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

BY

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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, $CaCl_2$ 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 $CaCl_2$ 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-CO_2 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 ± 0.67%) antioxidant activity. The dichloromethane fraction of the crude secondary metabolite extract (CSME) showed the IC_{50} 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 IC_{50} 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-CO_2 system. Gamma-elemene satisfied Lipinski, Ghose, Veber, and Eagan 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 البروتياز والمستقلبات الثانوية كمواد مغرية للحوم ومثبطات تلف الطعام، على التوالي. تم إجراء كلا التحسنين باستخدام منهجية سطح الاستجابة .(RSM) في قسم تطرية اللحوم تم اختيار أربعة متغيرات مدروسة وهي الأس الهيدروجيني وتركيز كلوريد الكالسيوم ووقت الخلط والكتلة. تم الحصول على ظروف استخراج البروتياز الخام المثلى (R^2 = 0.9803) (CPE) لأعلى نشاط للبروتياز 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 4.89 ± 0.10 مجم / مل مع نشاط محدد 1.29 ± 0.05 وحدة / مجم. تم تطبيق CPE بنجاح كمغرض للحوم من خلال ملاحظة الحنان المتزايد من 5.37 ± 1.12 مللي جول إلى 2.19 ± 0.55 مللي جول من القوة اللازمة لتشويه اللحوم، وتقليل نطاق البروتين من أكثر من 49.8 كيلو دالتون إلى أقل من 22.4 كيلو دالتون تقريبًا، 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 (ن = 3) للنشاط التجريبي المضاد للأكسدة (71.52 ± 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 نشاطًا مضادًا للبكتيريا ضد *Pseudomonas aeruginosa* و *Bacillus subtilis* و *Staphylococcus aureus* بنسبة 25.40 ± 0.05 مم و 20.80 ± 0.18 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 إليمين (67.30٪). يشرح COSMO-RS آلية الاستخراج التي حدثت في نظام $SFE-CO_2$.استوفى جاما إليمين تشابه عقار ليبينسكي، وجوز، وفيبر، 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).

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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	Ethylenediaminetetra-acetic 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 Perdew 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
CV	Coefficient of variation	LDPE	Low density polyethylene
CYP	Cytochrome P450 enzymes	LER	Low extraction rate
DC	Diffusion-controlled	LUMO	Lowest Unoccupied Molecular Orbital
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
MS	Mass spectrometry
MlogP	Moriguchi model of octanol-water partition coefficient
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	Tetramethylethylene-diamine
THBQ	*Tert*-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
UV	Ultraviolet light
UV VIS	Ultraviolet Visible light
2-D	Two-dimensional
2 ME	2-mercaptoethanol
3-D	Three-dimensional

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