UNIVERSITI PUTRA MALAYSIA

STABILITY OF ALGERIAN BLACK SEED (Nigella sativa L.) IN RELATION TO ANTIMICROBIAL ACTIVITY UNDER DIFFERENT STORAGE CONDITIONS

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By

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Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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Chairman : Intan Safinar Ismail, PhD
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The present study is an effort to determine the stability of the main volatile constituents of *Nigella sativa* seeds stored under several conditions. Nine storage conditions which were set based on the ecological abiotic effects of air, humidity, heat and light, with six replicates each were prepared and analyzed with Headspace (HS)-Gas Chromatography-Mass Spectrometry (GCMS) for three time points of initial (1st day – 0), 14th (1) and 28th (2) day of storage. A targeted multivariate analysis of PCA revealed that the stability of the whole seeds main volatile constituents were better than those of the ground seeds in relation to the selected ecofactors. The findings showed that the air, humidity, heat and light are suggested to be in reverse relationship with the stability of *N. sativa* seeds. Based on the study, the condition which showed minimal changes towards the environmental effects is that whole seeds stored in a transparent vial with nitrogen gas flush. The disc-diffusion test antimicrobial activity of the different stored conditions of *N. sativa* samples were conducted on several human pathogens wherein the whole seed stored in limited air content (condition B and C) and exposed to light gave more promising results. However, the minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) results showed no significant difference among all conditions after 28 days storage. The antimicrobial metabolites were identified through correlation of antimicrobial activities (disc-diffusion test) with *N. sativa* whole seeds of different storage conditions by supervised multivariate data analysis (MVA) of Orthogonal Partial Least Squares (OPLS), which suggested that 7 significant variables responsible as chemical markers. Furthermore, the predictability of antimicrobial activity based on the whole seed model was done on 10 new observations of known GC-MS data whereby only fresh whole seed (MEOH) sample was positively predicted. Based on the present findings, *N. sativa* seeds need suitable storage parameters to preserve the volatiles for less quality deprivation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
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KESTABILAN BIJI HITAM ALGERIA (*Nigella sativa* L.) TERHADAP AKTIVITI ANTIMIKROBIAL DALAM KEADAAN PENYIMPANAN BERBEZA

Oleh

MUHAMMAD SAFWAN BIN AHAMAD BUSTAMAM

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Kajian yang dilakukan ini merupakan satu usaha untuk menentukan kestabilan komponen meruap utama dalam biji *Nigella sativa* yang disimpan di dalam beberapa keadaan. Sembilan keadaan penyimpanan yang telah ditetapkan berdasarkan kesan abiotik ekologi udara, kelembapan, haba dan cahaya, dengan enam ulangan setiap satu telah disediakan dan dianalisis dengan *Headspace* (HS) - Gas kromatografi Jisim Spektrometri (GC-MS) untuk tiga tempoh masa iaitu permulaan (1 hari - 0), 14 (1) dan 28 (2) hari penyimpanan. Analisis data multivariate secara sasaran menggunakan komponen prinsip analisis PCA mendedahkan kestabilan komponen meruap utama dalam biji mentah adalah lebih baik daripada biji kisar bedasarkan kofaktor yang dipilih. Hasil kajian menunjukkan bahawa udara, kelembapan, haba dan cahaya dicadangkan mempunyai hubungan bertentangan dengan kestabilan biji *N. sativa*. Berdasarkan kajian itu, keadaan yang menunjukkan perubahan yang minimum terhadap kesan alam sekitar adalah biji mentah yang disimpan di dalam botol telus dengan semburan gas nitrogen . Ujian cakera penyebaran aktiviti antimikrob terhadap sample *N. sativa* sampel yang disimpan dalam keadaan berbeza telah dijalankan ke atas beberapa patogen manusia dimana penyimpanan biji mentah dalam kandungan udara terhad (kondisi B dan C) yang jauh dari cahaya memberikan hasil lebih bagus. Walaubagaimanapun kepekatan minimum perencatan (MIC) dan kepekatan bakteria minimum (MBC) tidak menunjukkan perbezaan yang signifikan dalam semua keadaan selepas penyimpanan 28 hari. Metabolit antimikrob telah dikenal pasti melalui korelasi aktiviti antimikrob (ujian cakera penyebaran) dengan keadaan penyimpanan biji mentah *N. sativa* yang berbeza melalui analisis data multivariat (MVA) berpandu menggunakan Ortogon Dua Terkecil Separa (OPLS) lalu mencadangkan 7 metabolit penting sebagai penanda kimia. Tambahan pula, ramalan terhadap aktiviti antimikrob berdasarkan model biji mentah telah dilakukan pada 10 pemerhatian baru dengan mengetahui data GC-MS dan hanya sampel biji mentah segar (MEOH) menunjukkan ramalan positif. Berdasarkan kajian ini, biji *N. sativa* memerlukan parameter penyimpanan sesuai untuk memelihara komponen meruap daripada pengurangan kualiti.

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I certify that a Thesis Examination Committee has met on 11 March 2016 to conduct the final examination of Muhammad Safwan bin Ahamad Bustamam on his thesis entitled "Stability of Algerian Black Seed (*Nigella sativa* L.) in Relation to Antimicrobial Activity under Different Storage Conditions" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Declaration by Members of Supervisory Committee

This is to confirm that:

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
B. cereus	*Bacillus cereus*
B. subtiius	*Bacillus subtilus*
CFU	Colony forming unit
DMSO	Dimethylsulfoxide
HS	Head space
KCCM	Korean Culture Centre of Microorganism
K. pneumoniae	*Klebsiella pneumonie*
MBC	Minimal bactericidal concentration
MHA	Mueller Hinton agar
MIC	Minimal inhibitory concentration
MVA	Multivariate data analysis
OPLS	Orthogonal Partial Least Squares
S. aureus	*Staphylococcus aureus*
V. parahaemolyticus	*Vibrio parahaemolyticus*
VIP	Variable importance in the projection

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Since thousands of years, medicinal plants have been greatly used as folk medicine and as alternatives to replace modern medicine due to the negative long-term effects on the human health. The increasing demand of natural herbal product throughout the world leads the scientist to investigate the efficacy of various herbs. Medicinal plant-based products with their potential and safety have been scientifically proven and globally commercialized (Ahmad *et al*., 2013).

One of the famous medicinal plants which have been greatly studied until present is *Nigella sativa* or black seed. Since about 1400 years ago, it was mentioned in the Islamic literature by the Prophet Muhammad S.A.W that *N. sativa* was considered as one of the greatest forms of remedy for many diseases except death (Al-Bukhari, 1976). According to SCIFinder® statistics in the database of the Chemical Abstracts Service (CAS), about 960 articles have been published until 2011 related to various medicinal properties of *N. sativa* which include antihypertensive, antidiabetic, anticancer, gastroprotective, antioxidant, anti-inflammatory, antihistamine, antimicrobial, antitumor, anti-hepatotoxic, anti-nephrotoxic, respiratory and immunomodulatory effects (Liu *et al*., 2011).

Antibiotic is no lesser important as it has revolutionised mankind's health status by treating the life threatening infections. However with the increasing occurrence of bacterial resistance against available antibiotics, it has now become essential to look for newer antibiotics with less or no resistance. Most of the antibiotics available today are from natural origin particularly from various microbial and marine sources. Nevertheless, plants also produce compounds to protect themselves from microbial attacks. Strong *in vitro* evidence from scientific study indicated that essential oils of various aromatic plants including black seed could act as antibacterial agents against a wide spectrum of pathogenic bacteria (Edris, 2007). Consequently, this assay was used to determine the antimicrobial potential of *N. sativa* against several human pathogens.

Chemical constituents scientifically responsible for the pharmacological potentials of black seed have been reported widely. The seeds are very rich and diverse in volatile compositions and generally composed of several terpene classes. Benkaci-Ali *et al*. (2006) reported that the highest volatile constituents were represented by monoterpene hydrocarbons (76.52%) and ketones (17.31%), followed by several minor classes in traces amounts such as sesquiterpene hydrocarbons (1.77%), alcohols (1.61%), esters (0.23%), aldehydes (0.01%) and diterpene hydrocarbons (traces).

Hence, various extraction and analytical technique in studying the volatiles composition of *N. sativa* have been previously reported whereby among the many, hydro distillation (HD) was regularly applied (Bourgou *et al*., 2010; Toma *et al*., 2010; Wajs *et al*., 2008). Another techniques reported were steam distillation (SD) (Kokoska *et al*., 2008), solvent extraction (SE) (Kokoska *et al*., 2008; Nickavar *et al*., 2003) and supercritical fluid extraction (SFE) (Venkatachallam *et al*., 2010; Kokoska *et al*., 2008). However, to our best knowledge until now, there is no report on the application

of static headspace as solvent-free fractionation of volatile sample. The head space method was preferable to be used for volatile compound analysis since this method is less time and cost consuming. It is also a combination of extraction, concentration and sampling in one single step without any usage of organic solvent (Kohlert *et al*., 2002). The headspace technique could be a direct combination with Gas chromatography couple with mass spectrometry (HS-GC-MS). This combination has been proposed as a competitive fast-response analytical tool completed with structural information (mass spectrum) which is required for the analysis of multi- secondary volatile components suitable for the constituents qualitative and quantitative analysis (Soria *et al*., 2008).

Furthermore the comparisons between data set generated from the chromatography method are often studied using concepts of multivariate data analysis (MVDA) (Brereton, 2003). The method has been successful applied to compare different in origin and quality of food (Matos *et al*., 2007; Moreda-Pineiro *et al*., 2003), and plants (Qiu *et al*., 2007; Schulz *et al*., 2005). Thus, the differences of chemical constituents in *N. sativa* in several storage conditions have been analyzed by multivariate concept based on the peak areas from total ion chromatogram (TIC) of the mass spectrometry.

1.2 Problem Statements

Numerous bioactivity tests which include antihypertensive, antidiabetic, anticancer, gastroprotective, antioxidant, anti-inflammatory, antihistamine, antimicrobial, antitumor, anti-hepatotoxic, anti-nephrotoxic, and respiratory and immunomodulatory effect have been conducted on *Nigella sativa* seeds (Liu et al., 2011). Due to these many therapeutic claims of *N.* sativa, seed-based products mainly in health supplement forms are widely available in the market. The shelf life and content consistency of this plant seeds and oil are much affected by the volatiles stability which is important for the seed and its formulations' pharmacological efficacy. The primary step in the quality assurance of a standardized efficacious *N.* sativa seed-based product is the storage of the raw material or the whole seeds. Despite many people share the logical concept of these elements having negative influence on the volatile constituents, no scientific evidence was shown before. The degradation of the volatiles will be affecting the pharmacological potential including the antimicrobial effects since the volatiles contained the compounds having functional group such as phenolic hydroxyl group which responsible in attacking the cell membranes of the pathogens. Hence, HS-GCMS in combination with multivariate data analysis entailed assessing the effects of four ecofactors which are air, humidity, heat and light, on the main volatile constituents of the *Nigella sativa* seeds to pave guides on the optimum storage conditions as explained herein. The antimicrobial activity was then determined in correlation with the volatile compositions studied from the first part using metabolomics with multivariate data analysis.

1.3 Research Objective(s)

Based on the problem statement, the present study was aimed to achieve these objectives:

1. To determine the stability of the main constituents in *N. sativa* samples stored in several conditions at three time points; 1st, 14th and 28th day of storage.
2. To evaluate and compare the antimicrobial activity of *N. sativa* samples stored in different conditions against several human pathogens; *Bacillus subtilis*, *Bacillus cereus*, *Vibrio parahaemolyticus*, *Propionibacterium acnes*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus mutans*.
3. To suggest the chemical markers of the antimicrobial assay through correlation of antimicrobial activities with the composition of *N. sativa* at different storage conditions.

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LIST OF PUBLICATIONS

Journal Article

Ahamad-Bustamam , M. S., Hadithon, K. A., Mediani, A., Abas, F., Rukayadi, Y., Lajis, N., Shaari, K., and Ismail, I. S., (2016). Stability Study of Algerian *Nigella sativa* Seeds Stored under Different Conditions**.** *Journal of Analytical Methods in Chemistry* – Accepted for publication

Yusoff, N. A. H., **Bustamam, M. S**., Abas, F., Khatib, A., & Rukayadi, Y. (2014).Antimicrobial activity of Cosmos caudatus extracta against foodborne pathogens. *Journal of Pure and Applied Microbiology*, *8*(5), 1–8.

Poster and Proceeding

Bustamam, M. S., Ismail, I. S., Hadithon, K. A., Abas, F., Rukayadi, Y. 2013. Stability on storage conditions of Algerian *Nigella sativa*. In The 26th Symposium of Malaysia Analytical Sciences (SKAM 26) 4-5 December, Hilton Kuching, Kuching, Sarawak

Oral Presentation

Ahamad-Bustamam,M. S., Ismail, I. S., Hadithon, K. A., Mediani, A., Abas, F., Rukayadi, Y., and Lajis, N. (2016) Stability Study of Algerian *Nigella Sativa* in Different Storage Conditions Related to Antimicrobial Activity. In 1st International DOST-SEI ASTHRDP-NSC Scholars's Conference, 7-8 April, Philippine International Convention Centre, Metro Manilla

Ahamad-Bustamam,M. S., Ismail, I. S., Hadithon, K. A., Mediani, A., Abas, F.,Rukayadi, Y., and Lajis, N. (2016) GCMS-Volatile Constituents of Algerian *Nigella Sativa* of Different Storage Conditions in Relation to Antimicrobial Activity. In International Conference On Natural Product (ICNP), 15 - 17 March 2016, Permai Hotel, Kuala Terengganu

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