PHYTOCHEMICAL PROFILE, ANTIMICROBIAL ACTIVITY OF NUTMEG (Myristica fragrans Houtt.) SEED EXTRACT AND ITS EFFICACY AS PRESERVATIVE FOR BEEF AND SHRIMP

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FSTM 2019 30

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By

OSAMA MOHAMED BEN LAGHA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

May 2019

DEDICATION

This PhD research thesis is dedicated to all members of my family, especially to my: father, Mohamed Ben Lagha; mother, Rabeah Mohia; sisters, Eman, Esmahan, Elham; my brother, Ahmed and to my wife Esra and my three beloved kids Rinad, Mohamed and Rua.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Doctor of Philosophy

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Food preservation prevents the growth of foodborne pathogens, which can cause foodborne illness. Several methods already have been established for food preservation, such as using artificial food additives. However, application of artificial food additives in the long term could affect human health. This study aimed to evaluate extracts of nutmeg (*Myristica fragrans* Houtt.) seed against a range of foodborne pathogens: *Escherichia coli* ATCC25922, *Listeria monocytogenes* ATCC19112, *Bacillus cereus* ATCC33019, *Pseudomonas aeruginosa* ATCC9027, *Vibrio parahaemolyticus* ATCC17802, *Bacillus pumilus* ATCC14884, and *Staphylococcus aureus* KCCM12255. Then, identify the active antimicrobial compounds and phytochemical constituents in effective nutmeg seed extracts and evaluate their effectiveness as a preservative for raw beef and shrimp. The results showed that methanol extract of nutmeg seed exhibited antibacterial activity against Gram-negative and Gram-positive bacteria, with the diameter of the inhibition zone ranging between 9.02 ± 0.05–12.05 ± 0.50 mm. The nutmeg methanolic extract was more effective against *B. cereus, B. pumilus, L. monocytogenes, E. coli,* and *S. aureus,* with minimal inhibitory concentrations (MICs) of 0.15, 0.62, 0.62, 0.62 and 0.62 mg/mL, respectively, than *V. parahaemolyticus* and *P. aeruginosa*, with MICs of 2.5 and 1.25 mg/mL, respectively. However, this extract exhibited moderate minimal bactericidal concentration (MBC) against all tested bacteria. The kinetics of the time-kill assay of the methanolic extract *Myristica fragrans* Houtt. against the tested bacteria were not steady. In general, there no significant changes in viable cell count (Log_{10} CFU/mL) of bacteria treated with 0.5×MIC/0.5 h compared to the control. Additionally, the growth of *V. parahaemolytics* and *B. pumilus* bacteria showed steady kinetics when treated with 2×MIC. Complete killing of *B. cereus* cells treated with MIC, 2×MIC, and 4×MIC of the methanolic nutmeg extract was observed within 2, 2, and 4 h after treatment. Scanning electron microscope (SEM) analysis of representative pathogens showed that the 1% methanolic extract caused distortion of the bacteria, with changes in the external shape of the bacterial cells observed after 4 h, eventually leading to cell rupture after 24 h. Different solvent systems were evaluated by thin layer chromatography (TLC), hexane: ethyl acetate and chloroform: methanol in the ratios of 5:5, 8:2, 2:8, 7:3 and 9:1. The results showed that the solvent system of hexane: ethyl acetate (8:2) was the best solvent system for the distribution of the active compounds. In addition, all solvent systems showed positive results for the bioautography assay. Based on the microbial assay results, the active methanolic extract was analysed by gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). GC-MS, identifying 36 compounds, with myristicin being the major compound (10.34%), followed by terpinene-4-ol (7.22%), tetradecanoic acid (5.66%), safrol (1.80%) and eugenol (0.32%). Results from the score plot (PCA) derived from the GC-MS data showed that the hexane extract and methanolic extract were related and distinct in comparison to the chloroform extract. Furthermore, partial least squares (PLS) score plots derived from GC-MS data showed that the tested bacteria statistically were more sensitive to particular active compounds. LC-MS, identifying the presence of active compounds including, limonene, γ-thujaplicin, palmitic amid and gingerol. Regarding the application, factorial design was employed and two storage conditions, 4^{o}C and -18^{o}C were used. The samples of beef and shrimp treated by 0.5%,1.5%,3% and 5% (v/w). The nutmeg extract in 1.5% and above significantly reduced the number of microflora and foodborne pathogens in beef and shrimp samples, and thereby extending the shelf life. The percentage of the reduction values in beef was between 71.81% and 99.75%. Meanwhile, the results ranging between 94.50% to 98.6% for the shrimp samples. Results showed that 1.5% of nutmeg extract maintained the thiobarbituric acid reactive substances (TBARS) and total volatile basic nitrogen (TVB-N) value at a minor level compared to the control samples. Regarding the sensory evaluations, 1.5% and 3% nutmeg extracts applied to raw and cooked samples were more acceptable to the panellists than the 5% nutmeg extract. In conclusion, nutmeg extract has the potential to be developed as a natural antimicrobial in food system as a natural alternative to synthetic preservatives.

Keywords: antimicrobial activity, *Myristica fragrans* Houtt., nutmeg, natural food preservative.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PROFIL FITOKIMIA, AKTIVITI ANTIMIKROB DARIPADA EKSTRAK BIJI BUAH PALA (*Myristica fragrans* Houtt.) DAN KEBERKESANANNYA SEBAGAI BAHAN AWET DAGING LEMBU DAN UDANG

Oleh

OSAMA MOHAMED BEN LAGHA

Mei 2019

Pengerusi : Profesor Madya Yaya Rukayadi, PhD
Fakulti : Sains dan Teknologi Makanan

Pemeliharaan makanan menghindar pertumbuhan patogen bawaan makanan. Pertumbuhan mikroorganisma dalam produk makanan boleh menyebabkan penyakit bawaan makanan. Beberapa kaedah telah diwujudkan untuk pemeliharaan makanan seperti penggunaan bahan tambahan makanan tiruan. Walau bagaimanapun, penggunaan bahan tambahan tiruan untuk jangka masa panjang telah menjejaskan kesihatan manusia. Oleh itu, pembangunan bahan makanan semula jadi yang berasal dari sumber tumbuhan semakin popular pada masa kini. Dalam kajian ini, aktiviti antibakteria terhadap ekstrak biji benih *Myristica fragrans* Houtt. diekstrak daripada metanol dan etanol dikaji terhadap tujuh spesies bakteria: *Escherichia coli* ATCC25922, *Listeria monocytogenes* ATCC19112, *Bacillus cereus* ATCC33019, *Pseudomonas aeruginosa* ATCC9027, *Vibrio parahaemolyticus* ATCC17802, *Bacillus Pumilus* ATCC14884 dan *Staphylococcus aureus* KCCM12255. Keputusan menunjukkan bahawa cabutan ekstrak pala methanolic mempamerkan aktiviti antibakteria terhadap bakteria Gram-negatif dan Gram-positif. Diameter zon inhibisi adalah sekitar 9.02 ± 0.05–12.05 ± 0.50 mm. Ekstrak pala diperoleh daripada metanol telah menunjukkan kepekatan perencatan minimum (MIC) penting. Nilai MIC ekstrak tersebut adalah 0.15, 0.62, 0.62, 0.62 and 0.62 mg/mL terhadap *B. cereus*, *B. pumilus*, *L. monocytogenes*, *E. coli* dan *S. aureus*. Manakala nilai MIC terhadap *V. parahaemolyticus* dan *P. aeroginosa* adalah2.50 dan 1.50 mg / mL. Secara keseluruhan, ekstrak menunjukkan nilai kepekatan bakteria minimum (MBC) yang sederhana terhadap semua bakteria yang diuji. Masa membunuh asai *Myristica fragrans* Houtt. terhadap bakteria yang diuji menunjukkan kinetik yang tidak stabil. Secara umum, bakteria yang dirawat dengan ekstrak kepekatan 0.5 × MIC / 0.5 h tidak menunjukkan perubahan ketara dalam jumlah sel yang berdaya maju (Log_{10} CFU / mL) berbanding dengan kawalan. Selain itu, kinetik pertumbuhan bakteria yang paling stabil adalah *V. parahaemolytics* dan *B. pumilus* apabila dirawat oleh 2 × MIC. Pembunuhan lengkap *B. cereus* yang dirawat dengan MIC, 2 × MIC, dan 4 × MIC

ekstrak diamati dalam 2, 2 dan 4 jam selepas merawat dengan ekstrak pala. Penemuan mikroskop elektron scanning (SEM) terhadap patogen perwakilan menunjukkan bahawa ekstrak metanol pada 1% mempunyai kesan herotan. Perubahan dalam bentuk luaran bakteria diperhatikan selepas 4 jam, yang menyebabkan sel-sel bakteria luka dan menyebabkan letupan sel selepas 24 jam. Analisis TLC menggunakan sistem pelarut yang berbeza digunakan heksana: etil asetat dan kloroform: metanol 5: 5, 8: 2, 2: 8, 7: 3 dan 9: 1. Keputusan menunjukkan bahawa sistem pelarut 8: 2 heksana: etil asetat adalah sistem pelarut terbaik untuk pengedaran sebatian aktif pada kertas TLC. Di samping itu, semua sistem pelarut menunjukkan keputusan positif untuk ujian bioautografi. Berdasarkan kajian ini, analisis ekstrak metanol oleh GC-MS mengenal pasti 36 sebatian, beberapa metabolit seperti: myristicin dianggarkan sebagai sebatian utama14. 88% dan sebatian lain diperhatikan sebagai sebatian kecil, contohnya: eugenol 0.291% -0.321%, safrol 1.45% -1.80%. Hasil daripada plot plot (PCA) yang diperolehi daripada data GC-MS, menunjukkan bahawa ekstrak heksana dan ekstrak metanol berkaitan dan berbeza berbanding ekstrak kloroform. Selain itu, plot skor (PLS) yang diperoleh daripada data GC-MS menunjukkan bahawa kedua-dua bakteria perwakilan lebih sensitif terhadap sebatian. Mengenai permohonan itu, reka bentuk faktorial digunakan dan dua keadaan penyimpanan, 4ºC dan -18ºC digunakan. Sampel daging lembu dan udang dirawat sebanyak 0.5%, 1.5%, 3% dan 5% (v / w). Ekstrak pala pada 1.5% dan ke atas mengurangkan jumlah mikroflora dan patogen yang terdapat dalam sampel daging lembu dan udang, dan dengan itu memperluaskan kehidupan rak. Peratusan nilai penurunan daging lembu adalah di antara 71.81% dan 99.75%. Sementara itu, hasilnya adalah dari 94.50% hingga 98.61% untuk sampel udang. Keputusan menunjukkan bahawa 1.5% ekstrak pala memelihara bahan-bahan reaktif asid thiobarbituric (TBARS) dan nilai nitrogen asas (NTV) yang tidak menentu pada tahap kecil berbanding dengan sampel kawalan. Mengenai penilaian deria, 1.5% dan 3% nutmeg ekstrak yang digunakan untuk sampel mentah dan dimasak lebih mudah diterima oleh ahli panel daripada ekstrak nutmeg 5%. Kesimpulannya, ekstrak pala mempunyai potensi untuk dikembangkan sebagai antimikrobial semula jadi dalam sistem makanan sebagai alternatif semulajadi untuk pengawet sintetik.

Kata kunci: aktiviti antimicrob, *Myristica fragrans* Houtt., Pala, pengawet makanan

ACKNOWLEDGEMENTS

To Allah The Merciful for sustaining me and always rewarding me with the faith and patience under challenging times, I am thankful, grateful and honoured by your generosity upon my life.

To my ever encouraging father, mother, sisters, brother, and brothers in law. I really thank you all for always finding the right things to say when I needed to hear. The patience and emotional support which you have given to me was and continues to be a fundamental strengthening agent in my life.

To Assoc. Prof. Dr. Yaya Rukayadi, thank you for the support and wisdom which you so willingly gave to me, know that you will always be greatly appreciated and respected by me.

Thanks to my co-supervisors, Assoc. Prof. Dr. Intan Safinar Ismail and Assoc. Prof. Dr. Nor Khaizura Mahmud @ Ab Rashid for sharing their knowledge, experience, support, and motivation.

Last but not least, thank you, my beloved wife, for supporting and taking care of our life and our family.

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ANOVA	Analysis of variance
CDC	Centres of Disease Control and prevention
CFU	Colony forming unit
CHX	Chlorhexidine
CLSI	Clinical and Laboratory Standards Institute
DMSO	Dimethyl sulfoxide
GC-MS	Gas Chromatography-Mass Spectrometry
GRAS	Generally Regarded as Safe
IBS	Institute of Bioscience
LC-MS	Liquid Chromatography-Mass Spectrometry
MBC	Minimum Bactericidal Concentration
MHB	Mueller Hinton Broth
MIC	Minimum Inhibitory Concentration
MOH	Ministry of Health
m/z	Mass/charge ratio
NIST	National Institute of Standards and Technology
NRCS	Natural Resource Conservation Service
OD	Optical Density
PBS	Phosphate Buffered Saline
PCA	Principal Component Analysis
PLS	Partial Least Squares
ppm	Parts per million
rpm	Revolutions per minute

SEM	Scanning Electron Microscopy
spp.	Species
TBARS	Thiobarbituric Acid Reactive Substances
TVB-N	Total Volatile Bases Nitrogen
TLC	Thin-Layer Chromatography
TCBS	Thiosulfate Citrate Bile Salts sucrose
UPM	Universiti Putra Malaysia
USFDA	United State of Food and Drug Administration
WHO	World Health Organization
XLD	Xylose Lysine Deoxylate

CHAPTER 1

INTRODUCTION

1.1 Background

The deterioration of many foods is strongly related to microbial activity by food spoilage bacteria, which is considered the initial trigger for food quality reduction, spoilage, and economic loss. Artificial chemicals have been extensively utilised for food preservatives, with recent attention directed towards natural preservatives (Smith-Palmer et al., 1998). A considerable number of plants are beneficial and of pharmaceutical nature, specifically possessing antioxidant, anti-tumour, and anti-infectious activities. Indeed, the health advantages of food for the treatment of health issues have been realised worldwide (Twarog & Kapoor, 2004). Bioactive substances are considered responsible for the previous healing features.

Presently, the rise in the level of resistance of a range of bacterial pathogens is of concern, raising issues regarding successful treatment. Plants have long been used for medicinal applications (Gold & Moellering, 1996). It is believed that approximately 80% of the global society understands they can rely on vegetation formulations as prescription drugs to match their health and wellbeing demands. Natural herbs and spices are thought to be harmless and have been confirmed powerful treatment for certain health conditions. Presently, there has been intensive investigation of potential organic food preservatives, which possess antioxidant and antimicrobial activities as well as preserving the quality, hence shelf life of perishable food and nutrients (Fratianni et al., 2010).

The consumption of food contaminated by pathogens can cause illness caused and has a major economic and public health impact worldwide (Gandhi & Chikindas, 2007). Approximately 76 million cases of illness by foodborne pathogens have been recorded yearly in the United States, with estimated costs ranging between \$6.5 and \$34.9 billion for medication and lost productivity (Buzby & Roberts, 1997; Mead et al., 1999). The World Health Organization (WHO) estimated that foodborne and waterborne diarrhoeal diseases together are responsible for the death of around 2.2 million humans, with approximately 1 million people in the United Kingdom (UK) experiencing foodborne illness yearly.

Unfortunately, studies have found that synthetic chemicals consist of toxic, mutagenic, clastogenic and genotoxic compounds (Farag et al., 1989). In contrast, essentially natural active compounds can be derived from plant sources, such as bay leaves, lemongrass, clove and basil, as well as from animal's sources. Plant essential oils are of great interest to the food industry due to their potential antimicrobial effects, as they are highly available and generally safe, not containing chemicals (Burt, 2004a). Consequently, this has led to the use of natural sources in food production to reduce the reproduction of foodborne pathogens as they cause no health issues for consumers (Omoruyi & Emefo, 2012).

The dried seed kernel of "*Myristica fragrans* Houtt." (Family *Myristicaceae*), commonly known as nutmeg, contains volatile oils, starch, fats, and mucilage. The fixed oil contains trimyristin and myristic acid, while terpenes and alkenyl benzene derivatives are components of the volatile oil, with myristicin, safrole, and elemicin constituting about 80% of the alkylbenzene derivatives (El-Alfy et al., 2010). Traditionally, nutmeg has been used in cooking as a spice, as well as in alternative medicine as a stimulant, antidiarrheal, carminative, stomachic, tonic, and as an aphrodisiac (El-Alfy et al., 2010). Pharmacological actions attributed *in vitro* and *in vivo* studies and have occurred anaesthetic effects in a wide array of nutmeg. There is an obligation to develop alternative safer, cheaper drugs which possess active antimicrobial agents for therapeutic administration to treat bacterial infections, with no undesirable effects of currently available drugs (El-Alfy et al., 2010).

This study aims to evaluate the antimicrobial activity of nutmeg and its application as natural food preservative agent in beef and shrimp, which can reduce the spoilage of the shrimp and beef as well as enhance the sensory attributes of the raw material. Moreover, it is anticipated that this research will significantly contribute more information about how the mechanism of action of the nutmeg extracts, thereby helping to understand the difference between natural antimicrobial products and synthetic preservatives.

1.2 Problem Statement

The consideration in recent years has focused on processed food, but synthetic preservatives which have been used in foods for decades probably have negative health consequences (Namiki, 1990). Several chemical preservatives have been used in the food industry as antimicrobial / antioxidant agents, such as sodium benzoate, benzoic acid, sodium sorbate, potassium sorbate, sodium nitrite and sulphur dioxide (Sharma, 2015; Silva & Lidon, 2016). However, these synthetic preservatives are responsible for many health risks, including cancer, urticaria and contact dermatitis, allergies, asthma and skin rashes (Sharma, 2015). Additionally, some countries have become stricter regarding residual drugs and antibiotics contained in exported food. For instance, shrimp shipments have been refused and considered unsafe for consumers (Hayes & Veal, 2014). Furthermore, consumers are now demanding healthier foods with natural components rather than chemical preservatives.

Natural preservatives were utilised from sources such as bacteria, fungi, plants, and animals, possessing the capability to maintain the safe practices of food credited to their valuable antimicrobial activity with a vast assortment of foodborne pathogens (Gyawali & Ibrahim, 2014a). The phytochemical constituents include monoterpenes, sesquiterpenes, terpenoids, alcohols, aldehydes, ketones, phenolics, esters, and other complex aromatic and aliphatic compounds, which are responsible for the antimicrobial and antioxidant properties of essential oils (Bertoli et al., 2011). However, certain characteristics influence the antimicrobial properties, such as botanical origin, cropping months, time of flowering, and the extraction technique. For example, Chorianopoulos et al. (2006) reported that essential oils (EOs) from the aromatic plants of the family Lamiaceae (*Satureja montana*) were at their maximum bactericidal efficiency when extracted at the time of flowering.

Structure and functional groups of the oils are other significant factors affecting the antimicrobial activity. Usually, phenolic groups are considerably efficient (Chorianopoulos et al., 2006), with the proportion of the chemical component, composition and concentration determining the biological influences of EOs. For instance, higher concentrations of terpenoids in EOs result in higher antifungal activity compared with EOs rich in monoterpenes and sesquiterpenes, whereas EOs containing higher amounts of thymol and carvacrol showed more significant membrane damage in bacteria compared to those EOs that had less phenolics (Kordali et al., 2005).

Ingredients, like lipid and protein in meat, are commonly thought to influence their quality. Lipid oxidation can often be a contributing factor to the decrease in quality through the development of a bitter flavour, thereby reducing the shelf life and nutrient content (Králová, 2015). Despite several studies on the antimicrobial activity of EOs from various plants and spices, the *in vitro* findings did not easily apply EOs for food systems, such as beef or shrimp, for preservative purposes. Generally, beef and shrimp are natural decomposable foods. To achieve the same effect in meat compared with *in vitro* assays, a greater concentration of EOs is required (Burt, 2004b). Typically, higher concentrations of the EOs should be applied to attain considerable antimicrobial activity that causes transformation in the quality, taste, and aroma of the meat (Gutierrez et al., 2009). Nonetheless, even though some EOs are used in low concentrations for food preservation, numerous EOs have a strong aroma and flavour, which if used to preserve meat could affect the quality, thus be undesirable for consumers. Therefore, improved concentrations for food application, and undesirable outcomes on acceptability of beef and shrimp should be investigated.

1.3 Objectives

The objectives of this study are:

1. To extract the seed of *Myristica fragrans* Houtt. by methanol and determine the antimicrobial activities of nutmeg *Myristica fragrans* Houtt. seed extracts against a range of foodborne pathogens.
2. To identify the active antimicrobial compounds and phytochemical constituents in the *Myristica fragrans* Houtt. seed extracts. and investigate the metabolomics approach of selected fractions of the *Myristica fragrans* Houtt. seed extract.

3. To evaluate the effect of the active nutmeg, *Myristica fragrans* Houtt. seed extracts at different concentrations, and exposure times at different storages condition on microflora, oxidative activity (TBARS), total volatile bases nitrogen (TVB-N) and sensory attributes on raw beef and shrimp.

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