

CHARACTERIZATION OF FUNCTIONAL FOOD
ADDITIVES FROM CASHEW APPLES (*Anacardium
occidentale*)

BY

MUHAMMAD SHAHRAIN BIN SHUHAIMEN

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International Islamic University Malaysia

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ABSTRACT

In this study, cashew apple (*Anacardium occidentale*) was selected as a natural alternative against synthetic food additives. This study was divided into two sections, which were the optimization of the extraction of protease and secondary metabolites as meat tenderizers and food spoilages' inhibitors, respectively. Both optimizations were made using Response Surface Methodology (RSM). In the meat tenderizing section, four studied variables, namely, pH, CaCl₂ concentration, mixing time, and mass were chosen. The optimal crude protease extract (CPE) extraction conditions ($R^2 = 0.9803$) for the highest protease activity were obtained at pH 6.34, 7.92 mM CaCl₂ solution, 5.51 min mixing time, and 19.24 g sample mass. The validation test ($n = 3$) showed that there is no significant difference (Tukey test; $p < 0.05$) between the statistical (6.30 units/mL) and experimental (6.49 ± 0.23 units/mL) protease activity. The total protein content of the extract was 4.89 ± 0.10 mg/mL with specific activity of 1.29 ± 0.05 unit/mg. The CPE was successfully applied as a meat tenderizer by observing the increasing tenderness from 5.37 ± 1.12 mJ to 2.19 ± 0.55 mJ of force needed to deform meat, and decreasing protein band from over ~ 49.8 kDa to under ~ 22.4 kDa, after been treated with the CPE using texture analyser and SDS-PAGE, respectively. In a concurrent study for food spoilages' inhibitors, the secondary metabolites of cashew apple were extracted using SFE-CO₂ by optimizing pressure, time, and temperature. Optimal extraction conditions ($R^2 = 0.9858$) that yield the highest DPPH radical scavenging activity (70.34%) were obtained at 288.98 bar, 66.21 min, and 36.98 °C. The statistical result was in reasonable agreement with the validation test ($n = 3$) of experimental ($71.52 \pm 0.67\%$) antioxidant activity. The dichloromethane fraction of the crude secondary metabolite extract (CSME) showed the IC₅₀ value of 0.58 mg/mL and 0.08 mgGAE/mL of total phenolic content. The CSME showed antibacterial activity against *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Staphylococcus aureus* with 25.40 ± 0.05 mm, 20.80 ± 0.18 mm, and 7.20 ± 0.04 mm, respectively. A non-competitive mixed inhibition type against tyrosinase, with IC₅₀ value of 0.02 v/v of the extract in ethyl acetate. Based on GC-MS, the most abundant of secondary metabolites identified was gamma-elemene (67.30%). COSMO-RS explains the extraction mechanism occurred in the SFE-CO₂ system. Gamma-elemene satisfied Lipinski, Ghose, Veber, and Egan drug-likeness by ADME pharmacokinetic analysis and showed strong binding interactions against protein receptor (bacterial and tyrosinase).

خلاصة البحث

في هذه الدراسة، تم اختيار تفاح الكاجو (*Anacardium occidentale*) كبديل طبيعي ضد المضافات الغذائية الاصطناعية. تم تقسيم هذه الدراسة إلى قسمين، وهما الاستغلال الأمثل لاستخلاص البروتينات والمستقلبات الثانوية كمواد مغرية للحوم ومثبطات تلف الطعام، على التوالي. تم إجراء كلا التحسين باستخدام منهجية سطح الاستجابة (RSM) في قسم تطوير اللحوم تم اختيار أربعة متغيرات مدروسة وهي الأس الهيدروجيني وتركيز كلوريد الكالسيوم ووقت الخلط والكتلة. تم الحصول على ظروف استخراج البروتين الخام المثلى ($R^2 = 0.9803$) (CPE) لأعلى نشاط للبروتين عند الرقم الهيدروجيني 6.34، و 7.92 ملي مولار من محلول $CaCl_2$ ، و 5.51 دقيقة من وقت الخلط، و 19.24 جم من كتلة العينة. أظهر اختبار التحقق ($n = 3$) أنه لا يوجد فرق كبير (اختبار Tukey؛ $p < 0.05$) بين نشاط الأنزيم البروتيني الإحصائي (6.30 وحدة / مل) والتجريبي (6.49 ± 0.23 وحدة / مل). كان محتوى البروتين الكلي للمستخلص 0.10 ± 4.89 مجم / مل مع نشاط محدد 0.05 ± 1.29 وحدة / مجم. تم تطبيق CPE بنجاح كمغرض للحوم من خلال ملاحظة الحنان المتزايد من 1.12 ± 5.37 ملي جول إلى 2.19 ± 0.55 ملي جول من القوة اللازمة لتشويه اللحوم، وتقليل نطاق البروتين من أكثر من 49.8 كيلو دالتون إلى أقل من 22.4 كيلو دالتون تقريباً، بعد معالجته باستخدام CPE باستخدام محلل النسيج و SDS-PAGE، على التوالي. في دراسة متزامنة لمثبطات تلف الطعام، تم استخلاص المستقلبات الثانوية لتفاح الكاجو باستخدام $SFE-CO_2$ عن طريق تحسين الضغط والوقت ودرجة الحرارة. تم الحصول على ظروف الاستخراج المثلى ($R^2 = 0.9858$) التي تنتج أعلى نشاط لكسح جذور DPPH (70.34%) عند 288.98 بار و 66.21 دقيقة و 36.98 درجة مئوية. كانت النتيجة الإحصائية متوافقة بشكل معقول مع اختبار التحقق من الصحة ($n = 3$) للنشاط التجريبي المضاد للأكسدة ($71.52 \pm 0.67\%$). أظهر جزء ثنائي كلورو ميثان من مستخلص المستقلب الثانوي الخام (CSME) قيمة IC_{50} البالغة 0.58 مجم / مل و 0.08 مجم / GAE مل من إجمالي المحتوى الفينولي. أظهر CSME نشاطاً مضاداً للبكتيريا ضد *Pseudomonas aeruginosa* و *Bacillus subtilis* و *Staphylococcus aureus* بنسبة 0.05 ± 25.40 مم و 0.18 ± 20.80 مم و 7.20 ± 0.04 مم على التوالي. نوع مثبط مختلط غير تنافسي ضد التيروزيناز، بقيمة IC_{50} تبلغ 0.02 تيرابايت / حجم المستخلص في أسيتات الإيثيل. بناءً على GC-MS، كانت أكثر المستقلبات الثانوية التي تم تحديدها وفرة هي جاما إيمين (67.30%). يشرح COSMO-RS آلية الاستخراج التي حدثت في نظام $SFE-CO_2$. استوفى جاما إيمين تشابه عقار ليبينسكي، وجوز، وفير، وإيجان من خلال تحليل الحرائك الدوائية ADME وأظهر تفاعلات ارتباط قوية ضد مستقبلات البروتين (البكتيرية والتيروزيناز).

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology).

.....
Mohammad Norazmi Ahmad
Supervisor

.....
Erna Normaya Abdullah
Co-Supervisor

.....
Muhammad Nor Omar
Co-Supervisor

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology).

.....
Noor Hasniza Md Zin
Internal Examiner

.....
Jinap Selamat
External Examiner

This thesis was submitted to the Department of Biotechnology and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology).

.....
Mardiana Mohd Ashaari
Head. Department of
Biotechnology

This thesis was submitted to the Kulliyah of Science and is accepted as a fulfilment of the requirement for the degree of Master of Science (Biotechnology).

.....
Shahbudin Saad
Dean, Kulliyah of Science

DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated to whomever it may concerns.

I love to see your smiles. Yes, You!

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LIST OF ABBREVIATIONS

| | | | |
|-----------------|--|--------|---|
| ABS | Abbott Bioavailability Score | EDTA | Ethylenediaminetetraacetic acid |
| ADME | Absorption, distribution, metabolism and excretion | EtOAc | Ethyl acetate |
| | | EtOH | Ethanol |
| | | FAO | Food and Agriculture Organization of the United Nations |
| ADP | Adenosine diphosphate | | |
| ANOVA | Analysis of variance | FCR | Folin Ciocalteu reagent |
| APS | Ammonium persulfate | FDA | United States Food and Drug Administration |
| BBB | Blood brain barrier | | |
| BHA | Butylated hydroxyanisole | FER | Falling extraction rate |
| | | F-test | Fisher's statistical test |
| BHT | Butylated hydroxytoluene | FT-IR | Fourier Transform Infrared Spectroscopy |
| BP5 | 5% phenylmethyl polysiloxane | GAE | Gallic Acid Equivalent |
| | | GC | Gas chromatography |
| BP86 | Becke Perdeuw 86 | GC MS | Gas Chromatography-Mass Spectrometry |
| BSA | Bovine serum albumin | | |
| CCRD | Central composite rotatable design | GI | Gastrointestinal |
| | | GMO | Genetically modified organism |
| CE | Catechin Equivalent | | |
| CER | Constant extraction rate | GRAS | Generally Regarded as Safe |
| CNS | Central nervous system | | |
| CNSL | Cashew nutshell liquid | HBA | Hydrogen bond acceptors |
| CO ₂ | Carbon dioxide | | |
| CDC | Centers for Disease Control and Prevention | HBD | Hydrogen bond donors |
| | | HDL | High density lipoprotein |
| COSMO-RS | Conductor like Screening Model for Real Solvents | HOMO | Highest Occupied Molecular Orbital |
| CPE | Crude protease extract | IIUM | International Islamic University Malaysia |
| CSME | Crude secondary metabolites extract | LDL | Low density lipoprotein |
| | | LDPE | Low density polyethylene |
| CV | Coefficient of variation | | |
| CYP | Cytochrome P450 enzymes | LER | Low extraction rate |
| | | LUMO | Lowest Unoccupied Molecular Orbital |
| DC | Diffusion-controlled | | |
| DCM | Dichloromethane | | |
| DF | Degree of Freedom | MD | Molecular docking |
| DFT | Density Functional Theory | MEP | Molecular Electrostatic Potential |
| DNA | Deoxyribonucleic acids | MHA | Muller-Hinton agar |
| DPPH | 2,2 diphenyl-1-picrylhydrazyl | MHB | Muller-Hinton broth |
| | | MHC | Myosin heavy chain |

| | | | |
|---------------------|--|--------|---------------------------|
| MLC | Myosin light chains | UV | Ultraviolet light |
| MS | Mass spectrometry | UV VIS | Ultraviolet Visible light |
| MlogP | Moriguchi model of octanol-water partition coefficient | 2-D | Two-dimensional |
| | | 2 ME | 2-mercaptoethanol |
| | | 3-D | Three-dimensional |
| MR | Molar refractivity | | |
| MW | Molecular weight | | |
| PBS | Phosphate buffer saline | | |
| PG | Propyl gallate | | |
| P-gp | P-glycoprotein | | |
| PPO | Polyphenol oxidase | | |
| PRESS | Predicted residual sum of squares | | |
| RCF | Relative centrifugal force | | |
| RNS | Reactive nitrogen species | | |
| RoB | Rotatable bonds | | |
| ROS | Reactive oxygen species | | |
| RSM | Response Surface Methodology | | |
| SD | Standard deviation | | |
| SDS | Sodium dodecyl sulfate | | |
| SDS-PAGE | Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis | | |
| SF | Supercritical fluid | | |
| SFE | Supercritical fluid extraction | | |
| SFE-CO ₂ | Supercritical fluid extracted using carbon dioxide solvent | | |
| TCA | Trichloroacetic acid | | |
| TEMED | Tetramethylethylenediamine | | |
| THBQ | <i>Tert</i> -butylhydroxy quinone | | |
| TPC | Total phenolic content | | |
| tPSA | Topological polar surface area | | |
| TZVP | Triple zeta valance potential | | |
| USA | United States of America | | |
| USDA | United States Department of Agriculture | | |

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Due to increasing jargons used to describe foods of exceptional health benefits and the ever-increasing demands of consumers for quality and safe food, scientists are obliged to investigate the effectiveness of the claimed food additives. Organoleptic characteristics have become important criteria for food selection by consumers (Bandara, Silva, Maduwanthi, & Warunasinghe, 2016; Lipinska, Tomaszewska, & Kołozyn-Krajewska, 2019). Off-texture of food, food browning, and microbial infection on food are among the factors that negatively-influence the organoleptic characteristics of food (Petruzzi, Corbo, Sinigaglia, & Bevilacqua, 2017).

The texture of food such as regarding tenderness and toughness of meat products governs its palatability and hence, acceptability and satisfaction by the consumers (Feldkamp, Schroeder, & Lusk, 2005; Koohmaraie & Geesink, 2006; Hanis, Jinap, Nasir, & Alias, 2013). Over the years, the demand for meat products has been increasing and tender meat is mostly preferred in contrast to tough meat. Food browning is a chemical process in food that involves an oxidation reaction which may be non-enzymatic (lipid peroxidation) or enzymatic (caused by polyphenol oxidase (PPO) such as tyrosinase) browning of food. Food browning turns the food color to brown and give off-flavor and off-odor which is disliked by consumers.

These issues and concerns relating to the quality and safety of food can be improved and corrected using food additives. However, commercial food additives in the market today have some other shortcomings such as being derived from animal and

microbial sources that invoke halal adulteration issues (Shah, Mir, & Paray, 2014), genetically modified organism (GMO)-produced food additives that invoke ethical issues (Grunert et al., 2001; Lähteenmäki et al., 2002), and synthetically-produced food additives that have adverse health issues (Meier, Gomez, Kirichenko, & Thompson, 2007; Dwyer-Nield et al., 2010; Gultekin & Doguc, 2013). Therefore, an alternative source of food additives which are more natural and socially-acceptable that could rectify these concerns are highly demanded and sought after in the market of the food industry.

The incorporation of Response Surface Methodology (RSM) to optimize the extraction of food additives from plant could reduce cost and time while providing statistical analysis of the interactions between the tested variables (Dutta, Dutta, & Banerjee, 2004; Thys, Guzzon, Cladera-Olivera, & Brandelli, 2006; Fakhfakh-Zouari, Haddar, Hmidet, Frikha, & Nasri, 2010). With the advancement of technologies by time, new computational and analytical methods have been invented and introduced to further elaborate and demonstrate properties and functionalities of foods. Such new information helps us to further understand the actual reactions and mechanisms behind certain claimed health benefits (Ramachandran, Gopakumar, & Namboori, 2008). This study combines method in extracting potential multifunctional food additives from cashew apples (*Anacardium occidentale*) by optimization, to characterizing the extracted compounds by the experimental and computational approaches.

1.2 PROBLEM STATEMENT

Food safety and quality deteriorations are serious concerns for the market and consumers. Present-day commercial food additives that are used to improve the safety and quality of foods have carried several limitations. Commercial food additives in the