UNIVERSITI PUTRA MALAYSIA

EVALUATION OF LOCAL SPICES AND HERBS AS MARINATING INGREDIENTS FOR THE REDUCTION OF HETEROCYCLIC AMINES IN GRILLED BEEF

SHABNAM SEPAHPOUR

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By

SHABNAM SEPAHPOUR

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

May 2017

DEDICATION

This thesis is dedicated to

My Dear parents

Who taught me to be strong and tolerant
&
Whose souls are my eternal guardian angels

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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Heterocyclic aromatic amines (HCAs) are an important group of food mutagens and potential carcinogens which are formed during heat processing. Phenolic compounds, ubiquitous in plants are well known for their free radical scavenging activities which can inhibit the formation of HCAs. There is no study which have investigated the effects of different proportions of spices/herbs on the reduction of HCAs in grilled beef. This research has been conducted to determine the antioxidant property of selected herbs and spices, optimize the proportion of their combination to achieve the highest reduction of HCAs, identify the main bioactive inhibitors of HCAs formation and explore the level of mutagenicity and toxicity of marinated grilled beef. Selected herbs and spices (turmeric, curry leaf, lemon grass and torch ginger) were evaluated for their total phenolic compound (TPC), total phenolic content (TFC), DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP). The bioactive compounds of the samples were identified using high performance liquid chromatography (HPLC). Nineteen combinations of selected herbs and spices (turmeric, curry leaf, lemon grass and torch ginger) based on a simplex centroid mixture design were used to marinate beef in order to determine the optimal proportion which achieve the highest reduction of HCAs. The results showed that the combination of turmeric and lemon grass exhibited the highest reduction of HCAs. The optimum mixture of crude extract of these herbs was fractionated to determine the main inhibitors of PhIP formation. Six fractions were collected and applied into PhIP model system containing glucose, phenylalanine and creatinine. Liquid chromatography-mass spectrometry quadruple time of flight (LC-MS/MS Q-TOF) and orthogonal partial least-squares (OPLS) analysis was used to correlate PhIP reduction to different metabolite profiles of each fraction. *In vivo* and *in vitro* experiments were respectively carried out using zebrafish embryo and *Salmonella* Typhimurium (Ames test), to determine whether total HCAs reduction in optimized marinated grilled beef exhibited comparable reduction of toxicity and mutagenicity. The results of experiment showed that turmeric possessed the signifinatly highest TPC (172.10 mgGA/dw), TFC (380.7 mgQE/dw), DPPH antioxidant activity (47.35%) and FRAP (55.79 mgQE/gdw basis) which was followed by curry leaf, torch ginger and lemon grass. The combination of turmeric with lemon grass (50:50 w/w) showed the significanyly highest reduction of total HCAs at 94.7%. Using the response optimizer, the optimal proportion of spices and herbs for reduction of total HCAs was found to be a combination of turmeric:lemon grass with the proportion of 52.42%:47.57%. OPLS results indicated that the fraction 5 exhibited remarkable inhibitory activity in the formation of PhIP. The potential inhibitor compounds in this fraction were identified as luteolin, curcumin, luteolin 6-C-Glucosyl (Isoorientin), luteolin 6-C-Pentosyl-8-C-pentosyl, Pentosyl-8-C-deoxyhexosyl luteolin, apigenin 7-rutinoside, 2''-O-Rhamnosyl isoorientin, Embigenin 2″-(2″-acetylrhamnoside), apigenin 7-rutinoside-4'-glucoside, luteoin 7-(6″″-acetylollosyl-(1->3)-glucosyl-(1->2)glucoside, Kaempferol 3-rutinoside-7-sophoroside. The results of experiment also showed that the mixture of turmeric and lemon grass (52.42%:47.57%) can be used in the diet of zebrafish without any detrimental effect on liver, kidney, fertility, growth rate and marinades decreased the mutagenic activity in the marinated grilled samples compared with those of the unmarinated samples. The results of the mutagenic activity demonstrated that this optimized marinade formula significantly ($p < 0.05$) diminished mutagenicity of grilled beef in bacterial Ames test.

It can be concluded that all selected herbs and spices utilized in this study possess antioxidant activity which can reduce total HCAs concentration in grilled beef. It is evidenced that combination of turmeric and lemon grass (52.42%:47.57%) gave satisfactory results for the maximum reduction of total HCAs and mutagenicity without any detrimental effect on zebrafish embryo.

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

PENILAIAN REMPAH DAN HERBA TEMPATAN KE ATAS PENURUNAN AMINA HETEROSIKLIK DALAM DAGING LEMBU PANGGANG

Oleh

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Amina aromatic heterosiklik (HCAs) adalah kumpulan utama mutagen dan karsinogen makanan yang berpotensi terbentuk semasa proses pemanasan. Sebatian phenolik yang wujud di dalam tumbuhan dikenali dengan kebolehan untuk memerangkap radikal bebas yang mana boleh menghalang pembentukan HCAs. Tiada kajian dilakukan berkaitan kesan campuran rempah/herba ke atas penurunan HCAs dalam daging lembu panggang. Matlamat khusus kajian ini adalah untuk menentukan activiti antioksidan herba dan rempah terpilih, mengoptimumkan nisbah gabungan herba dan rempah untuk mencapai penurunan tertinggi bagi HCAs, mengenal pasti pengabalan bioaktif utama pembentukan HCAs dan meneroka tahap mutagen dan ketoksikan daging lembu panggang diperap. Kajian ini telah dijalankan untuk menentukan kesan rempah / herba terpilih pada pengurangan amina heterocyclic (HCAs) dalam daging lembu panggang. Herba terpilih dan rempah (kunyit, daun kari, serai dan bunga kantan) telah dinilai untuk kandungan phenolik, kandungan flavonoid, keupayaan memerangkap radikal bebas menggunakan asay DPPH dan kuasa penurunan antioksidan ion ferik menggunakan asay FRAP. Sebatian bioaktif dalam sampel dikenalpasti dengan menggunakan high performance liquid chromatography, (HPLC). Sembilan belas kombinasi rempah/herba telah (kunyit, daun kari, serai dan bunga kantan) digunakan berdasarkan rekabentuk simplek campuran sentroid telah digunakan untuk menentukan kadar yang optimum yang mencapai penurunan tertinggi sebanyak HCAs. Keputusan menunjukkan kombinasi kunyit dengan serai mempamerkan kadar penurunan HCAs yang tinggi. Campuran optimum ekstrak mentah herba ini telah difraksikan untuk mendapatkan penghalang utama pembentukan PhIP menggunakan HPLC. Enam fraksi telah dikumpul dan digunakan ke dalam sistem model PhIP yang mengandungi glukosa, penilalanin dan kreatinin. Liquid chromatography-mass spectrometry quadruple time of flight (LC-MS/MS Q-TOF) dan orthogonal partial least-squares (OPLS) analisis telah digunakan untuk mengaitkan penurunan pembentukan PhIP dalam fraksi yang berbeza bagi profail

metabolik berbeza untuk setiap fraksi yang diperolehi daripada campuran ekstrak kunyit dan serai. Eksperimen *in vivo* dan *in vitro* telah dilakukan untuk menentukan sama ada jumlah penurunan HCAs dalam daging yang diperap menggunakan perkadaran rempah/herba yang telah dioptimakan mempamerkan penurunan yang setanding bagi ketoksikan dan kemutagenan dalam embrio ikan zebrafish dan dalam *Salmonella* Typhimurium (Ujian Ames) untuk menentukan sama ada jumlah pengurangan HCAs dalam dioptimumkan daging lembu panggang diperap mempamerkan pengurangan setanding ketoksikan dan mutagen. Keputusan eksperimen menunjukkan bahawa kunyit memiliki TPC tertinggi (172.10±1.40 mgGA/berat kering), TFC (380.7±5.5 mgQE/berat kering), aktiviti antioksidan menggunakan assay DPPH (47.35±2.57%) and kuasa penurunan antioksidan ion ferik menggunakan asay FRAP (55.79±0.39 mgQE/g berat kering) tinggi dan diikuti oleh daun kari, bunga kantan dan serai. kombinasi kunyit dengan serai (50:50 b/b) telah menurunkan 94.7% kandungan HCAs (204.7 to 10.86 ng/g). Menggunakan pengoptimuman respon, perkadaran optimum rempah/herba untuk penurunan kandungan HCAs adalah 52.42%:47.57% (kunyit:serai). Keputusan OPLS menunjukkan fraksi 5 mempamerkan aktiviti penghalangan pembentukan PhIP yang luar biasa. Sebatian penghalang yang berpotensi dalam fraksi ini dikenalpasti sebagai luteolin, kurkumin, luteolin 6-C-Glucosyl (Isoorientin), luteolin 6-C-Pentosyl-8-C-pentosyl, Pentosyl-8-C-deoxyhexosyl luteolin, apigenin 7-rutinoside, 2"-O-Rhamnosyl isoorientin, Embigenin 2″-(2″-acetylrhamnoside), apigenin 7-rutinoside-4'-glucoside, luteoin 7-(6‴-acetylollosyl-(1->3)-glucosyl-(1->2)glucoside, Kaempferol 3-rutinoside-7-sophoroside. Keputusan juga menunjukkan campuran boleh digunakan dalam diet ikan zebrafish tanpa kesan yang memudaratkan ke atas hati, buah pinggang, kesuburan, kadar pertumbuhan dan perapan telah menurunkan aktiviti metagenik dalam sampel yang diperap berbanding sampel kawalan yang tidak diperap.

Ia dapat disimpulkan bahawa semua herba dan rempah terpilih yang digunakan dalam kajian ini mempunyai aktiviti antioksidan yang boleh mengurangkan kepekatan jumlah HCAs dalam daging panggang. Ia telah terbukti bahawa gabungan kunyit dan serai (52.42%:47.57%) memberi keputusan yang memuaskan bagi pengurangan maksima jumlah HCAs dan kesan mutagen tanpa sebarang kemudaratan pada embrio zebrafish.

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

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LIST OF ABBREVIATIONS

ACs	amino-carbolines
AOAC	Association of Official Analytical Chemist
AαC	2-Amino-9*H*-pyrido[2,3-*b*]indole
BaP	Benzo[a]pyrene
BHA	Butylated hydroxyanisole
C	Carbon
C-C	Carbon-Carbon
CE	Crude extract
DEG	Diethylene glycol
dG	Deoxyguanosine
DMIP	2-Amin-1, 6-dimethylimidazo[4,5-*b*]pyridine
DNA	Deoxyribo nucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EGCG	Epigallocatechin gallate
ESI	Electrospray ionization
ESI	Electro spray ionization
FID	Flame ionization detector
FRAP	Ferric reducing antioxidant power assay
g	Gram
GAE	Gallic acid equivalent
GC-MS	Gas chromatography mass spectrometry
Glu-P-1	2-Amino-6-methyl-dipyrido[1,2-*a*:3_,2_-*d*]imidazole
Glu-P-2	2-Amino-dipyrido[1,2-*a*:3-,2-*d*]imidazole

h	Hour
H	Hydrogen
HCAs	Heterocyclic amines
HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
HSD	Tukey's honestly significant different
I.D.	Internal Diameter
IAAs	Imidazo-azaarenes
IARC	The International Agency for Research on Cancer
IQ	2-amino-3-methylimidazo[4,5-f] quinolone
IQ	2-Amino-3-methylimidazo[4,5-*f*]quinolone
IQx	2-Amino-3-methylimidazo[4,5-*f*]quinoxaline
kPa	Kilopascal
LC-MS	Liquid chromatography-mass spectrometry
LC-MS/MS	Liquid chromatography- mass spectrometry/mass spectrometry
LOD	Limit of detection
LOQ	limit of quantification
M	Molar
MeAαC	2-Amino-3-methyl-9*H*-pyrido[2,3-*b*]indole
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinolone
MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline
mg	Milligram
min	Minute
ml	Milliliter

mM	Millimolar
MRM	Multiple reaction monitoring
MS^2	mass spectrometry/mass spectrometry
MVDA	Multivariate data analysis
N2	Nitrogen
NaOH	Sudium hydroxid
ND	Not detected
ng	Nanogram
nm	Nanometer
OH^-	Hydroxide
OPLS	Orthogonal projections to latent structures
PDA	Photodiode array detector
Phe-P-1	2-Amino-5-phenylpyridine
PhIP	2-amino-1- methyl-6-phenylimidazo[4,5-b]pyridine
ppb	Parts-per-billion
Q-TOF	Quadruple time-of-flight
Quercetin equivalents	QE
TCA	Trichloroacetic acid
TFC	Total flavonoid compounds
TMIP	2-Amino-1,5,6-trimethylimidazo[4,5-*b*]pyridine
TPC	Total phenolic content
Trp-P-1	3-Amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole
Trp-P-2	3-Amino-1-methyl-5*H*-pyrido[4,3-*b*]indole
UHPLC	ultra-high performance liquid chromatography

UV	Ultraviolet
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/ Weight
α	Alfa
β	Beta
γ	Gama
δ	Sigma
µg	Microgram
%	Percentage
°C	Degree celcius
µg	Microgram
µl	Microliter
µm	Micrometer
µmol	Micromole
4,7,8-TriMeIQx	2-Amino-3,4,7,8-tetramethyl-3H-imidazo[4,5-f]quinoxaline
4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline
4-CH_2OH-8-MeIQx	2-Amino-4-hydroxymethyl-3,8-dimethylimidazo[4,5-*f*]quinoxaline
4-OH-PhIP	2-Amino-1-methyl-6-(4-hydroxyphenyl)imidazo[4,5-*b*]pyridine
7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline
7,9-DiMeI*g*Qx	2-Amino-1,7,9-trimethylimidazo[4,5-*g*]quinoxaline

CHAPTER 1

INTRODUCTION

1.1 Background of study

Heterocyclic amines (HCAs) are mutagenic and/or carcinogenic compounds form during thermal processing of meat and fish products at the temperatures over 150 ºC. Based on the temperature of formation (Gibis & Weiss, 2012) they are classified into two groups, polar or thermic HCAs and nonpolar or pyrolytic HCAs. Polar or thermic HCAs which are known as amino imidazo-azaarenes, generate at temperatures above 150 °C through Maillard reaction between reducing sugars, especially glucose, and different free amino acids and creatine or creatinine. Non-polar or pyrolytic HCAs which are known as amino-carbolines form by pyrolysis of free amino acids at temperatures above 300 ºC (Kizil, Oz, & Besler, 2011).

The International Agency for Research on Cancer (IARC) classified HCAs as a probable human carcinogens (2-amino-3-methylimidazo[4,5-f] quinoline (IQ) (class 2A) and possible human carcinogens (class 2B) (2-amino-1- methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), (2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline (MeIQ), and 2-amino- 3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx) (Gibis, Kruwinnus, & Weiss, 2015).

Several epidemiological studies on cancer provide strong evidences which show an association between high consumption of heat processed meat and high risk of cancer in breast, prostate, lung, stomach (Alaejos, Pino, & Afonso, 2008) and colon (Butler et al., 2008). Moreover, many of HCAs have been shown to be mutagenic in Ames/*salmonella* test. It is demonstrated that some HCAs are 100 fold more mutagenic than aflatoxin B1 and over 2,000-fold more than benzo[a]pyrene (BaP) in *in-vitro* Ames test (Kizil et al., 2011).

In the past years, several studies were performed to develop some strategies which could beneficially reduce or inhibit HCAs formation in real meat matrix or chemical model systems. Microwave pretreatment prior to deep-frying and grilling of chicken and beef showed significant reduction of HCAs concentration (Jinap et al., 2013). It has been reported that the formation of HCAs can be reduced by using natural or synthetic antioxidants in chemical model or real meat systems. It has been reported that synthetic or natural antioxidants such as butylated hydroxyanisole (BHA), α-tocopherol, eugenol (Moon & Shin, 2013), epigallocatechin gallate (EGCG), naringenin and quercetin-3-O-glucoside (Cheng, Chen, & Wang, 2007) fresh garlic and anion (Gibis, 2007), black paper powder (Oz & Kaya, 2011), virgin olive oil (Persson, Graziani, Ferracane, Fogliano, & Skog, 2003) and extracts of hibiscus (Gibis & Weiss, 2010), green tea (Quelhas et al., 2010), rosemary and grape seed (Ahn & Grün, 2005) reduce HCAs.

PhIP (2-Amino-1-methyl -6- phenylimidazo[4,5 - b]pyridine) is the most abundant HCAs occurred in high concentration in meat product which is cooked at high temperatures. Carcinogen and mutagen PhIP is originated from the condensation of creatinine with Millard reaction product of glucose and phenylalanine (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012; Yu, Chen, & Yu, 2016). *Salmonella* mutagenicity test (Ames test) (Apostolides, Balentine, Harbowy, & Weisburger, 1996; Singla & Kaur, 2003; Zhang et al., 2011) has demonstrated that PhIP is mutagenic compound for *S.*Typhimurium strain TA98 and TA 100 and several other studies have shown that PhIP induced tumors in prostate (Cross et al., 2005), colon (Nicken, Schröder, von Keutz, Breves, & Steinberg, 2013) and colorectal tissue (Rohrmann, Hermann, & Linseisen, 2009).

Therefore, inhibition or mitigation of PhIP formation has been the goal of numerous research projects. Several studies have shown that the application of plant extracts rich in phenolic compounds in real meat system or chemical model system could suppress the PhIP formation (Ahn & Grün, 2005; Cheng et al., 2009; Moon & Shin, 2013). Conversely, Cheng et al. (2007) indicated that some polyphenols such as rutin, chlorogenic acid, hesperidin, carnosic acid and rosmarinic acid increase PhIP concentration in model system, therefore, phytochemical screening of plant extract is a good way to achieve information about the constituents which are key inhibitory factors in PhIP formation.

1.2 Importance of study

Since 1939 when Widmark, the Swedish chemist observed that extracts of heat processed horse meat induced carcinogenic tumor on the mice skin, many studies on the evaluation of mutagenicity and carcinogenicity of heat processed (fried, grilled, barbequed) meat have been conducted. They can be generally categorized into three classes: (i) those studying the mechanism of HCAs formation in real meat or chemical model systems and possible mechanism of their mutagenicity in *S.* Typhimurium (Ames test) and carcinogenicity in rodents, (ii) those isolating, characterizing and quantifying HCAs from foods and (iii) those investigating the reduction of HCAs using different ways. To date, many studies have been conducted to mitigate HCAs generation in food by different strategies such as reducing cooking temperature and time (Oz, Kaban, & Kaya, 2010; Puangsombat et al., 2012), microwave pretreatment (Jinap et al., 2013; Felton, Fultz, Dolbeare, & Knize, 1994) and using synthetic or natural antioxidants (Gibis, 2007; Gibis & Weiss, 2010; Damašius et al., 2011). It has been reported that the concentration of HCAs in meat products can be reduced by adding spices and herbs as marinade ingredient prior to heat processing. There are many publications which used single synthetic antioxidants or single herbs, spices or fruit extracts for the reduction of HCAs; however, no publication has been found on evaluation of using mixture of herbs and spices.

According to Food Consumption Statistics of Malaysia (2010), consumption of beef and chicken among Malaysian has increased considerably. The intakes of beef and chicken for Malaysian were estimated to be 104.16 g/day (Jing, 2008). This level of meat consumption assures high probability of Malaysian exposure to HCAs in the harmful level that leads to cancer. Malaysian regularly employ many high temperature cooking methods such as grilling, barbequing, deep frying and roasting to prepare meat. Chicken and beef satay are good examples of popular foods among Malaysians which get pre prepared at high temperatures.

The latest study on dietary exposure to HCAs in cooked meat and fish among Malaysians was done by Jahurul et al. (2010). The results showed that the dietary intake of HCAs in foods consumed by people in Selangor, Malaysia was 553.7 ng/capita/day and the intake of PhIP was the highest (30.6 ng/g), followed by MeIQx and MeIQ. The results reveal that grilled meat and fish products were the major contributors to the exposure of HCAs. Several studies (Ahn & Grün, 2005; Gibis & Weiss, 2012; Oz & Kaya, 2011; Quelhas et al., 2010; Ruan et al., 2014; Sabally, Sleno, Jauffrit, Iskandar, & Kubow, 2016; Zöchling, Murkovic, & Pfannhauser, 2002) showed that marinating meat by some spices and herbs before grilling can reduce HCAs formation.

The reduction of HCAs using local spices and herbs and their combination have not been investigated. Hence, this study is aimed to find associations between using combination of local spices and herbs on reduction of HCAs in grilled beef.

1.3 Objectives

The specific objectives of this study are as follow:

1. To determine the antioxidant properties and bioactive compounds of selected local spices and herbs.
2. To optimize the best concentration of combination of local spices and herbs and evaluate the effectiveness for the reduction of HCAs in grilled meat.
3. To identify the compounds in mixture extracts of optimized spices and herbs which responsible for the reduction of PhIP formation in model system.
4. To determine the reduction of toxicity and mutagenicity of the optimized marinated grilled beef

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