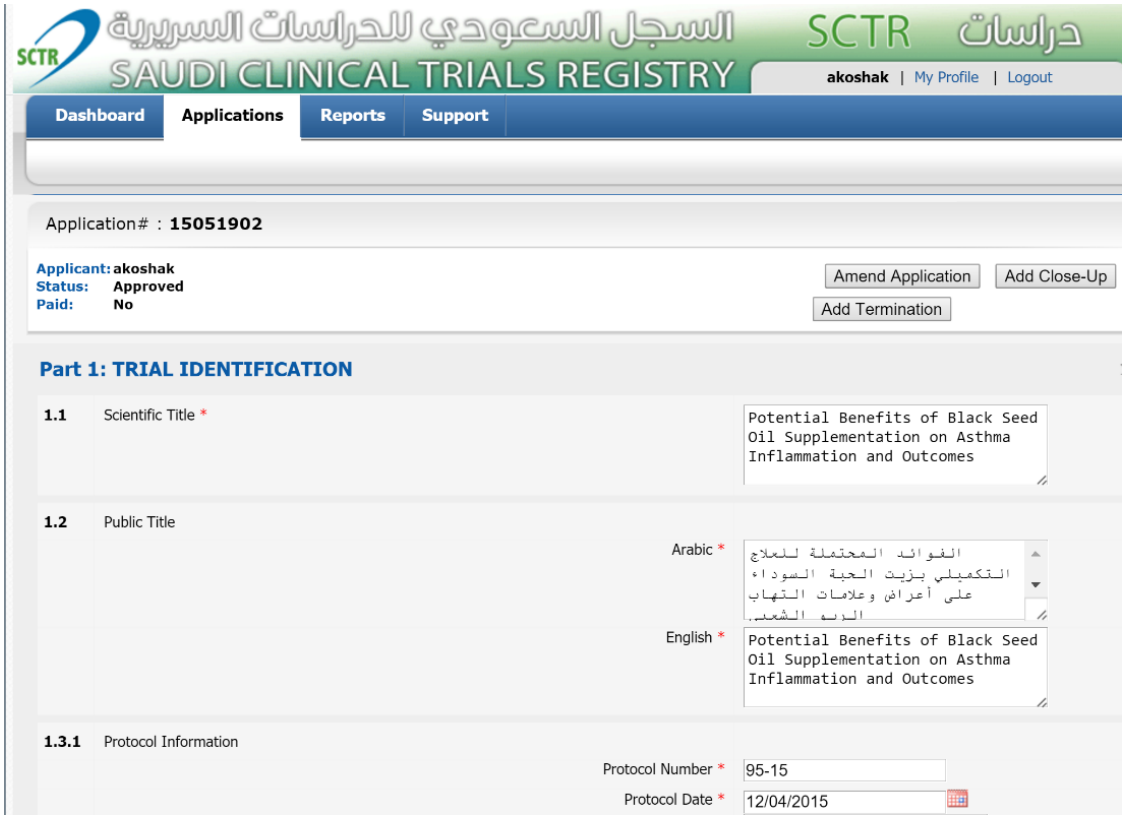


Figure 25. The trial registration within the National Health Service website.

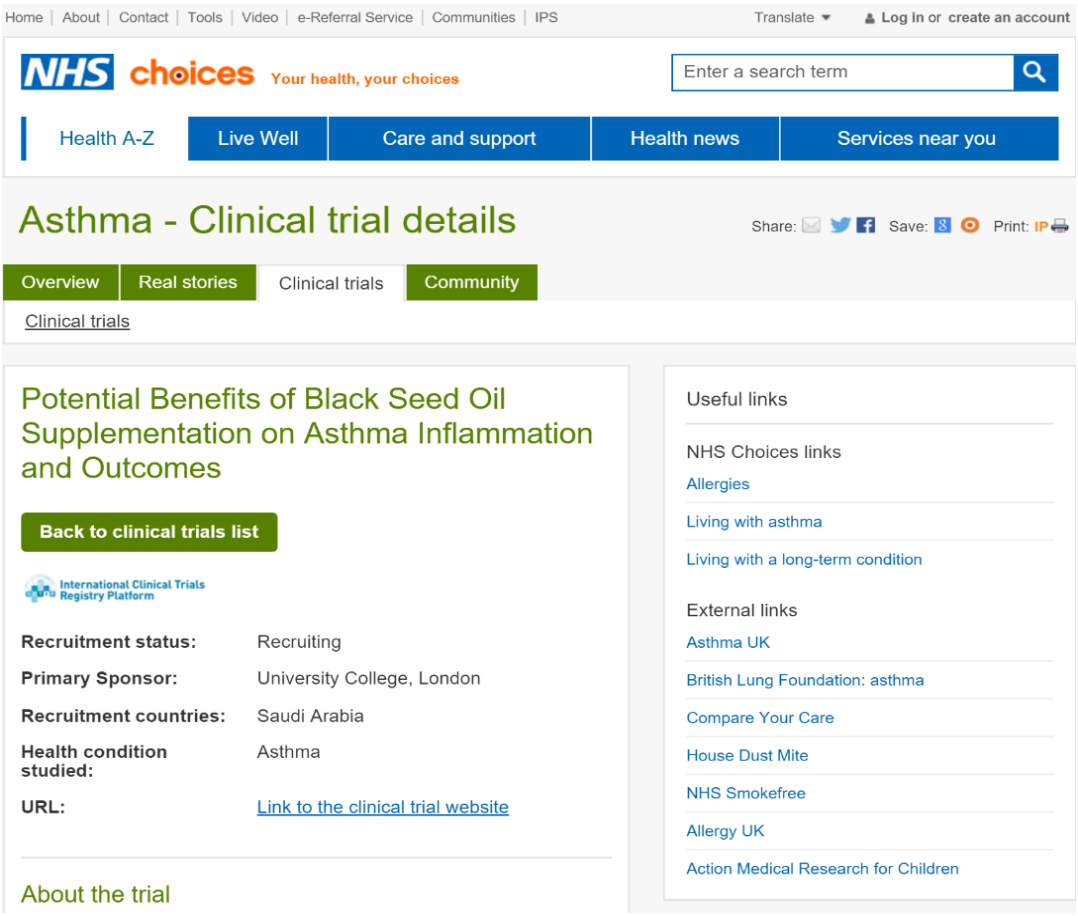


Figure 26. The trial registration within the World Health Organization website.

4.3.2.2 Safety reporting

According to good clinical practice guidelines, adverse events (AEs) need to be reported to ethical committee(s) within relevant time frame (Immediately for serious AEs, and 10 days for non-serious AEs) by the primary investigator once becoming aware of the event as Related (resulted from the administration of any of the research procedures) or Unexpected (not listed in the protocol as an expected occurrence).

4.3.2.3 Data handling

The clinical data were collected and analysed by the primary investigator within six months following the end of the study. Patients' personal data were managed in a confidential way. Sensitive personal data were stored in a secure location (locked filing cabinet) and digitally saved at UCL' secure servers (Data Save Haven) with limited access to the primary investigator as well as the PhD supervisors for further analysis. Each participant was anonymised by assigning an ID number, which used as an identifier in the main dataset. The results of the study were written up as part of a PhD thesis and used for publication in peer-reviewed journals or scientific conferences.

4.3.2.4 Safety of investigators and patients

All clinical procedures including patient diagnosis, primary and secondary outcome measurements were carried by fully qualified medical doctors (Prof Emad Koshak, Prof Siraj Wali and Prof Omer Alamoudi) in collaboration with a registered pharmacist (Abdulrahman Koshak) inside the medical clinics and laboratories of King Abdulaziz University Hospital.

4.3.2.5 Collection of blood samples from patients

Trained medical staff at a specialised phlebotomy area within King Abdulaziz University Hospital were responsible for collecting blood samples from patients.

4.3.2.6 Herbal product adverse effects and interactions

Overall, adverse effects of NS are rare, although potential adverse effects including decreased blood coagulation, decreased blood sugar and decreased blood pressure may occur (NaturalMedicines, 2014). Therefore, subjects at high risk for occurrence of side effects such as a patient at risk of bleeding were excluded from the study. Participants were briefed to look out for any appearance of adverse events and advised to stop taking the supplement with immediate contact for their physician if any adverse event appeared.

4.3.2.7 Funding and payments

There was no direct payment or any kind of reimbursement to participants. Participation was voluntary by both the participants and the co-investigators. Participants' interventions, laboratory analysis and diagnostic tests were free of charge. All costs occurred were covered by the PhD project bench fees. The trial was conducted in a Saudi governmental setting in which healthcare is provided free of charge.

4.3.2.8 Other ethical considerations

UCL's Risk Assessment Procedures were followed. The research did not include children or vulnerable adults such as individuals with a learning disability or cognitive impairment or individuals in a dependent or unequal relationship. Participants did not participate in any activities that may be potentially stressful or harmful in connection with this research. There was no invasive procedure.

The questionnaire used don't raise any topics or issues that might be sensitive, embarrassing or upsetting for participants. The study was covered by UCL's insurer company.

4.3.3 Recruitment clinics

Patients were recruited from the outpatient clinics at King Abdulaziz University Hospital (Figure 28). These clinics were managed by five different physicians who served as co-investigators in this trial (Table 10). The role of co-investigators was to identify eligible patients for the trial and refer them to the primary investigator. The primary investigator attended all these clinics to manage the recruitment of patients and the measurement of outcomes.

Table 10. The clinics of co-investigators from which patients recruited.

Co-investigator name	Outpatient clinic	Time
Prof Emad Koshak	Allergy and Asthma	Sunday, Tuesday, Thursday 6pm-9pm
Prof Siraj Wali	Respiratory	Wednesday 9am-12pm
Prof Omar Alamoudi	Respiratory	Monday 9am-12pm
Dr Abdulrahman Demerdash	Respiratory	Monday 9am-12pm Wednesday 9am-12pm
Dr Majdi Qotob	Family Medicine / Allergy	Tuesday 9am-3pm

Figure 27. The study site reception and clinics at King Abdulaziz University Hospital.

4.4 Results and discussion

4.4.1 Patients

Between Jun 1, 2015 and Dec 30, 2015, 140 patients with asthma were assessed for eligibility. Of them, 80 patients were enrolled and randomised (1:1) into two groups, the treatment and placebo groups. In each group, 10 patients did not complete the study. Reasons for drop-out were mainly due to non-medical reasons such as loss of interest and inability to attend. Four patients had medical related reasons such as adverse events and asthma exacerbation. At the beginning of the study, a large proportion of patients dropped out since they lived far away from the study site. Therefore, it was decided to exclude patients who are living outside the city. Also, patients were encouraged to come for follow-up by providing them with an extra check-up by the medical team. The recruitment flow chart and details of drop-out reasons are provided in Figure 28. The baseline demographics of the recruited patients are presented in Table 10. All participating patients were previously diagnosed with asthma and were on regular asthma medications that were adjusted and optimised according to GINA guidelines for standard asthma management (Global Initiative for Asthma, 2017). These medications were inhaled short-acting beta agonists (salbutamol), inhaled long-acting beta agonists (salmeterol or formoterol), inhaled corticosteroids (budesonide or fluticasone), oral anti-leukotrienes (montelukast).

Figure 28. Flow diagram of patient's enrolment.

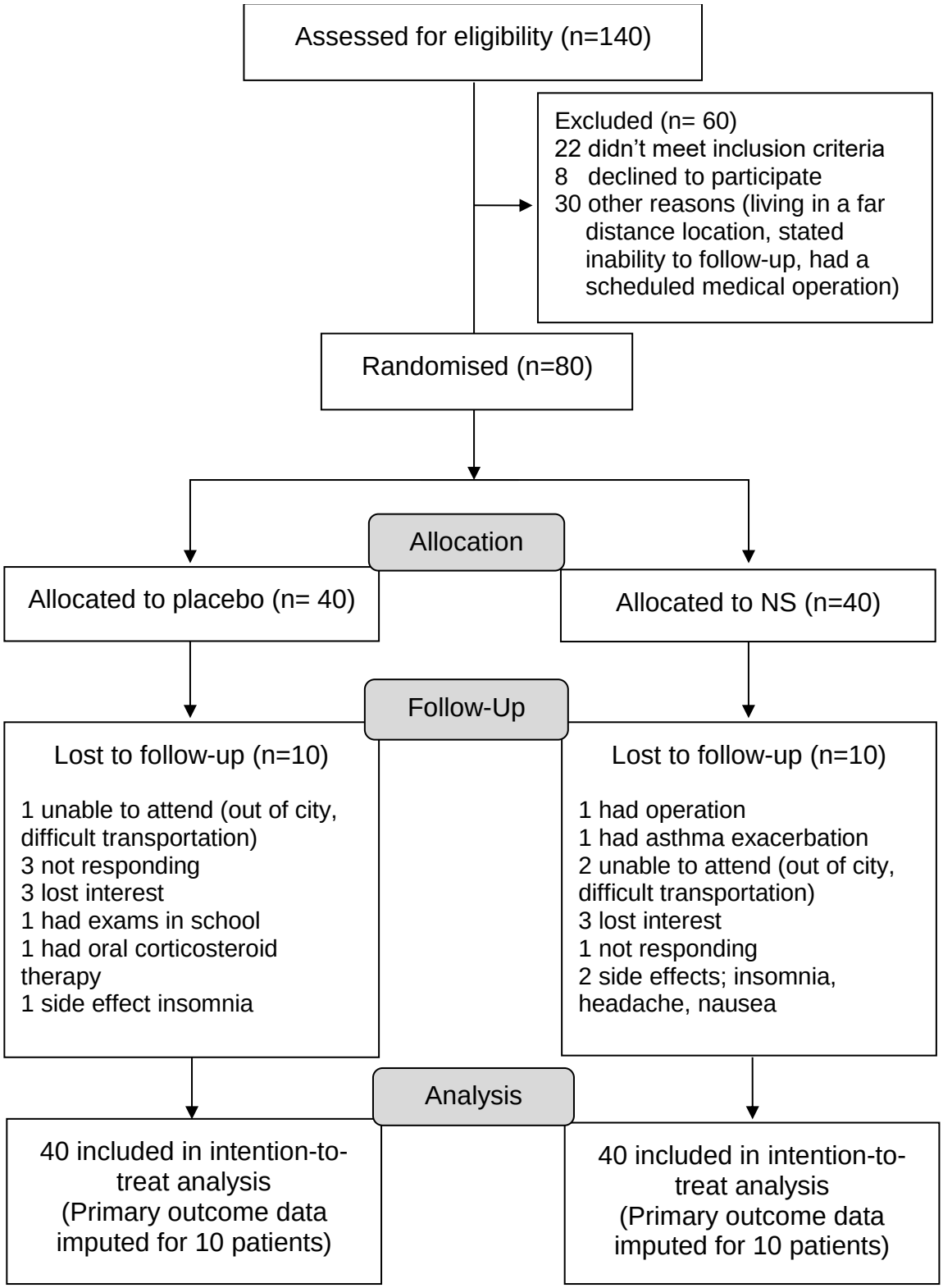


Table 11. Baseline demographics of subjects.

Variable	Treatment (n=40)	Placebo (n=40)
Age	39 (13)	42 (15)
Men	15 (38%)	18 (45%)
Women	25 (62%)	22 (55%)
BMI	28 (5)	30 (8)
Predicted FEV1%	74 (17)	72 (16)
Total ACT	16.0 (3.9)	16.5 (3.6)
Blood eosinophil count, Cells/uL	350 (187 – 711)	300 (135 – 415)
Total IgE, IU/mL	164 (112 – 468)	224 (57 – 682)
Changed medications	45%	40%
Added medications	12%, Montelukast 10mg 14%, Symbicort 160/4.5mcg 2%, Theophylline 250mg	10%, Montelukast 10mg 12%, Symbicort 160/4.5mcg 4%, Theophylline 250mg
Values represented as mean (SD), n (%), or median (IQR).		

4.4.2 Compliance with medications

Medication compliance was assessed by counting the remaining capsules and was referred as “good” if the patient adhered to 80-100% of study treatment regimen. Treatment adherence was assessed by direct questioning of patients during the treatment period and at the follow-up visit. Ultimately, 95% of patients fulfilled this criterion and had a good compliance. Few patients (5%) had poor compliance as they adhered to less than 80% of the treatment regimen.

4.5 Efficacy

4.5.1 Asthma Control Test

Inspection of Q-Q plots revealed that Asthma Control Test (ACT) scores were close to normally distributed for both groups. Therefore, independent t-test was used for significance testing between means with a 95% confidence interval (CI).

The baseline scores of the mean total ACT were not significantly different between the treatment (n=40) and placebo group (n=40). All randomised 80 patients were statistically analysed for the primary outcome by using the intention-to-treat method of analysis. This method was used to include the withdrawn patients in the analysis by imputing the missing data of 10 patients using SPSS statistical software.

As a primary outcome, the mean total ACT score in the treatment group was significantly higher than the placebo group at the end of the study, as shown in Table 12.

In addition, the five individual questions of ACT (ACT sub-scores) were analysed as secondary outcomes. Significant improvement was found in the 1st and 5th questions of the ACT which reflects a significant improvement in daily functioning and overall self-assessment of asthma control. However, there was a non-significant improvement in general asthma symptoms, the frequency of shortness of breath and use of rescue medications (2nd, 3rd and 4th questions of the ACT) as shown in Table 13.

Our findings in asthma symptom control, measured by the ACT, were consistent with symptoms improvement in previous clinical studies of NS in

asthmatic patients. NS oil enhanced scores of subjective improvement in clinical symptoms of allergic conditions including asthma (Kalus *et al.*, 2003). An aqueous extract of NS significantly improved the severity of asthma symptoms (Boskabady *et al.*, 2007). The administration of powdered NS among asthmatic children with immunotherapy significantly increased ACT scores (Sugiono *et al.*, 2013).

Table 12. Scores of total Asthma Control Test.

Parameter	Pre-treatment			Post-treatment		
	Treatment (n=40)	Placebo (n=40)	p	Treatment (n=40)	Placebo (n=40)	p
Total ACT	16.0 (3.9)	16.6 (3.6)	0.44	21.1 (3.1)	19.6 (3.9)	**0.04**
Values represented as mean (SD)						

Table 13. Sub-scores of Asthma Control Test.

Parameter	Pre-treatment			Post-treatment		
	Treatment (n=40)	Placebo (n=40)	P	Treatment (n=30)	Placebo (n=30)	P
Q1 (daily functioning)	3.4 (0.9)	3.3 (1.1)	0.62	4.4 (0.6)	3.8 (0.9)	**0.01**
Q2 (frequency of shortness of breath)	3.1 (1.0)	3.4 (1.0)	0.22	4.2 (0.7)	3.9 (0.9)	0.28
Q3 (night symptoms)	3.3 (1.3)	3.6 (1.2)	0.27	4.4 (0.7)	4.0 (1.1)	0.14
Q4 (use of rescue medications)	3.8 (1.3)	3.7 (1.3)	0.77	4.5 (0.7)	4.2 (1.2)	0.17
Q5 (overall self-assessment of asthma control)	2.7 (0.7)	2.7 (0.9)	1.00	4.1 (0.6)	3.5 (0.9)	**0.01**

Values represented as mean (SD). Q1-Q5 are the individual questions of ACT. *Q1=In the past 4 weeks, how much of the time did your asthma keep you from getting as much done at work, school or at home?, Q2=During the past 4 weeks, how often have you had shortness of breath?, Q3= During the past 4 weeks, how often did your asthma symptoms (wheezing, coughing, shortness of breath, chest tightness or pain) wake you up at night or earlier than usual in the morning?, Q4= During the past 4 weeks, how often have you used your rescue inhaler or nebulizer medication?, Q5= . How would you rate your asthma control during the past 4 weeks?.

Values represented as mean (SD).

4.5.2 Pulmonary function test

Inspection of Q-Q Plots revealed that pulmonary function test scores were not close to a normal distribution for both groups. Therefore, Mann-Whitney U Test was used for significance testing between medians with a 95% confidence interval (CI).

At the end of the study, there was a trend for an improvement in the predicted FEV1% and PEF% in the treatment group (n=25) versus the placebo group (n=24). However, the changes in pulmonary function were not statistically significant (Table 14). Eleven patients in both groups (5 in the treatment group and 6 in the placebo group), were not able to successfully perform the pulmonary function test due to breathing difficulty.

Table 14. Changes in pulmonary function test.

Parameter	Change in placebo group (n=24)	Change in treatment group (n=25)	p
FEV1 as % predicted	1.00 (-2.00 to 5.00)	4.00 (-1.25 to 8.75)	0.170
PEF as % predicted	2.00 (0.00 to 14.50)	6.50 (0.25 to 22.75)	0.279
FEF25-75% as % predicted	3.00 (-6.50 to 16.50)	2.50 (-7.75 to 18.75)	0.992
Values represented as median (IQR). FEV1: Forced expiratory volume in 1 second. PEF: Peak expiratory flow. FEF 25-75%: Forced expiratory flow between 25-75%.			

Alternatively, subgroup analysis of patients with below normal predicted FEV1% (<80%) at baseline had a significant improvement in the treatment subgroup (n= 14) vs placebo subgroup (n= 15) by median= 8% (IQR= 3 to 13%) vs median= 1% (IQR= -2 to 5%) (p= 0.018), respectively. Their mean total ACT

scores were 21.93 (SD= 1.9) for the treatment group vs 19.07 (SD= 3.3) for the placebo group with p=0.01. Also, their median change in PEF (% predicted) scores were 6.5 (IQR= 1.0 to 14.5) for the treatment vs 2.0 (IQR= 0.0 to 10.0) for the placebo with p=0.25.

The baseline characteristics of the subgroups are shown in Table 15.

Table 15. Baseline characteristics of subgroups with below normal predicted FEV1% (<80%)

Variable	Treatment (n=14)	Placebo (n=15)
Age	41 (8.8)	46 (15)
Men	5 (36%)	4 (27%)
Women	9 (64%)	11 (73%)
BMI	28 (5)	30 (5)
Predicted FEV1%	63 (10)	62 (11)
Total ACT	17.6 (3.5)	16.7 (3.2)
Blood eosinophil count, Cells/uL	448 (163 to 820)	271 (130 to 350)
Total IgE, IU/Ml	151 (53 to 369)	305 (58 to 718)
Values represented as mean (SD), n (%), or median (IQR).		

In a previous clinical study, an aqueous extract of NS significantly improved pulmonary function parameters in the treatment vs. placebo group only after 3 months treatment but all patients in this study had an abnormal pulmonary function at baseline (Boskabady *et al.*, 2007). Also, an aqueous extract of NS showed a significant improvement in pulmonary function parameters in short time effect (30 min until 150min), but the results were not compared to placebo, and all patients (15 asthmatics) had an abnormal pulmonary function at baseline

(Boskabady, Mohsenpoor and Takaloo, 2010). In a non-RCT open-label clinical study, NS seeds combined with bee honey improved pulmonary function, but this study was not placebo controlled and included only 5 asthmatics (Al Ameen *et al.*, 2011).

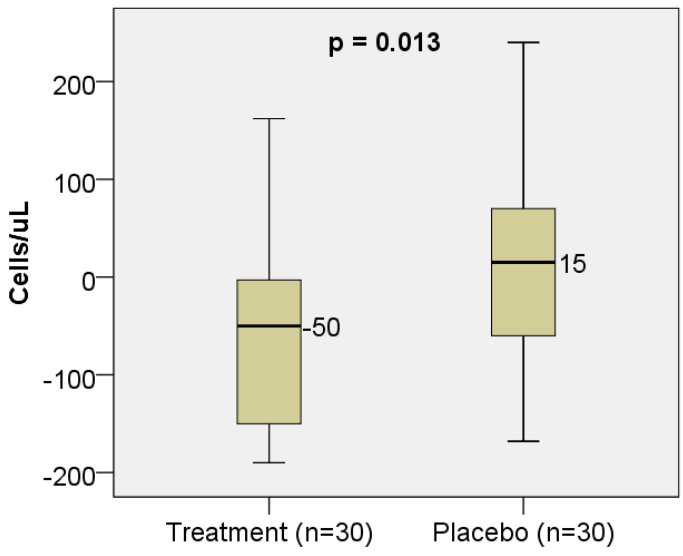
4.5.3 Blood biomarkers

4.5.3.1 Absolute blood eosinophil count

Inspection of Q-Q Plots revealed that the absolute blood eosinophil count scores were not close to a normal distribution for both groups. Therefore, Mann-Whitney U Test was used for significance testing between medians with a 95% confidence interval (CI).

There was a significant reduction in the absolute blood eosinophil count in the treatment group (n=30) compared to the placebo group (n=30) by a median of -50 (IQR= -1 to -155) versus a median of 15 (IQR= -60 to 87) cells/uL (p= 0.013) as shown in Figure 29.

Figure 29. Change in absolute blood eosinophil count level from baseline.



The following is a subgroup analysis of patients who had high absolute blood eosinophil count (Eosinophilia) at baseline (>350 cells/uL). Eosinophilia was found in 16 patients (%) in the treatment group and 15 patients in the placebo group (%). The analysis of this subgroup showed a median change for the blood eosinophil count by -145 (IQR= -42 to -422) cells/uL for the treatment (n=16) versus 20 (IQR= -130 to 140) cells/uL for the placebo (n=15) with p=0.03. Also, their total ACT scores after treatment were 21.5 (SD=2.3) for the treatment group vs 19.5 (SD=4.0) for the placebo group with p=0.1. In addition, the change in median FEV1 (% Predicted) were 6.5 (IQR= 3.2 to 12.2) for the treatment group vs 2.0 (IQR= -2.0 to 4.0) for the placebo group with p=0.009.

To our knowledge, this is the largest RDBPCT study discovered a remarkable reduction of peripheral blood eosinophil count by NS oil in asthmatic patients. In a previous clinical trial, there was a non-significant decrease of blood eosinophils within NS oil group. However, this study included 3 asthmatic patients only and did not statistically compare the results of blood eosinophil count between groups (Kalus *et al.*, 2003).

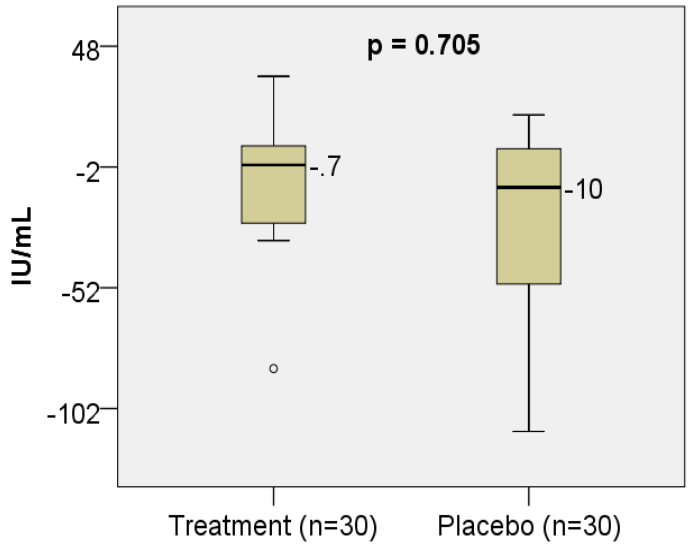
4.5.3.2 Total serum immunoglobulin E

Inspection of Q-Q plots revealed that total serum immunoglobulin E (IgE) scores were not close to a normal distribution for both groups. Therefore, Mann-Whitney U Test was used for significance testing between medians with a 95% confidence interval (CI).

There was no significant change found on the total serum IgE level between both groups (n=30 each) as shown in Figure 30. This was consistent with findings of Kalus *et al*. (2003) who reported a non-significant IgE changes within NS oil group. However, the study of Kalus et al. (2003) included only 3

asthmatic patients and did not statistically compare the total serum IgE changes between groups. In another clinical trial, there was a significant reduction of total serum IgE in the group of NS, but this study was conducted in children, was not double-blinded placebo-controlled, and NS was combined with probiotics and immunotherapy (Sugiono *et al.*, 2013).

Figure 30. Change in total serum immunoglobulin E level from baseline.



4.5.3.3 Serum inflammatory mediators

Inspection of Q-Q Plots revealed that serum inflammatory mediators scores were not close to a normal distribution for both groups. Therefore, Mann-Whitney U Test was used for significance testing between medians with a 95% confidence interval (CI).

In the treatment group (n=26), there was a trend for upregulation of the serum inflammatory mediators IL-10, IP-10, IFNγ and IL-12p70 from baseline scores in comparison to placebo (n=27) (Figures 31-34). However, no significant changes were found in the other inflammatory mediators between groups (Table 16). Therefore, NS may have a role in upregulating the serum levels of Th1 cell

promoting factors (IFNγ, IL-12p70), Th1 secreted cytokines (IL-10, IFNγ) and IFNγ-induced protein (IP-10) when compared to placebo. This trend of Th1 cytokines domination may be useful to counteract the elevation of Th2 cytokines found in asthmatic patients and hopefully restore Th1/Th2 balance (Ray and Cohn, 2000). In a RSBPCT, powdered NS increased serum IFNγ in patients with asthma (Sale*m et al*., 2017). In an RDBPCT, NS oil increased serum level of IL-10 in patients with rheumatoid arthritis (Hadi *et al.*, 2016).

Scientific studies showed that modifying cytokine profile of Th2 cells by induction of Th1 responses may benefit Th2-related diseases such as asthma (Teixeira *et al.*, 2005). IFNγ showed a positive role in allergic-related diseases and reduced level of IFNγ was associated with severe asthma (Teixeira *et al.*, 2005). IFNγ showed a reduction of blood eosinophil count in patients with severe steroid-dependent asthma (Barnes *et al.*, 2009). The anti-inflammatory cytokine (IL-10) may have a beneficial role for the control of asthma and allergy (Urry, Xystrakis and Hawrylowicz, 2006). IL-12 cytokine may have a potential role in asthma therapy, and several in-vivo studies showed that IL-12 could inhibit allergic airway inflammation in asthma (Leonard and Sur, 2003). Inhalation of IL-12 appeared to be inhibiting allergic inflammation in murine models (Leonard and Sur, 2003). IL-12 might reduce eosinophilic inflammation as administration of recombinant human IL-12 to asthmatic patients resulted in a decrease of blood and sputum eosinophils count and improved airway hyperresponsiveness (Barnes *et al.*, 2009).

Figure 31. Change in serum interleukin-10 level from baseline.

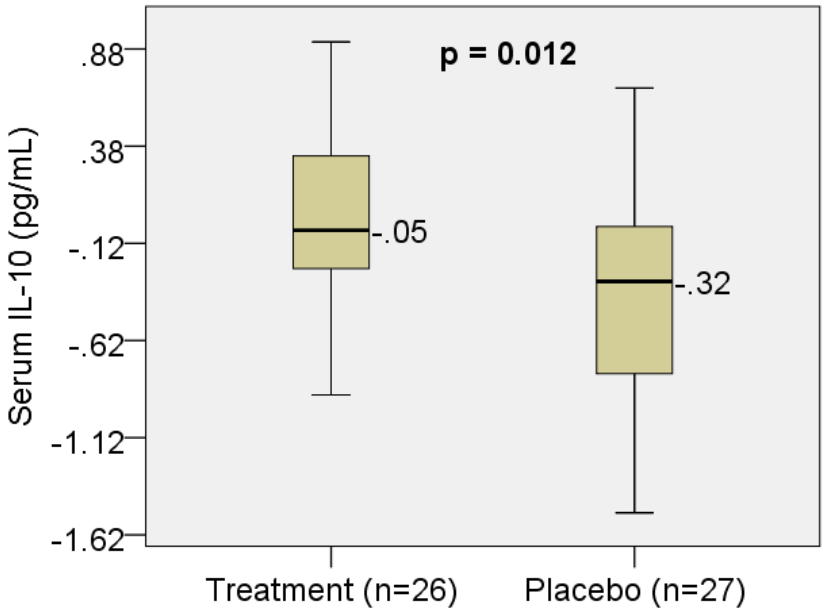


Figure 32. Change in Interferon-gamma-inducible protein 10 level from baseline.

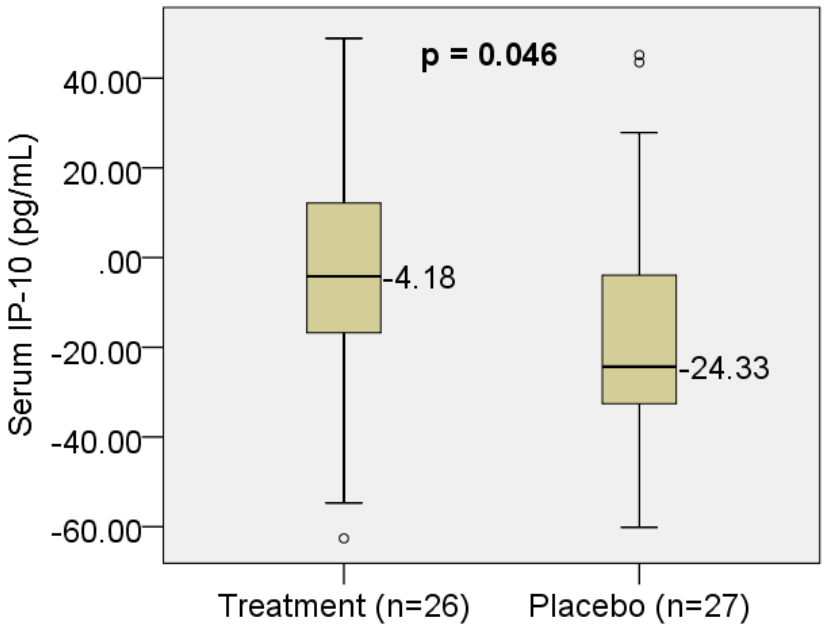


Figure 33. Change in serum Interferon gamma level from baseline.

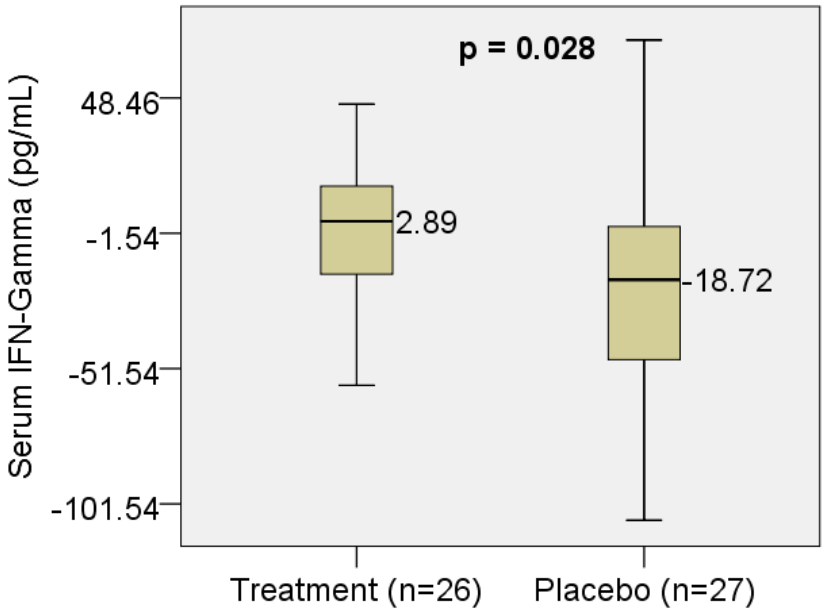


Figure 34. Change in serum interleukin-12p70 level from baseline.

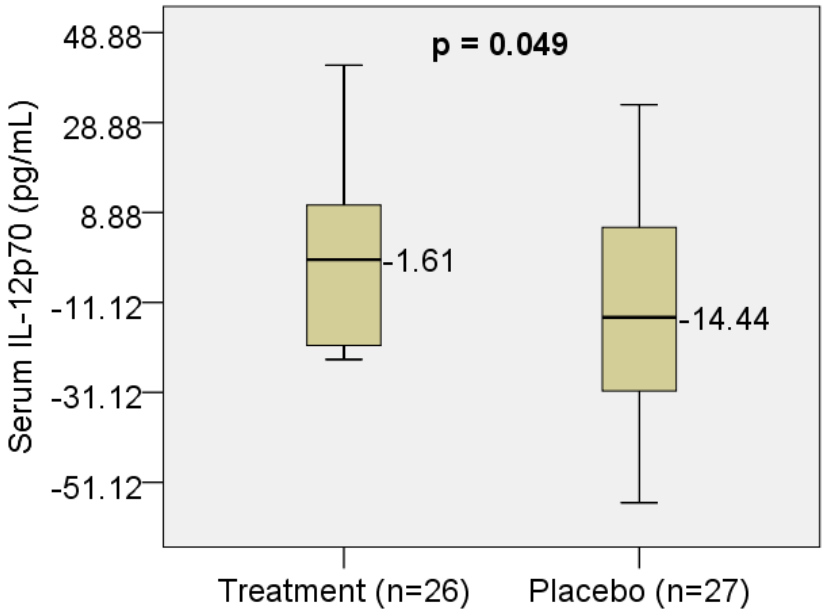


Table 16. Changes in serum inflammatory mediators.

Mediator	Median changes from baseline		
	Placebo (pg/mL)	Treatment (pg/mL)	p
IL-1β	-0.2 (-0.8 – 0.4)	-0.3 (-0.5 – 0.1)	0.972
IL-1α	-6 (-17.8 – 6.6)	-1.8 (-8.5 – 8.7)	0.207
IL-4	-6.2 (-28.2 – 2.4)	0.8 (-24.8 – 23.6)	0.113
IL-6	-7 (-18.7 – 6)	-3 (-7.3 – 4.6)	0.319
IL-8	-2.4 (-3.9 – 0.4)	0.1 (-4 – 1.7)	0.286
IL-10	-0.3 (-0.9 – 0)	0 (-0.2 – 0.3)	0.019
IP-10	-24.3 (-34 – 0)	-4.1 (-17.4 – 12.8)	0.046
IL-12p70	-14.4 (-32 – 6.5)	-1.6 (-20.8 – 10.8)	0.048
IL-13	-1.4 (-2 – 0.2)	-0.3 (-1.9 – 2.5)	0.140
IL-17A	-1.2 (-3.2 – 0)	-1.2 (-2.4 – 0.8)	0.499
INF-α	-0.5 (-2.1 – 0.4)	-0.1 (-1.2 – 0.9)	0.220
INF-γ	-18.7 (-51.8 – 1.8)	2.8 (-17 – 15.9)	0.039
TNF-α	-24.8 (-103.8 – 24.5)	-14 (-50 – 46.1)	0.188
sICAM-1	-30820 (-192561 – 81074)	5104 (-24230 – 68982)	0.182
MIP-1α	-9.6 (-43.3 – 10.9)	-1.1(-14.3 – 11.3)	0.423
MIP-1β	-55.1 (-132.6 – 133.7)	-14.6 (-84.6 – 63.8)	0.656
MCP-1	-24.5 (-116.2 – 90.6)	5.7 (-94.9 – 86.9)	0.477
P-Selectin	-17758 (-93719 – 17533)	1345 (-72982 – 120603)	0.466
E-Selectin	-5426 (-18032 – -1794)	-634 (-8206 – 2202)	0.024

4.6 Safety and tolerability

Three patients (two from the treatment group and one from the placebo group) reported three adverse events including; stomach upset, headache, and insomnia. These adverse events were mild and self-limited (did not require any treatment or hospitalisation). The study treatment is considered to be tolerable as only two patients (one from the treatment group and one from the placebo group) discontinued the study due to adverse events.

4.7 Study limitations

This clinical study had several limitations. The duration of the intervention is relatively short (four weeks only), and the sample was comparatively small. This was due to time limitation of the PhD study. A relatively high drop-out rate which resulted from reasons not related to study design or medication, but rather due to unexpected reasons not considered initially including the geographical mobility of some of the study participants. Also, the lack of incentives for participants (due to limited funding) along with the socioeconomic status of some the participants played a role in patient retention rate. The time of using bronchodilator medication before measuring pulmonary function was not considered and this may affect the final results of pulmonary function parameters. The choice of outcomes was dependent on its applicability and availability at the study site. Additional asthma outcomes and biomarkers such as FeNO, Sputum eosinophils, allergen-specific IgE and urinary LTE4 may be considered in the future studies (Szefler *et al.*, 2012).

4.8 Conclusion

In this study, four weeks of add-on (complementary) treatment with NS oil significantly improved asthma symptom control over placebo and showed a trend toward pulmonary function improvement with an acceptable safety and tolerability profile in adult asthmatic patients. Remarkably, NS oil showed a reduction of the biomarker blood eosinophilia. In addition, the changes in the serum cytokines levels indicated a potential immunomodulatory effect of NS toward the upregulation of Th1 specific cytokines which may result in balancing the impaired Th1/Th2 cytokines balance in the context of asthma. However, significant changes in total serum IgE did not occur.

These findings provided an evidence base for the probable benefits of NS oil supplementation in the clinical management of asthma. To our knowledge, this is the largest sample size RDBPCT of using NS oil in patients with asthma and compared the effect of NS oil on blood eosinophils between the two groups (treatment versus placebo). In the future, studies should follow patients for a longer period with a larger group of patients and consider further outcomes in a multicentre trial to establish a stronger evidence of NS oil benefits in patients with asthma.

5 Chapter 5 General discussion and conclusion

5.1 General discussion

In the era of evidence-based medicine, well-designed clinical studies are essential to evaluate the safety and efficacy of herbal medicines for the treatment of human diseases. This project addressed some vital needs for conducting clinical trials using herbal medicines. Many clinical trials using herbal medicines often have drawbacks that limit the scientific rationale for the clinical use of herbal medicines. A key problem of clinical research with herbal medicines is the quality and the lack of a clear characterisation of the studied material. Specifically, the variability of herbal preparations used may lead to a discrepancy in the results and conflicting conclusions about the safety and efficacy of herbal medicines. This project addressed this problem by combining a phytochemical and pharmacological characterisation with a clinical trial of a herbal medicine. In addition, this project has overcome the financial challenge for clinical trials especially using herbal medicines by conducting the trial in a local government university hospital in which healthcare is provided free of charge.

The general approach of this study comprised of three phases. The first phase was to assess and identify the weaknesses in the evidence for the clinical use of NS in asthma (Chapter 1). The second phase was the pre-clinical characterisation (including chemical and pharmacological) of NS preparations and identifying the most suitable preparations for a clinical trial (Chapter 2 and 3). The last phase was the designing and conducting a randomised double-blind placebo-controlled clinical trial on the supplementary use of NS in patients with asthma.

Our project was focused on asthma disease as one of the leading chronic respiratory illnesses. Due to the limitations of conventional asthma medications, patients with asthma may tend to use herbal medicines as one of the modalities of unconventional treatment approaches. *Nigella sativa* (NS) is one of the most popular medicinal plants among different communities, especially in the Arab and Islamic countries. The religious significance and several medicinal benefits associated with NS made it a culturally and commercially valuable plant. In Saudi Arabia, NS is one of the most frequently used non-standard treatments among asthmatic. However, the evidence for the use of NS in asthma is not well established. Therefore, this project came to establish a scientific basis for the clinical use of NS in patients with asthma via addressing the current limitations of the scientific evidence and overcoming these limitations with unique mixed methods approach of combined chemical, pre-clinical pharmacological and clinical investigations.

The literature review provided an important update on the evidence of the use of NS in asthma disease (Chapter 1). Based on the literature review, it became apparent that NS had empirical evidence for its anti-asthmatic effect in traditional medical textbooks as well as experimental pre-clinical and clinical studies. However, these experimental studies had limitations such as quality of study design, sample size, investigated outcomes, and most importantly the variability of the investigational NS preparation and its chemical characterisation among different studies. Hence, the evidence base for the clinical use of NS in asthma is limited and unclear. A specific approach for the chemical and pharmacological characterisation of an NS preparation was necessary to find a suitable high-quality active preparation of NS that can be used in a clinical trial.

In the second chapter of phytochemical characterisation, ten NS preparations (extracts 1-10) were obtained via either a specific method of extraction using different percentages of combined water/ethanol extraction solvents (extracts 1-6) or inclusion of ready-made commercial NS products (extracts 7-10). All NS preparations were chemically characterised by quantification of the main active compound TQ. This experiment was important to provide an overview of the variability in the chemical composition and allowed to assess the level of the key active compound TQ among different NS preparations. The level of TQ was highest in the oily extracts, especially the supercritical fluid extract (no. 7) and the commercial oil capsules by Marnys, Spain (no. 10). However, preparations extracted with water or combination of water/ethanol did not contain a substantial amount of TQ due to the poor stability and solubility of TQ in aqueous solutions. This variability in the chemical composition was due to the method of preparations, which is a vital element that can affect the pharmacological activity of NS.

In the third chapter, several *in vitro* models of inflammation/immunomodulation were used for the pharmacological characterisation of the NS preparations. The effect of NS preparations on the inhibition of inflammatory mediators (IL-6, IL-2, PGE2) and inflammatory signalling pathway (p38 MAPK) was investigated in human T-lymphocyte cells and monocytes as well as A549 lung epithelial cell line. The results of this experiments revealed that thymoquinone-rich extracts (especially extract no. 7 and 10) appeared to be the most potent active extracts in the context of anti-inflammatory/immunomodulatory activities. The variability of the *in vitro* activity of NS preparations was most probably due to the differences in the chemical composition of NS preparations. Our results provided a direct pre-clinical

evidence that NS inhibited the release of some cytokines with inflammatory properties which are found to be upregulated patients with asthma, and relatively NS had corticosteroid like effect in abolishing this upregulation to induce remission of asthmatic symptoms. Interestingly, NS also induced the release of PGE2 cytokine which thought to have a bronchodilatory effect in bronchial epithelium. This experiment was a very useful tool that enabled us to identify the most active NS preparations that can be selected for a clinical trial. In order to achieve the effective concentration (100mcg/ml) *in vivo*, it is necessary to know the bioavailability of TQ. Due to the lack of knowledge on the clinical bioavailability of TQ, it is difficult to translate our *in vitro* effective dose into an oral human dose.

In the fourth chapter, the design of the clinical trial was developed based on the existing literature for outcomes selection taking into consideration the co-investigators recommendation. In the Saudi Arabian context, extract 10 was the most logistically suitable for a clinical trial because it is licensed with Saudi Food and Drug Authority. Our RDBPCT showed that the NS oil group had a statistical significant improvement in the total ACT score. Although the total ACT score increased significantly more in the treatment group than in the control group, the difference between the groups did not reach the clinically important change of 3 points. The improvement of total ACT score in the placebo group is most probably due to changing the conventional medications of patients. This effect was accompanied by a marked normalisation of blood eosinophilia, which is considered a biomarker of asthma. Also, a trend of pulmonary function improvement (predicted FEV1%) was seen in the NS oil group. However, there were no significant changes in serum total IgE. These insignificant outcomes may reach statistical significance by administration of a higher dose of NS for a longer

period with larger sample size. Interestingly, this study was the first clinical trial explored the effect of NS on a wide range of serum inflammatory cytokines in asthmatics, which early studies suggested that some of these serum cytokines might be associated with asthma control. The serum cytokines IL-10, IFNγ, IP-10 and IL-12p70 had a trend of increase in the NS group compared to placebo but not significant. This might point out that NS may affect the Th1/Th2 cytokines balance by probable upregulation of Th1 cytokines which can consequently counteract the Th2 elevation in asthma. The result of this study is consistent with previous clinical studies in asthma symptom control. Interestingly, this study discovered a potential effect of NS on improving blood eosinophilia so that NS may be used as an anti-eosinophilic agent in several diseases.

Novel therapeutic approaches for asthma management are targeting disease-specific phenotypes. Severe eosinophilic (allergic) asthma is one of the difficult asthma groups that may have limited response to available treatments (Pavord *et al.*, 2017). Currently, late-onset eosinophilic asthma is one of the common asthma phenotypes associated with very poor quality of life and frequent exacerbations (Groot, Brinke and Bel, 2015). Therefore, NS may be a potential controller or add-on treatment option for the allergic phenotype of asthma which is associated with eosinophilic airway inflammation if patients had limited response to inhaled corticosteroids.

The findings of this study are important because it provided a higher quality of clinical evidence for the benefits of the complementary (add-on) use of NS on asthma symptoms and blood eosinophilia.

In the GINA guided stepwise approach for asthma management, the introduction of NS is potentially suggested from step 2 as a controller medication

to reduce airway inflammation or add-on option for patients with severe asthma. Step 1 is usually mild intermittent asthma that can be managed by as-needed short-acting beta$_2$-agonist (Global Initiative for Asthma, 2017).

If the efficacy of NS is well-established as a complementary treatment option for patients with asthma, it may offer cost-effectiveness benefits through reducing the use of high-cost asthma medications or preventing its side-effects. For example, in Saudi Arabia, the cost of commonly used controller medications such as Symbicort 80 mcg/4.5 mcg turbohaler (budesonide, formoterol) is 129.55 SAR (approx. £26) for an average one month supply. Another example is Singulair 5 mg (montelukast) costs 123.6 (approx. £24) for an average one month supply. On the other hand, the used NS capsules in our trial (Marnys Cuminmar 500 mg) costs 50 SAR (approx. £10) for an average of one to two months' supply.

This research project had several limitations. First, the targets used in the *in vitro* screening of the NS preparations were considered to be general inflammatory targets that have some relationship with asthma. This was due to the limitations in funding and the capabilities of the laboratory in which this screening was conducted. It would be more appropriate to investigate classical targets in asthma disease such as to compare the effect of NS preparations on the release of Th2 cytokines IL-4, IL-5, and IL-13 in *in vitro* models.

Second, the choice of the investigational NS product was dependent on two major factors which are high *in vitro* activity and logistical suitability within the clinical trial site. If we base our choice on the most potent preparation, the supercritical fluid preparation (extract 7) would be the first choice. However, it would be more complex and time-consuming to implement extract 7 in the clinical trial because it was not available in capsules form, not registered with any

pharmaceutical regulatory framework and had no safety data. Therefore, the commercial oil product by Marnys, Spain (extract 10) which a relative high *in vitro* activity and being registered within the Saudi regulatory framework of herbal medicinal products (SFDA) was chosen for the clinical trial. This facilitated getting the logistical approval to conduct the clinical trial in Saudi Arabia, and it was more acceptable by the collaborating clinical team as well as the patients to use a product that already been in the Saudi pharmaceutical market.

Third, the dose of NS oil used in this trial was determined based on the manufacturer recommendation (2-3 capsules of 500mg daily) as well as previous clinical trials. Thus, a dose-escalating clinical trial could be a helpful step to find an optimal dose prior to the clinical efficacy trial.

Fourth, the trial was relatively short in duration, small in sample size and the choice of outcomes was limited to the applicability and logistical factors mainly. These limitations stem from the fact that the clinical trial was conducted mainly by a single researcher (the PhD student) who was responsible for running the trial and data collection at a single centre. Also, there were some logistical reasons that limited the number of participants such as lack of incentives for the participants and difficult transportation for some participants (can't afford transportation cost or living in far areas). Thus, providing patients with incentives, compensation for transportation and extra medical care might be useful to minimise patient withdrawal. After few patients dropped out due to difficult transportation, only patients living the same city or who assured they can come for follow-up were recruited. Given the timeframe of a PhD study and the difficulty in convincing the patient to come for a second or third follow-up, it was difficult to have longer duration trial and patients were given four weeks only. The asthma biomarkers choice was based on its feasibility and recommendation by

collaborators. For example, sputum eosinophils and urinary leukotrienes are which were not used in this trial due to the complexity of implementation.

In the future, a well-designed clinical trial characterised by larger sample size (more than 80 patients), longer duration (more than 4 weeks), including multiple trial sites and additional outcomes (such as fractional exhaled nitric oxide, sputum eosinophils, serum allergen-specific IgE and urinary Leukotriene E4 (Szefler *et al.*, 2012) is recommended to establish a stronger evidence for the clinical use of NS oil in patients with asthma. This may result in better outcomes especially in pulmonary function and serum total IgE. Also, elucidating the mechanism of action would be an interesting topic of exploration in future studies. Also, there is a high demand to establish the clinical bioavailability and pharmacokinetic parameters of NS especially for the main active compounds such as thymoquinone. Thus, the next suggested step is to conduct a clinical pharmacokinetic and dose-finding study to optimise the dosage of oral NS oil followed by a longer-term trial to investigate other outcomes such as frequency of exacerbations and asthma medications. In the future, this trial may be combined with other randomised clinical trials of NS in asthma into a meta-analysis study to provide a higher level of evidence for the use of NS in patients with asthma.

5.2 General conclusion

Asthma is a leading chronic disease characterised by suboptimal control regardless of the availability of conventional medications. This project evaluated the use of the herbal medicine *Nigella sativa* oil in asthma as an add-on treatment. NS is considered a traditional remedy for asthma, but the scientific evidence for the clinical use of NS in patients with asthma is not well established

and had several limitations which encouraged us to conduct a properly designed clinical trial using a chemically and pharmacologically characterised NS preparation. Clearly, there is a link between the phytochemistry and pharmacological activity of herbal preparations. Combining pre-clinical characterisation (both chemical and pharmacological) with a well-designed clinical trial is a very important step for investigating the safety and efficacy of herbal medicines. We found a variability in the chemical composition as well as the pharmacological activity of different NS preparations. The oily preparations especially extract 7 (a supercritical fluid extract) and extract 10 (a commercial product by Marnys, Spain) contained the highest concentrations of the key active volatile oil compound TQ. Both extracts showed the best anti-inflammatory / immunomodulatory activity in *in vitro* inflammatory models. Our randomised, double-blind, placebo-controlled phase-II clinical trial of NS, using a commercial herbal product registered in Saudi Arabia, provided a higher level of evidence for the clinical improvement of asthma symptoms control and blood eosinophilia by NS in patients with asthma. In the future, a multicentre clinical trial with longer duration, larger sample size, and additional biomarkers is required to establish a stronger level of evidence for the clinical use of NS in patients with asthma. In the end, this project provided an example of a scientific methodology for the assessment of the clinical efficacy of a herbal medicine considering the specific needs of clinical research using a herbal derived material.

6 Publications

6.1 Original papers

- Koshak, A., Wei, L., Koshak, E., Wali, S., Alamoudi, O., Demerdash, A., Qutub, M., Pushparaj, P. N. and Heinrich, M. (2017) '*Nigella sativa* Supplementation Improves Asthma Control and Biomarkers: A Randomized, Double-Blind, Placebo-Controlled Trial', *Phytotherapy Research*. doi: 10.1002/ptr.5761.

- Koshak, A., Koshak, E. and Heinrich, M. (2017) 'Medicinal benefits of *Nigella sativa* in bronchial asthma: A literature review', Saudi Pharmaceutical Journal. doi: 10.1016/j.jsps.2017.07.002.

6.2 Conference poster presentations

- The 9th Joint Natural Products Conference 2016, Copenhagen, Denmark

Koshak, A., Wei, L., Koshak, E., Wali, S., Alamoudi, O., Demerdash, A., Qutub, M., Pushparaj, P. and Heinrich, M. (2016) 'Potential benefits of *Nigella sativa* oil supplementation on asthma inflammation: a randomised, double-blind, placebo-controlled, exploratory phase-II clinical trial', *Planta Medica*. 81(S 01), pp. S1–S381. doi: 10.1055/s-0036-1596928.

- The UCL Institute of Immunity and Transplantation Annual Symposium 2016, London, UK

Koshak, A., Wei, L., Koshak, E., Wali, S., Alamoudi, O., Demerdash, A., Qutub, M., Pushparaj, P. and Heinrich, M. (2016) 'Immunomodulatory effects of *Nigella sativa* oil supplementation in patients with asthma: an RCT', in *The UCL Institute of Immunity and Transplantation Annual Immunology Symposium with Pears Lecture*. Royal Free Campus, University College London, London, UK.

- European Academy of Allergy and Clinical Immunology Congress 2015, Barcelona, Spain

Koshak, A., Fiebich, B., Koshak, E. and Heinrich, M. (2015) 'Comparative anti-inflammatory / immunomodulatory effect of different extracts of the medicinal

plant *Nigella sativa*', in *European Academy of Allergy and Clinical Immunology Congress*. Barcelona, Spain: EAACI Online Library. Available at: http://eaaci.multilearning.com/eaaci/2015/barcelona/104874/emad.koshak.comparative.anti-inflammatory.immunomodulatory.effect.of.different.html?f=p6m3e814o10431 (Accessed: 6 March 2017).

6.3 Conference oral presentations

- World Integrative Medicine Congress 2017, Berlin, Germany
- European Respiratory Society International Congress 2016, London, UK

Koshak, A., Wei, L., Koshak, E., Wali, S., Alamoudi, O., Demerdash, A., Qutub, M., Pushparaj, P. and Heinrich, M. (2016) 'LATE-BREAKING ABSTRACT: The benefits of *Nigella sativa* oil supplementation on asthma inflammation: A randomised, double-blind, placebo-controlled, phase II trial', *European Respiratory Journal*. European Respiratory Society, 48(suppl 60), p. OA4830. doi: 10.1183/13993003.congress-2016.OA4830.

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8 Appendix

Figure 35. HPLC chromatogram of *Nigella sativa* extract 1.

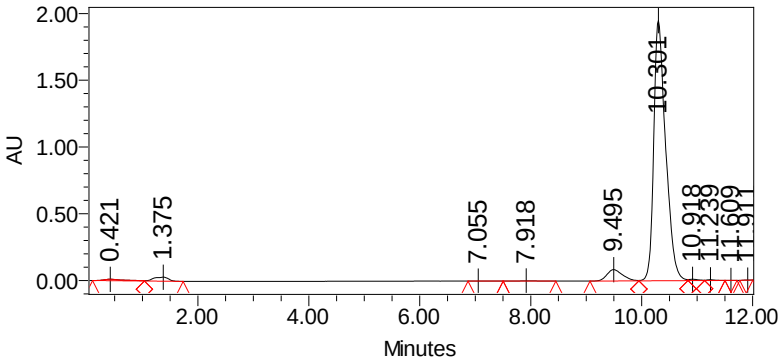

Figure 36. HPLC chromatogram of *Nigella sativa* extract 2.

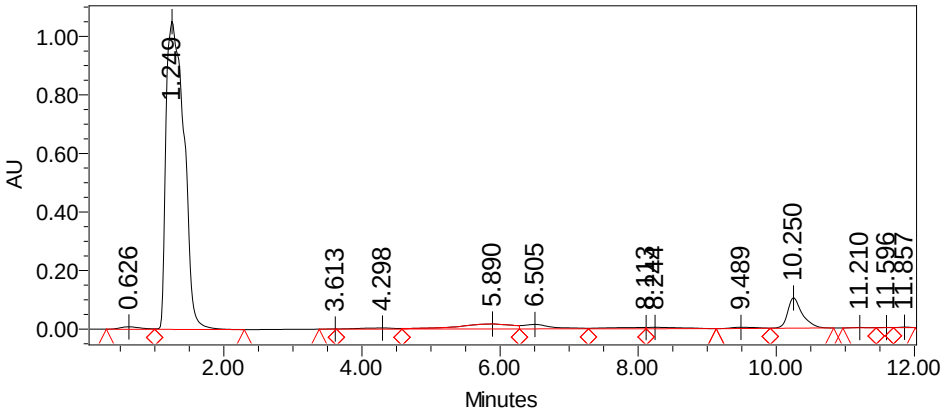

Figure 37. HPLC chromatogram of *Nigella sativa* extract 3.

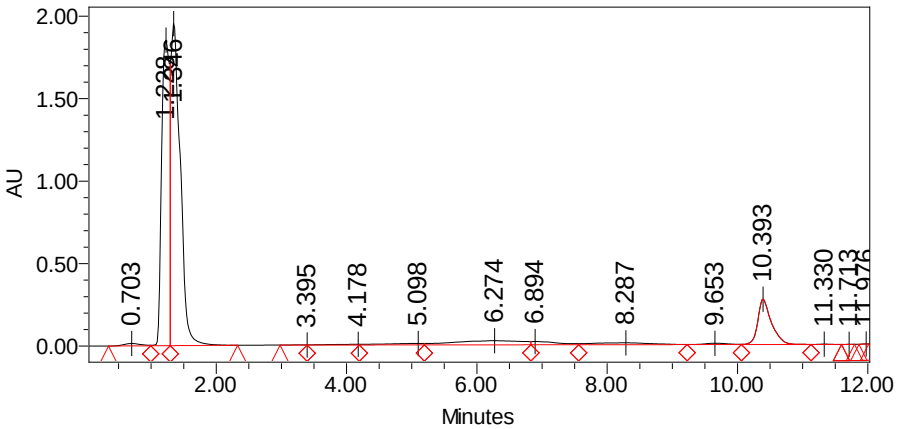

Figure 38. HPLC chromatogram of *Nigella sativa* extract 4.

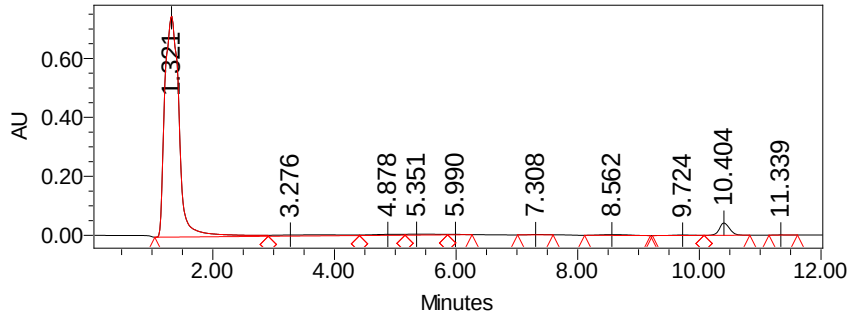

Figure 39. HPLC chromatogram of *Nigella sativa* extract 5.

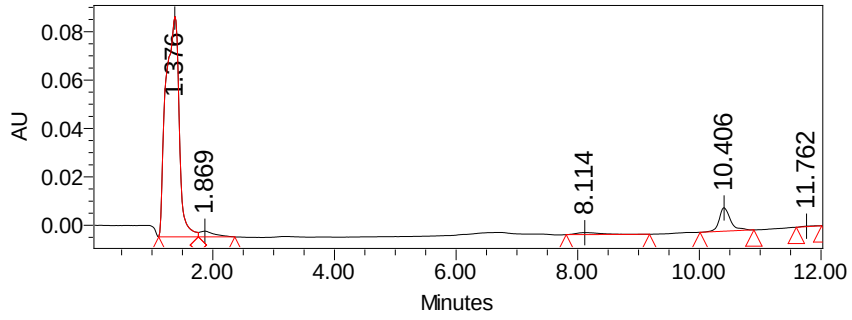

Figure 40. HPLC chromatogram of *Nigella sativa* extract 7.

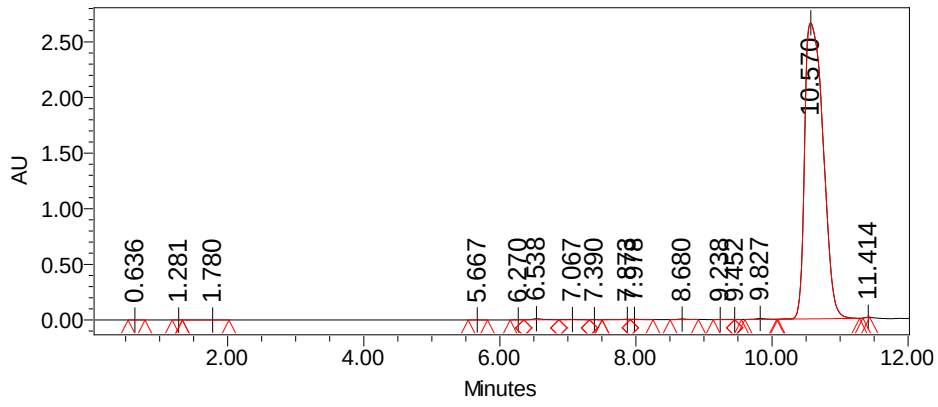

Figure 41. HPLC chromatogram of *Nigella sativa* extract 9.

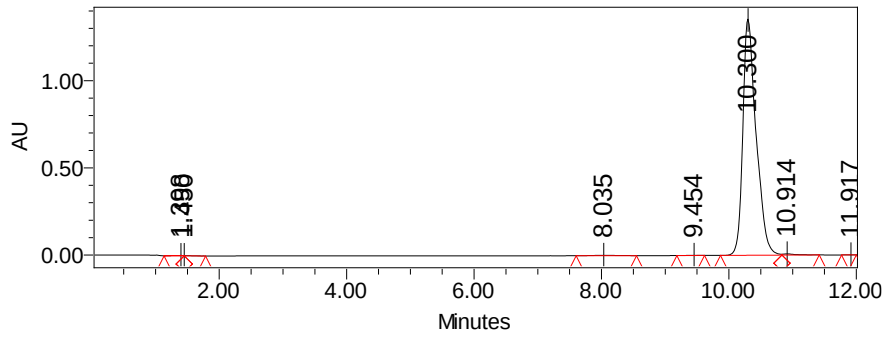

Figure 42. HPLC chromatogram of *Nigella sativa* extract 10.