ANTIOXIDANT ACTIVITY AND ANTIOXIDANT PROTEINS DYNAMIC SIMULATION OF MALAYSIA UPLAND RICE GENOTYPES

NURUL HAFIFI BINTI KAMDI

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To my beloved parents *Hjh Supiah binti Abd Kadir & Allahyarham Hj Kamdi bin Kayat* for their sacrifice, patience and endless love. *Al-fatihah* for Abah.

To His abundant love and blessings, Alhamdulillah.

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ABSTRACT

Upland rice is known as a type of rice specifically grown in hilly area that thrive under minimum irrigation. Upland rice is high in antioxidants compared to wetland rice. Due to that, the consumption of upland rice as an alternative carbohydrate has been associated with a reduced incidence of chronic diseases such as diabetes in Asia. However, the study of antioxidant activity for Malaysia upland rice via *in vitro*, *in vivo* or *in silico* is still lacking. In the present study, the antioxidant activity assessments were performed on pigmented upland rice (*Hitam*, *Bario* and *Udang*), non-pigmented (*Wai* and *Putih*) and control wetland varieties (MR220, MR219 and several commercially available white and pigmented rice). Three antioxidant assays namely ferric reducing antioxidant power (FRAP) assay, DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging and superoxide dismutase (SOD) enzyme assay were implemented on raw and cooked rice extracts. Results showed that all three pigmented upland rice exhibited higher antioxidant activity than other wetland rice cultivars. *Hitam* rice had the highest activities of FRAP, DPPH and SOD assays with the value of 9.63 ± 0.52 Fe^{2+}mmol/100g**,** 78.34% radical scavenging activity (RSA) and 35.72 U/g, respectively. *Udang* and *Bario* rice exhibited lower antioxidant activity than *Hitam*. Meanwhile, white rice had the lowest total content of antioxidants. Data elucidated that an average of 75% of the total antioxidant and enzymatic activities were decomposed or inactivated after all the rice were cooked. To study the stability effect of antioxidant proteins towards high temperature in order to mimic the cooking process, *in silico* protein modelling were conducted on three unique upland rice antioxidant proteins chosen from an earlier upland rice seed profiling data. The molecular dynamic (MD) simulation of manganese superoxide dismutase (SOD [Mn]), dehydroascorbate reductase (DHAR), and glyoxalase (GLX) were executed at different temperatures (310 K, 318 K and 373 K) using GROMACS. Trajectory results on the 50 ns simulation at those temperatures were presented in the RMSD (structural stability) and RMSF (structural flexibility) plots, gyration graphs (Rg) for structural compaction, secondary structure analysis as well as bonds analysis. Results showed that all three antioxidant protein structures were stable at 310 K and 318 K but, least stable at 373 K. These findings corresponded to the analysis of secondary structure, structure flexibility and types of bonds from the proteins three-dimensional (3D) model. This study also revealed that SOD [Mn] protein from *Triticum aestivum* was more stable at 310K and 373K than upland rice (*Oryza sativa)* and *Zea mays* when all simulated proteins were compared. This information could provide insights into a better understanding the roles of SOD [Mn] catalytic action and protein-protein interactions which might also be applied to other antioxidant proteins yet to be discovered. Taken together, the findings present valuable knowledge on antioxidant activity and thermostability of antioxidant proteins in upland rice. These findings suggest that upland rice might be a good source of natural antioxidant and a potential source of nutraceuticals in the future.

ABSTRAK

Padi bukit merupakan sejenis padi yang ditanam di kawasan berbukit dan memerlukan kadar pengairan yang minimum. Padi bukit adalah tinggi dengan antioksidan berbanding padi tanah lembap. Oleh kerana kandungan antioksidan yang tinggi dalam padi bukit, pengambilannya sebagai sumber karbohidrat alternatif dihubungkaitkan dengan pengurangan penyakit kronik seperti diabetis di Asia. Walau bagaimanapun, kajian secara *in vitro*, *in vivo* mahupun *in silico* tentang antioksidan padi bukit di Malaysia masih lagi berkurangan. Dalam kajian ini, penentuan aktiviti antioksidan telah dijalankan ke atas padi bukit berpigmen (*Hitam*, *Bario* dan *Udang*), tidak berpigmen (*Wai* dan *Putih*) serta varieti kawalan dari tanah lembap (MR220, MR219 serta beberapa beras putih dan berpigmen di pasaran komersial). Tiga cerakinan antioksidan telah dilaksanakan ke atas ekstrak padi mentah dan padi yang telah dimasak iaitu asai kuasa penurunan ferik (FRAP), asai penyahbebas radikal DPPH (2,2-difinil-1-pikrilhidrazil) dan asai enzim superoksida dismutase (SOD). Hasil kajian menunjukkan ketiga-tiga padi bukit berpigmen mempunyai aktiviti antioksida yang tinggi berbanding padi tanah lembap. Padi *Hitam* mempamerkan aktiviti FRAP, DPPH dan SOD yang paling tinggi dengan mencatat bacaan masing-masing pada nilai 9.63 ± 0.52 Fe^{2+}mmol/100g**,** 78.34% aktiviti penyahbebas radikal (RSA), dan 35.72 U/g. Padi *Udang* dan *Bario* menunjukkan aktiviti antioksidan yang rendah berbanding *Hitam*. Manakala, aktiviti antioksidan beras putih adalah yang paling rendah. Data menunjukkan bahawa sebanyak purata 75% aktiviti antioksidan dan enzim adalah terurai atau tidak aktif setelah beras dimasak. Untuk mengetahui tentang kesan suhu tinggi yang menyerupai proses masakan terhadap kestabilan protein antioksidan, pemodelan protein *in silico* telah dijalankan ke atas tiga protein antioksidan padi bukit unik yang dipilih dari data kajian awal pemprofilan protein. Simulasi dinamik molekular (MD) bagi mangan superoksida dismutase (SOD [Mn]), dihidroaskorbat reduktase (DHAR), dan *glyoxalase* (GLX) telah dijalankan pada suhu berbeza (310 K, 318 K dan 373 K) menggunakan perisian GROMACS. Analisis trajektori ke atas simulasi selama 50 ns pada tiga suhu berbeza telah dibentangkan dalam bentuk plot RMSD (kestabilan struktur) dan RMSF (kelenturan struktur), graf legaran (Rg) untuk kepadatan struktur, analisis struktur sekunder, serta analisis ikatan. Hasil kajian menunjukkan kestabilan ketiga-tiga struktur protein antioksidan pada suhu 310 K dan 318 K manakala, kurang stabil pada suhu 373 K. Semua penemuan kajian ini menyokong kestabilan struktur sekunder, kelenturan gelung dan jenis ikatan model tiga dimensi (3D) protein. Kajian ini juga menunjukkan SOD [Mn] protein dari spesis *Triticum aestivum* adalah paling stabil pada suhu 310 K dan 373 K berbanding spesis padi bukit (*Oryza sativa)* dan *Zea mays* melalui perbandingan simulasi protein. Maklumat ini boleh dijadikan asas kepada memahami peranan SOD [Mn] sebagai pemangkin dan interaksi antara protein serta boleh juga diaplikasikan kepada protein antioksidan lain yang bakal ditemui. Secara keseluruhannya, keputusan kajian ini memberikan pengetahuan yang bermakna ke atas aktiviti antioksidan dan termostabilan protein dalam padi bukit. Dapatan kajian ini mencadangkan bahawa padi bukit boleh digunakan sebagai sumber antioksidan semulajadi serta berpotensi sebagai sumber nutraseutikal pada masa akan datang.

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LIST OF SYMBOLS

$RO^{\bullet}$	-	Alkoxy radicals
α	-	Alpha
Å	-	Angstrom
β	-	Beta
°C	-	Degree celcius
<	-	Less than
>	-	More than
δ	-	Delta
g	-	Gram
γ	-	Gamma
$OH^{\bullet}$	-	Hydroxyl radical
H_2O_2	-	Hydrogen peroxide
h	-	Hour
K	-	Kelvin
L	-	Liter
mL	-	Milliliter
M	-	Molar
µM	-	Micomolar
µL	-	Microliter
µg	-	Microgram
µmol	-	Micromole
mmol	-	Millimole
mg	-	Milligram
min	-	Minute
nm	-	Nanometer
ns	-	Nanosecond

NO	-	Nitric oxide
NO_2	-	Nitrogen dioxide
NO_3^-	-	Peroxynitrite
$^1O_2$	-	Singlet oxygen
$O2^{\bullet-}$	-	Superoxide radicals
U	-	Unit
%	-	Percent
v/v	-	Volume per volume

LIST OF ABBREVIATIONS

1D	-	One-dimensional
3D	-	Three-dimensional
ACN	-	Acetonitrile
ALC	-	Acetylcarnitine
AMBER	-	Assisted Model Building with Energy Refinement
ANOVA	-	Analysis Of Variance
APX	-	Ascorbate Peroxidase
ATP	-	Adenosine Triphosphate
BHT	-	Butylated Hydroxytoluene
BLAST	-	Basic Local Alignment Search Tool
BRF	-	Black Rice Fraction
CAT	-	Catalase
CE	-	Combinatorial Extension
CHD	-	Coronary Heart Disease
CVD	-	Cardiovascular Diseases
DHAR	-	Dehydroascorbate reductase
DNA	-	Deoxyribonucleic Acid
DPPH	-	2,2-diphenylpicrylhydrazyl
ESBRI	-	Evaluating the Salt Bridge in Proteins
ESPript	-	Easy Sequencing in PostScript
ETC	-	Electron Transport Chain
ET	-	Electron Transfer
FAO	-	Food and Agriculture Organization
FRAP	-	Ferric Reducing Antioxidant Power
FR	-	Free Radicals
GR	-	Glutathione reductase

GLX	-	Glyoxalase
GOPX	-	Guaicol Peroxidase
GPX	-	Glutathione Peroxidase
GROMACS	-	GROningen Machine for Chemical Simulation
GSH	-	Glutathione
GST	-	Glutathione-*S*- Transferase
HAT	-	Hydrogen Atom Transfer
IRRI	-	International Rice Research Institute
I-TASSER	-	Iterative Threading ASSEmbly Refinement
LA	-	Lipoic Acid
LDL	-	Low – Density Lipoprotein
LMWA	-	Low Molecular Weight Antioxidants
LOMETS	-	Local Meta-Threading Server
MARDI	-	Malaysian Agricultural Research and Development Institute
MD	-	Molecular Dynamics
MG	-	Methylglyoxal
MS	-	Mass Spectrometry
MDHAR	-	Monodehydroascorbate Reductase
mtDNA	-	Mitochondrial Deoxyribonucleic Acid
NADPH	-	Nicotinamide Adenine Dinucleotide Hydrate
NAMD	-	Nanoscale Molecular Dynamics Program
NMR	-	Nuclear Magnetic Resonance
NCBI	-	National Center for Biotechnology Information
PCD	-	Program cell death
PDB	-	Protein Data Bank
PSI	-	Photosystem I
PSII	-	Photosystem II
QA	-	Quinone A
QB	-	Quinone B
RAMPAGE	-	Ramachandran Plot Assessment
RNA	-	Ribonucleic Acid
RNS	-	Radical Nitrogen Species

ROS	-	Radical Oxygen Species
RS	-	Radical Species
RSA	-	Radical Scavenging Activity
SAVES	-	Structure Analysis and Verification
SOD	-	Superoxide Dismutase
SOD [Mn]	-	Manganese Superoxide Dismutase
SPCE	-	Simple Point Charge Extend
TPC	-	Total phenolic contents
TPTZ	-	2,4,6-tripyridyl-s-triazine
UV	-	Ultraviolet
WHO	-	World Health Organization
WRF	-	White Rice Fragment
XOD	-	Xanthine Oxidase

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Rice *(Oryza sativa)* is one of the most vital food crops for human consumption compared to any other cereals. Over 3 billion of people around the world depended on rice as their primary staple food over any other grain types. Rice contributes around 50% of human total daily calories. FAO declared that in many developing countries rice has provide 27% energy supply, 21% protein intake and 3% of dietary fat (Kennedy *et al.*, 2003). Besides that, it also said to provide consumers with moderate levels of proteins, minerals, vitamin and fibre. However, due to the downstream process of getting edible rice (milling process), these nutrients content were reduced, with the exception of carbohydrates. Hence, rice is normally consumed as carbohydrate source in human dietary regime (Abbas *et al.*, 2011). There are 24 different species in *Oryza* genus, but only two species are cultivated which is *O. glabberima* also known as African rice grown abundantly in West of Africa and *O. sativa* in other part of the rice producers. Those species are originally irrigated however due to the evolutionary process, they become adapted to many different ecosystem as lowland and upland (Agrawal *et al.*, 2006). Irrigated rice is a common type of rice cultivated worldwide which covers almost 90% of production while upland rice growth are very limited as it only accommodate around 11% of total global production and is grown on 14 million hectares over 150 million hectares of rice cultivated land (Sohrabi *et al.*, 2012).

In Malaysia particularly, rice can be found in two types of growing conditions, they are wetland (irrigated) which requires high water level that covers almost 87% of rice planted in the nation and the remaining is composed of upland rice that thrives in minimum rain-fed level, naturally well drained soil without surface water accumulation and with the ability to survive under dry condition (Najim *et al.*, 2007). Findings from previous studies of 50 varieties of upland rice germplasm by Sohrabi and colleagues (Sohrabi *et al.*, 2012) showed that Malaysia has a vast number of upland rice varieties with genetic diversity yet to be explored. Based on the recent studies, it is reported that upland rice has better ability to resist to many stress response compared to other varieties for example drought, salinity and low light stresses (Atlin *et al.*, 2006; Silveira *et al.*, 2015). It is presumed that this unique characteristics of upland rice may be due to the powerful antioxidant machinery they had in combating the output of the stress responses which is usually in the form of radical oxygen species (ROS) (Srivalli *et al.*, 2003). Despite the better characteristics, upland rice cultivation in Malaysia has always been neglected due to the low grain yield at about 0.46 to 1.1 tonne/ha compared to wetland rice which can reach up to 10-11tonne/ha per season (Sohrabi *et al.*, 2012). Nevertheless, the rapid advancement of biotechnology would shed some hopes of improvement in the production of upland rice. Moreover, the lack of vegetative growing land for wetland rice is the issue nowadays, most of the arable land were developed into new housing and industrial-township. Statistic data from IRRI in mid-2017 showed a dropped in total wetland rice cultivation areas in Malaysia that was from 517,586 h/a in 2011 to 514,381 in 2014 and then was further dropped to 510,000 h/a in 2016, a drop of 1.47% in five years. Hence, there is a potential to start exploring our local upland rice varieties to accommodate the reduced in wetland space which eventually assist in high yield rice production in the near future.

In the present year, chronic diseases such as cancer, cardiovascular diseases (CVD), diabetes, hypertension, and stroke accounts for almost half of deaths number in Malaysia that often be correlated with sedentary life and unhealthy diet. These diseases are implicated by the excessive free radicals that is produced over the protective capacity of antioxidant defence system or in other words called imbalance redox homeostasis during metabolism (Waris and Ahsan, 2006). Aside from having a thrifty and physically active lifestyle, several studies has reported that these diseases can be controlled with managing daily diet regimes of an individual with consumption of fruits, vegetables, legumes and whole grains contributing to a reduced risk of getting such diseases (Liu, 2003; Boeing *et al.*, 2012; Hartley *et al.*, 2012). This could be supported by the presence of natural antioxidant compounds in these food in such a way that attracts more research interest in studying antioxidants from natural resources. The assortment of endogenous antioxidants for instance superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and other exogenous antioxidants complex in the body works either by inhibiting the fatal effect of the radical species or protect the biological system from the excessive destruction induced by the free radicals (Obrenovich *et al.*, 2010; Wang *et al.*, 2011). Extensive research were currently focus on antioxidant properties and nutrient fortification in rice since it is found closer and almost exclusive for human consumptions (McLean *et al.*, 2002). It is found that this staple food has received the upmost attention because of its potent antioxidant properties particularly in pigmented rice (Reddy *et al.*, 2016). This properties were attributed mainly by their phytochemicals contents associated with providing health benefits in reducing chronic diseases (Liu, 2007). In recent scientific and industrial (dietary, pharmaceuticals and cosmetic purpose) research, there is also an increasing interest in the measurement and applications of plant antioxidants mainly due to their strong biological activity when compared to the synthetic antioxidants that may probably promotes carcinogenesis. Breakthroughs in food science and increasing concerns of global micronutrient malnutrition problems has also led to a new focus on micronutrient density in staple food especially rice. Thus, there is a need to deepen our knowledge on this staple sources in order to exits for safe, economic, powerful antioxidants to replace the easily found commercialized synthetic antioxidant in the market nowadays.

1.2 Problem Statement

Rice productivity in Malaysia has increases at a stagnant pace each year, while the growing demands from the market needs an extra support from other neighbouring countries such as Thailand, Vietnam, and Pakistan (Kennedy and Burlingame, 2003). Besides providing carbohydrates, rice also is a source of good protein, minerals and other nutrition. Generally, there are two types of rice grown in Malaysia; the well cultivated wetland type that requires flooded area and upland rice that thrives under minimum water level cultivated mainly for home consumption and sometimes as a source of economy for rural people in Pahang, Sabah and Sarawak. Previous studies reported a higher value of antioxidant activities in upland rice from other Asian countries such as Thailand and the Philippines (Faiz *et al.*, 2015). However, there are lack of studies regarding antioxidant activity in local upland rice and those studies were also limited in raw rice form. Thus, this study will provides the information on antioxidant activity before and after the rice is cooked which would also be useful in pharmaceuticals and nutraceuticals field of research. Presently, there is a paucity in the study on the temperature stability of antioxidant proteins of upland rice by using MD simulations. Therefore, this study will also provide preliminary information on the ability of those proteins to adapt with the scorching temperature which would be helpful in pharmaceuticals, food-base products, or even breeding program in the near future.

1.3 Objectives of the Research

The followings are the objectives of this research: -

1. To determine the antioxidant activity of raw and cooked seed of Malaysian upland rice quantitatively via Ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Superoxide dismutase enzyme (SOD) assays.

2. To simulate three chosen antioxidant proteins that is superoxide dismutase (SOD), dehydroascorbate reductase (DHAR) and glyoxalase (GLX) under different temperature of 37^{o}C, 45 ^{o}C and 100 ^{o}C (310 K, 318 K and 373 K) respectively.

3. To simulate superoxide dismutase (SOD) protein from upland rice with wheat *(T. aestivum)* and corn *(Z. mays)* for comparison of behaviour of SOD protein among monocot plants.

1.4 Scope of the Research

Several types of Malaysian pigmented upland rice genotypes known as Hitam, Bario, Udang and non-pigmented Wai and Putih along with control varieties of MR220, MR219 and several commercially available white, red and brown rice were used for this study and antioxidant compounds were extracted by using a developed method utilizing 80% (v/v) methanol extraction. *In vitro* antioxidant activity determination was assessed by several developed antioxidant test known as FRAP, DPPH as well as one antioxidant enzyme assay known as SOD assay. The *in silico* protein study was performed based on the spectral results obtained from previous study by (Lee, 2015) on upland rice seed and searched against *Oryza sativa* database for protein identifications. Three different antioxidant proteins were chosen for homology modelling via i-Tasser server and molecular dynamics (MD) simulation using GROMACS version 5.0.4 on three different temperature (311 K, 318 K and 373 K)

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