UNIVERSITI PUTRA MALAYSIA

IDENTIFICATION OF METABOLIC SIGNATURES ASSOCIATED WITH ANTI-INFLAMMATORY EFFECTS OF *Pheonix dactylifera* L. USING NMR AND MS-BASED METABOLOMICS

NUR ASHIKIN ABDUL HAMID

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By

NUR ASHIKIN ABDUL HAMID

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 thesis is dedicated to my beloved parents and my much-loved grandfather.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

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Chairman : Associate Professor Faridah Abas, PhD
Institute : Bioscience

Phoenix dactylifera L. (date palm) is an essential agricultural crop in most arid regions of the world and have been used in traditional medicine to treat illnesses. The extracts of *P. dactylifera* pulps and seeds are reported to possess valuable pharmacological attributes including antioxidant, anti-fungal and anti-inflammatory activity. However, there is still a lack of scientific data to support the potency of *P. dactylifera* in treating inflammation. Thus, the primary purpose of this study is to fill in the current research gap concerning the mechanism underlying the anti-inflammatory effects of this fruit crops. There are many date varieties available in the market with various phenotypic features, consumers' preferences and price ranges. Eighteen varieties of dates from two date producing countries were selected including Algerian Lahmira (LM), Timjouhert (TM), Adham Talmine (AT), Deglet Talmine (DT), Adam Boullah (AB), Tinasser (TN), Deglet (DG), Deglet Nour (DN) and Takerbouch (TB) along with Saudi varieties of Berni (BR), Halaoua (HL), Shalabi (SB), Sogaai (SG), Sukkari (SK), Nebtat Ali (NB), Anbara (AN), Ajwa (AJ) and Medjoul (MJ). The quality was evaluated based on the metabolite composition, physico-chemical characteristics and biological activities which include nitric oxide (NO) inhibition via the cell-based approach and NO scavenging abilities. The correlation between the phytochemicals and biological activities was achieved via ^{1}H nuclear magnetic resonance (NMR) based metabolomics approach. Algerian Deglet and Saudi Ajwa showed the most promising NO inhibition as compared to the rest of date varieties. The current study was then focusing on the variation in phytochemicals and biological activities between the seeds and pulps of *P. dactylifera.* The information on chemical constituents was further strengthen utilizing ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS) technique. The multi-platforms metabolomics of MS and NMR methods were adopted in exploring differences between the phytochemical in seed and pulp extracts. The seed of Algerian Deglet revealed a significantly lower IC_{50} values of NO inhibitory and DPPH scavenging with the IC_{50} values of 107.99 µg/mL

and 12.58 µg/mL, respectively. Among the metabolites that prominently contributed towards the observed bioactivities as suggested by Partial Least Squares analysis including catechin, ascorbic acid and serine. The proposed metabolic pathway indicated the higher amount of several metabolites in date seeds as compared to the pulps. The variation between the extracellular metabolites of RAW 264.7 cells from different passage numbers was determined along with the impact of different harvesting protocols on the intracellular metabolites. The trypsinized RAW cells from lower passage groups gave higher intensities of several metabolites including asparagine, serine and tryptophan. The Principal Component Analysis revealed variation between cells from different passage and harvesting methods as indicated by the formation of clusters in the score plot. The current study is the first to report on the passage and harvesting dependent effects using metabolomics workflow for the murine macrophage, RAW 264.7 cells. The overall bio-markers from the cell metabolome of the induced and treated cells and also bio-active compounds in date extracts linking to the anti-inflammatory property was obtained in the present study. The acquired data adds up to the existing knowledge on anti-inflammatory effects of date seed extracts by highlighting their capacity to assist the inversion of activated RAW 264.7 cells back to the normal state. This work demonstrated the potential application of metabolomics approaches as tools in evaluating quality of plant materials and also in providing better insight into cells biology.

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

PENGENALPASTIAN PENANDA METABOLIK YANG BERKAITAN DENGAN KESAN ANTI-RADANG *Pheonix dactylifera* L. MENGGUNAKAN METABOLOMIK BERASASKAN NMR DAN MS

Oleh

NUR ASHIKIN ABDUL HAMID

Mei 2019

Pengerusi : Profesor Madya Faridah Abas, PhD
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Phoenix dactylifera L. (kurma) adalah tanaman pertanian penting di kebanyakan kawasan kering di dunia dan telah digunakan dalam perubatan tradisional untuk merawat pelbagai penyakit. Ekstrak pulpa dan biji *P. dactylifera* telah dilaporkan mempunyai ciri-ciri farmakologi yang bernilai termasuk aktiviti antioksidan, anti-kulat dan anti-radang. Walau bagaimanapun, masih terdapat kekurangan data saintifik untuk menyokong kemujaraban *P. dactylifera* dalam merawat radangan. Oleh itu, tujuan utama kajian ini adalah untuk mengisi jurang penyelidikan semasa berkaitan dengan mekanisma yang mendasari kesan anti-radang tanaman buah ini. Terdapat banyak jenis kurma yang terdapat di pasaran dengan pelbagai ciri fenotip, pilihan pengguna dan julat harga. Lapan belas jenis kurma dari dua negara penghasil kurma telah dipilih termasuk Algeria Lahmira (LM), Timjouhert (TM), Adham Talmine (AT), Deglet Talmine (DT), Adam Boullah (AB), Tinasser (TN), Deglet (DG), Deglet Nour (DN) dan Takerbouch (TB) manakala dari Saudi iaitu Berni (BR), Halaoua (HL), Shalabi (SB), Sogaai (SG), Sukkari (SK), Nebtat Ali (NB), Anbara (AN), Ajwa (AJ) dan Medjoul (MJ). Kualiti dinilai berdasarkan komposisi metabolit, ciri fiziko-kimia dan aktiviti biologi yang merangkumi perencatan nitrik oksida (NO) melalui pendekatan berasaskan sel dan kebolehan pemerangkapan NO. Hubungan antara fitokimia dan aktiviti biologi dicapai melalui pendekatan metabolomik berasaskan resonans magnetik nuklear ^{1}H (NMR). Algerian Deglet dan Saudi Ajwa menunjukkan perencatan NO yang paling menjanjikan berbanding dengan jenis kurma lain. Kajian semasa kemudian memberi fokus kepada perubahan dalam fitokimia dan aktiviti biologi di antara biji dan pulpa *P. dactylifera*. Maklumat tentang unsur-unsur kimia dikukuhkan dengan penggunaan teknik kromatografi cecair ultra tinggi spektrometri jisim (UHPLC-MS). Metabolomik dari gabungan kaedah MS dan NMR telah digunakan untuk meneroka perbezaan antara fitokimia dalam ekstrak biji dan pulpa. Biji kurma dari Algerian Deglet mendedahkan nilai IC_{50} yang lebih rendah untuk perencatan NO dan pemerangkapan DPPH dengan nilai IC_{50} masing-masing 107.99

μg/mL dan 12.58 μg/mL. Antara metabolit yang menyumbang kepada bioaktiviti yang diperhatikan seperti yang dicadangkan oleh Analisis Separa Kuasa Dua termasuk katekin, asid askorbik dan serine. Laluan metabolik yang dicadangkan menunjukkan jumlah metabolit yang lebih tinggi dalam biji berbanding dengan pulpa. Perbezaan antara metabolit ekstraselular bagi sel RAW 264.7 dari nombor pasaj yang berlainan ditentukan dengan kesan protokol penuaian yang berbeza pada metabolit intraselular. Sel RAW yang diperoleh menggunakan kaedah tripsin dari kumpulan pasaj bawah memberikan intensiti yang lebih tinggi bagi beberapa metabolit termasuk asparagine, serine dan triptofan. Analisis komponen utama mendedahkan perbezaan antara sel daripada nombor pasaj berlainan dan kaedah penuaian yang berbeza seperti yang ditunjukkan oleh pembentukan kluster dalam skor plot. Kajian semasa adalah yang pertama melaporkan kesan pasaj dan kaedah penuaian menggunakan alur kerja metabolomik untuk makrofaj sel mencit RAW 264.7. Penanda bio keseluruhan dari metabolit sel yang teraruh dan dirawat serta sebatian bioaktif dalam ekstrak kurma yang berkaitan dengan anti-radang telah ditentukan melalui kajian semasa. Data yang diperolehi menambah pengetahuan sedia ada ke atas kesan anti-radang ekstrak biji kurma dengan menunjukkan keupayaannya untuk membantu pembalikan sel RAW 264.7 yang diaktifkan kembali kepada keadaan normal. Penyelidikan ini menunjukkan potensi penggunaan pendekatan metabolomik sebagai alat dalam menilai kualiti bahan tumbuhan dan juga dalam memberikan kefahaman yang lebih baik tentang biologi sel.

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

AB	Adham Boullah dates
AJ	Ajwa dates
AN	Anbara dates
AT	Adham Thalmine dates
ANOVA	Analysis of variance
BR	Berni dates
CD_3OD	Deuterated methanol-*d*4
CPMG	Carr–Purcell–Meiboom–Gill
CO_2	Carbon dioxide
CV-ANOVA	Cross-validation-analysis of variance
d	Day
D_2O	Deuterated deuterium oxide
DG	Deglet dates
DN	Deglet Nour dates
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FRAP	Ferric reducing antioxidant power
GCMS	Gas chromatography mass spectrometry
h	Hour
HL	Halaoa dates
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration of a substance

IFN-$_{\gamma}$	Interferon-gamma
iNOS	Inducible nitric oxide synthase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KH_2PO_4	Non-deuterated potassium dihydrogen phosphate
LCMS	Liquid chromatography mass spectrometry
LM	Lahmira dates
LPS	Lipopolysaccharide
MJ	Medjoul dates
min	Minute
NaOD	Sodium deuterium oxide
NaN_3	Sodium azide
NB	Nebtat Ali dates
NMR	Nuclear magnetic resonance
NO	Nitric oxide
NSAIDs	Non-steroidal Anti-inflammatory Drugs
OPLS-DA	Orthogonal Partial Least Squares–Discriminant Analysis
PBS	Phosphate buffered saline
PCA	Principal component analysis
PLS	Partial least squares
PLS-DA	Partial least square-discriminate analysis
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVDA	Multivariate data analysis
PC	Principal component
PRESAT	Pre-saturation

SB	Shalabi dates
SG	Sogaai dates
SK	Sukkari dates
TB	Takerbouch dates
TM	Timjouhert dates
TPC	Total phenolic content
TSP	Trimethylsilyl propionic acid-*d*4 sodium salt
UPLC-MS	Ultra-performance liquid chromatography mass spectrometry
VIP	Variable importance in the projection
^{1}H	Proton
2D	Two-dimensional

CHAPTER 1

INTRODUCTION

1.1 Background

Inflammation refers to an innate, immune defense mechanism in response to infection caused by foreign pathogens or injured cells besides initiating tissue wound healing (Lee et al., 2016). The inflammatory reaction involves with a series of well-controlled mechanisms that acted on several stimulating activities including chemical, microbial and immunological reaction (Tan et al., 2015). Macrophages play vital roles in the expression and release of growth factors and inflammatory mediators including nitric oxide (NO) and prostaglandin E2 (PGE2) (Lee et al., 2016). With regards to pro-inflammatory molecules, NO is biosynthesized in a range of mammalian cells through the reaction between L-arginine and oxygen, and is enzymatically assisted with the enzyme nitric oxide synthase (NOS). Inducible NOS (iNOS) plays a role in the immune defense system and can be stimulated quantitatively by inflammatory stimuli. Nonetheless, the excessive production of NO by iNOS triggers many harmful cellular responses and has been positively associated with the pathophysiology of various illnesses (Khoo et al., 2018; Wong and Lerner, 2015). There is a growing body of literature that recognizes NO as the key signaling molecules in inflammatory reactions, thus, suppression of this pro-inflammatory factor is fundamental for addressing issues linked to the treatment of inflammatory diseases (Lee et al., 2013).

Date palm, (*Phoenix dactilyfera* L.) is considered an ancient crop with significant nutritional, medicinal and economic value. Approximately, 3000 different cultivars of date palm have been utilized for global date production, by which about 60 are extensively cultivated (Moussouni et al., 2017). Algeria is considered to be one of the top producers of date palm fruits with an annual production of up to 468,000 tons (Kehili et al., 2016). The date palm is among the main crop been planted in the Algerian Sahara for both traditional and modern agricultural system and are commonly consumed in Algeria (Moussouni et al., 2017). Likewise, this fruit crop is one of the earliest crops to be cultivated in the Middle East. Saudi Arabia is one of the prominent countries that exports dates and has approximately 7 to 8 million palm trees (Al-Hooti et al., 1997). Nearly 400 different varieties of dates are available from Saudi Arabia, yet, only a limited number of date varieties have been utilized for scientific purposes. Further studies on date palm fruits from different origin of cultivation might probably allow for selection of varieties with the optimum quality attributes for consumption.

Date fruits are composed of a fleshy pericarp and a single seed. The fleshy date pulp has great value as a human dietary component due to its high content of crucial nutrients and the health benefits it offers. In contrast to the high consumption of date pulp, the date seed are typically discarded as by-products by the agriculture industry after the removal of seed from the pulp (Abu-Reidah et al., 2017). Furthermore,

enormous amounts of date seed can also be found on farms where date pastes are made and are normally used for limited applications such as animal feed (Chandrasekaran and Bahkali, 2013). Conventionally, date pulp and date seed have been used in various traditional systems of medicine to cure diseases. In the Ayurvedic text, date pulp was utilized as a treatment for fever, asthma and abdominal pains (Abdul Afiq et al., 2013). Likewise, the date seed powder was conventionally used to relieve toothaches (Adeosun et al., 2016).

From the medicinal viewpoint, date palm fruits have been shown to retain several biological activities, including antioxidant, anti-inflammatory and anti-carcinogenic activities (Al-Mamary et al., 2014; Farag et al., 2014; Saleh et al., 2011). These published data were focusing on the health promoting attributes of date palm fruits as the promising alternatives to synthetic drugs. Previously, several studies have established that the biological activities of date palm fruits vary with regard to the type of variety (Farag et al., 2014; Saleh et al., 2011). Extracts of date pulp and date seed were shown to possess strong antioxidant properties in several *in vitro* (Al-Farsi and Lee, 2011; Maqsood et al., 2015; Platat and M Habib, 2014) and *in vivo* studies (Mousalamy and Hussein, 2016; Orabi and Shawky, 2014; Takaeidi et al., 2014). While a small number of studies have examined the anti-inflammatory effects of date pulps for both *in vivo* and *in vitro* (Al-Farsi & Lee, 2011; Habib et al., 2013; Maqsood et al., 2015; Rahmani et al., 2014), there is still very little scientific understanding regarding the anti-inflammatory effects of date seeds (Orabi and Shawky, 2014; Takaeidi et al., 2014).

Recently, enormous efforts have been undertaken to provide knowledge on the phytochemicals and potential health benefits of date palm (Diboun et al., 2016; Farag et al., 2015; Kehili et al., 2016; Nehdi et al., 2010; Abu-Reidah, et al., 2017). Earlier studies revealed that date pulp contains anthocyanins, phenolics, carotenoids, procyanidins and flavonoids (Farag et al., 2014; Hong et al., 2006). There is an established correlation between the pharmacological attributes of plant materials and their chemical compositions, and specific phytochemicals have been recognized to possess antioxidant, anti-inflammatory and hepatoprotective activities (Jain, 2013). Similarly, date seed can also be regarded an important and affordable natural source of phenolic compounds and dietary fiber (Besbes et al., 2004; Mishra and Ahmed, 2016; Nehdi et al., 2010). The differences in the levels of metabolites in various plant organs have been documented previously (Gogna et al., 2015). Thus, an examination of the health-promoting chemical constituents of date pulp and date seed is believed to be meaningful in the context of exploring the potential pharmacological relevance of crop plants.

Metabolomics offers a powerful platform that can be used to gain a better perception of the chemical constituents of plant materials. Two analytical platforms that have been widely used in investigating the multilayered plant metabolome are ^{1}H nuclear magnetic resonance (NMR) and ultra-high performance liquid chromatography mass spectrometry (UHPLC-MS). Several earlier studies used these tools independently to yield complementary data; hence, the combination of NMR and UHPLC-MS in

metabolomics study is considered to be of importance in obtaining better insight into plant metabolite profiles (Tian et al., 2015). Nevertheless, no data are presently available concerning discrimination between the date pulp and seeds using these advanced approaches. Therefore, this study is conducted to fill the current research gap existing for these two interesting organs of *P. dactylifera*.

Metabolomics approaches have been successfully applied to various bio-fluids, including serum and urine, for the purpose of biomarker characterization, toxicity assessments and disease detection (Leon et al., 2013). Recent progress in metabolomics emphasizes the evaluation on the cell metabolomes of organism ranging from prokaryotes to eukaryotic cells. Cellular metabolomics may offer information on adjustments to biochemical reactions and mechanisms that occur within the cells (Čuperlović-Culf et al., 2010). The comprehensive investigation of cellular metabolism may offer important insight into the biological systems, granting the interpretation of molecular events related to the supplementation of plant extracts. Thus, metabolomics method might be an effective tool in exploring the metabolism of cells in relation to inflammation.

Nonetheless, the progression of cell metabolomics has been hindered by a number of concerns including passage number effects and the choice of harvesting and extraction techniques (Čuperlović-Culf et al., 2010; Muschet et al., 2016). In developing an experimental protocols for cell culture applications, passage number has been identified as a factor of high importance (Leon et al., 2013). Generally, passage number refers to the extent to which a cell line has been sub-cultured. The health of the cells begins to diminish at higher passages, hence may lead to the acquirement of misleading findings. Preventing passage-dependent effects from influencing the experimental data implies the verification of the optimal passage range for which a set of experiments can be conducted using a particular cell line (Hudges et al., 2007). In addition, adherently growing cells must be detached from the culture dishes or flasks prior to extraction. In contrast to metabolomics studies in which bio-fluids are assessed, cellular metabolomics necessitate an additional step in sample handling, which is cell harvesting. To acquire reliable metabolite profiles, an adequately robust and reproducible technique for cell harvesting is needed.

The current work is comprised of two major themes that is plant metabolomics followed with mammalian cell metabolomics. The study begins by focusing on 18 selected varieties of Algerian and Saudi Arabian date palm. The variation in the biological activities and phytochemical constituents in the pulps and seeds were then accomplished for the first time using multi-platform metabolomics based on ^{1}H NMR and UHPLC-MS techniques. The study also provides the first attempt in examining the impact of passage numbers and harvesting protocols on RAW264.7 cell metabolome via the application of NMR metabolomics. The NMR metabolomics method was also applied for the first time in exploring the effects of *P. dactylifera* extracts on the extracellular and intracellular metabolite profiles of the LPS-IFN-γ induced RAW cells.

1.2 Problem statements

Inflammation plays an important role in many illnesses, including rheumatoid arthritis and asthma, each of which has a high global incidence. The clinically useful drugs against inflammation demonstrate undesirable effects, which has led to a considerable interest in seeking out safer alternative therapeutic sources for these conditions. The pulps and seeds of date palms have been used in folk medicines to treat ailments including fever, asthma and toothaches. However, little is currently known concerning their mechanism of action in alleviating inflammation. Characterization of the mode of action is important for our increased understanding of the anti-inflammatory property of *P. dactylifera*. Therefore, it is of interest to perform further experimental studies to investigate the pharmacological potential of date pulp and date seed extracts and to provide scientific evidence as a justification for their conventional application.

Notwithstanding the array of scientific data showing that broad metabolic modifications can be produced by using cells of different passage numbers and various harvesting techniques, the existing data are scarce, particularly for murine macrophage cell lines. Inflammation, evidently would cause metabolic shifts yet much uncertainty still exists about the metabolic status of the extract-treated induced RAW 264.7 cells.

1.3 Objectives

This study seeks to examine the quality variation among selected 18 varieties of *Phoenix dactylifera* and also to gain a better insight on the mechanism of action for *P. dactylifera* in suppressing and inhibiting inflammation. To achieve these goals, several specific objectives were proposed and the metabolomics method was the central methodology used in all research objectives.

The five specific objectives are listed below:

1. To evaluate the phytochemicals of selected 18 varieties of Algerian and Saudi date palms and the correlation with the phenotypic features, proximate composition, NO-inhibitory/scavenging, DPPH scavenging activities for anti-inflammatory property and also total phenolic content via the ^{1}H-NMR metabolomics
   (From the screening activities in Objectives 1, Algerian Deglet and Saudi Ajwa were identified as having the highest activity, thus were selected for further study)
2. To determine the variation in the metabolome of *P. dactylifera* pulp and seeds and correlate their chemical profiles with biological activities using multi-platform metabolomics based on ^{1}H NMR and UHPLC-MS techniques.
3. To examine the influence of passage number and harvesting protocols on metabolite profiles of the murine RAW 264.7 macrophage cell line using NMR metabolomics

(From the results in Objective 3, the seed of Algerian Deglet was used for further analysis)

4. To investigate the effects of *P. dactylifera* seed extracts in LPS-IFN-γ-induced RAW 264.7 cells for the perturbed metabolic pathways and metabolic signatures via the application of metabolomics method

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LIST OF PUBLICATIONS

Abdul-Hamid, N.A., Maulidiani, M., Mediani, A., Izzatul, U., Yahya, I., Safinar, I., Tham, C.L., Shadid, K., Abas, F., 2018. Physicochemical characteristics, nutritional composition, and phytochemical profiles of nine Algerian date palm fruit (*Phoenix dactylifera* L .) varieties. J. Food. Biochem. 42(6), art. no. e12663

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