BIOTRANSFORMATION OF ARTONIN E BY LOCALLY ISOLATED MICROORGANISMS

LAI YEW SENG

A thesis submitted in fulfillment of the
requirements for the award of the degree of
Master of Science (Bioscience)

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

March 2013

A special dedication for my family, supervisors, lecturers and friends:-

ACKNOWLEDGEMENTS

In preparing this thesis, I came in contact with many people, researchers and academicians. They had contributed towards my further understanding and developed innovative thoughts on the research itself. In particular, I would like to express my deepest gratitude towards my supervisor, Dr. Shaza Eva binti Mohamad for her care, concern and guidance throughout the project. I am also very thankful to my co-supervisor, Dr Shajarahtunnur binti Jamil for her encouragement, guidance and advices.

I would like to express my sincere appreciation to all my lecturers, who have had given me advices throughout my study in UTM. I am also grateful to MOSTI (VOT 79292) for financial support, Faculty Bioscience and Bioengineering, UTM for giving me the chance to study in this field and Research Management Center for managing the distribution of funds for this project.

Special appreciation goes to all the laboratory assistants for their kindness and assistance during the project. Thanks to all my friends for their cooperation during the progress of this study. A meaningful gratitude dedicated to my beloved family for their understandings, support and inspirations throughout the project.

ABSTRACT

Biotransformation is a process that involves modification of the structure of a chemical compound by microbial activities. It has advantages over chemical synthesis as it is able to be operated at near room temperature, with non-extreme pH as well as it is a highly stereospecific reaction. The purpose of this research are to study the ability of selected microbes to transform artonin E, a flavonoid extracted from the bark of local plant, *Artocarpus tesymanii* and identify potential transformation products. 17 types of microbes were isolated from various sources, however 3 types of bacteria and 2 types of fungi were able to grow in the presence of artonin E. They were identified by 16S rRNA and Internal Transcrib Spacer Region for bacteria and fungi respectively. These microbes were *Bacillus cereus*, *Bacillus subtilis*, *Bacillus pumillus*, *Aspergillus fumigatus* and *Aspergillus aculeatus*. The transformation products were analysed by High Performance Liquid Chromatography (HPLC) and Thin Layer Chromatography (TLC). There was a significant peak which corresponded to the biotransformation product as shown in the HPLC chromatogram at retention time of approximately 3.9 minutes. The percentage area of the peak was shown to increase from 6.89% at day 0 to 11.80% at day 1 followed by decreasing of the percentage area of the remaining artonin E concentration in the reaction medium. This was further confirmed by TLC where a compound termed as P1 (transformation product) emerged at the R_f value of 0.37. Artonin E showed a moderate antioxidant property with IC_{50} value of 81.61 µg/mL while the biotransformation product showed very weak antioxidant property with IC_{50} more than 200 µg/mL. Sample was sent for structure elucidation using Nuclear Magnetic Resonance Spectroscopy (NMR) but due to limited concentration of sample produced in the transformation, the structure could not be fully analyzed.

ABSTRAK

Biotransformasi adalah proses yang melibatkan pengubahsuaian sebatian kimia oleh aktiviti mikrob. Ia mempunyai kelebihan berbanding sintesis kimia biasa kerana ianya dapat beroperasi pada suhu bilik dan pH yang tidak melampau dan tindakbalas yang dilakukan juga adalah sangat stereospesifik. Tujuan kajian ini ialah untuk mengkaji keupayaan mikrob terpilih untuk mengubah artonin E, flavonoid yang diekstrak daripada kulit tumbuhan tempatan iaitu *Artocarpus tesymanii* dan juga untuk mengenalpasti produk transformasi. 17 mikrob yang dipencilkan dari pelbagai sumber , tetapi hanya 3 jenis bakteria dan 2 jenis fungus telah dipilih di mana mikrob ini mempunyai kebolehan hidup dengan kehadiran artonin E serta telah dikenal pasti melalui analisis 16S rRNA dan Internal Transcrib Spacer. Mikrob ini adalah *Bacillus cereus, Bacillus subtilis, Bacillus pumillus, Aspergillus fumigatus* and *Aspergillus aculeatus*. Produk transformasi dianalisa menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC) dan Lapisan Kromatografi Nipis (TLC). Satu puncak ketara yang dipercayai produk biotransformasi ditunjukkan pada kromatogram HPLC pada masa kira-kira 3.9 minit. Terdapat peningkatan peratusan keluasan dari 6.89% pada hari 0 ke 11.80% pada hari pertama dan diikuti dengan penurunan peratusan keluasan baki kepekatan artonin E. Ini telah dibuktikan juga oleh TLC di mana satu sebatian yang dinamakan P1 muncul pada nilai R_f 0.37. Artonin E menunjukkan ciri-ciri antioksidan yang sederhana di mana nilai IC_{50} 81.61μg/mL, manakala produk biotransformasi menunjukkan ciri antioksidan yang sangat lemah berbanding artonin E dengan nilai IC_{50} ~~adalah~~ 200 μg/mL. Sampel telah dihantar untuk pengenalpastian struktur dengan menggunakan Spektroskopi Resonan Magnetik Nuklear (RMN) tetapi disebabkan kepekatan sampel yang dihasilkan dalam transformasi adalah terhad, struktur tidak dapat dianalisa sepenuhnya.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATION

ACN-----Acetonitril

bp ------- base pair

DNA ---- Deoxyribonucleic acid

DPPH--- 1,1-diphenyl-2-picrylhydrazyl

NMR-----Nuclear Magnetic Resonance

EDTA -- Ethylene-diamine tetraacetate

EtBr ---- Ethidium bromide

HPLC -- High performance liquid chromatography

ITS----- Internal Transcribed Spacer

OD ---- Optical density

PCR --- Polymerase chain reaction

PDA--- Potato Dextrose Agar

ppm-- - Part per million

R_f-------Value of Thin Layer Chromatography

rpm --- Rotation per minute

TLC---Thin Layer Chromatography

Tm --- Melting temperature

UV --- Ultraviolet light

mM ---- Millimolar

mg ----- Milligram

mL -------Millilitre

nm ------Nanometer

µg ------Microgram

µL ------Microlitre

µM-----Micromolar

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Intensive research on natural products has been carried out in great depth due to its therapeutic properties. In order to enhance its pharmacological activities, these natural products were studied in details by looking at potential functional groups that could be manipulated to improve its efficacy (Havsteen, 1983). Flavonoid, a polyphenolic compound that exists in the plant has been given particular attention.

It has been reported to possess bactericidal action (Havsteen, 1983) and also the ability to inhibit various enzymes such as glutathione reductase (Elliot *et al.*, 1992), mitochondria succinoxidase (Hodnick *et al.*, 1986), cyclooxygenase and lipoxygenase (Laughton *et al*., 1989) and to act as antioxidants due to its ability to chelate iron (Ueno *et al.*, 1984). There are a lot of previous studies showing the transformation of different sources of flavonoid with different types of microbes and enzymes (Robak and Gryglewski, 1988; Afanas'Ev *et al.*, 1989; Jovanovic *et al.*, 1994).

Biotransformation or biocatalysis specifically involves the chemical modification or the modification made by a microorganism on the structure of the compound. Biocatalysis is more favorable compared to chemical synthesis due to the fact that the microbial transformation can be operated in non-extreme pH condition, near room

temperature not forgetting its high stereospecificity. In addition, biotransformation is the useful tools for the production of medicinal chemicals from natural products (Demetzos *et.al*.,1997).

1.2 Problem Statement

Previous studies has shown that flavonoid possesses diverse physiological and pharmacological activities such as astrogenic, antilipoperoxidant, antitumor, antiplatelet, antiviral, antifungal, antibacterial, antihemolytic, anti-ischemic, antiallergic and anti-inflammatory.

Artonin E is a type of isoprenyl flavoid extracted from the bark of *Artocarpus teysmanii* and it was previously shown to have antimicrobial activity against *Escherichia coli* and *Bacillus subtilis* (Jagtap and Bapat, 2010). In addition, the artonin E was proven to give some potential transformation products as shown by Tang (2007).

Microbial biotransformation is one of the best choices to produce medicinal chemicals from the natural products (Demetzos *et al*., 1997). It can stimulate the production of biomass and microbial genetic systems that are generally well understood. In addition, it may mimic mammalian catabolism, and possibly allow the production of useful intermediates or metabolites in sufficiently large quantities to enable identification and usage in drug toxicity studies (Rathbone *et al*., 2002).

However, chemical synthesis which often produces many chemical groups such as hydroxyl groups, sulphate groups and others tend to be too randomly allocated (Barron D., *et. al*., 1988) and may be difficult to purify. Furthermore, it is also likely to generate toxic waste products (Seeger *et* al., 2003).

In this study, locally isolated microorganisms will be used to screen their

ability to transform the artonin E. The potential biotransformation product will be analysed by HPLC and TLC followed by investigation of their scavenging ability using the free radical scavenging activity (DPPH) assay.

1.3 Scope of Research

In this study, the microorganism will be isolated from different parts of several *Artocarpus sp*. such as the bark, the skin of the fruit and also the soil around the *Artocarpus* tree. These parts of the tree and surroundings will be selected as microorganisms from these sources could have the ability to grow in the presence of artonin E, by utilizing this compound as their natural carbon sources. Artonin E was provided by Dr. Shajarahtunnur Jamil, Department of Chemistry, Faculty of Science, UTM where the artonin E was isolated from the bark of *Artocarpus tesymanii*.

The isolated microorganisms that can grow in the presence of the artonin E will be characterized by gram staining. They will be further identified by 16S rRNA analysis and Internal Transcribed Spacer Region for fungi identification. The transformation of the artonin E will be carried out using locally isolated microorganisms. The transformed products will be subjected to Thin Layer Chromatography (TLC), High Performance Liquid Chromatography (HPLC). The structure of the biotransformation products will be elucidated by NMR spectroscopy. Antioxidant assay will be performed to evaluate the antioxidant property of the bitransformation products.

1.4 Research Objective

The objectives of this study are stated as below:

1. To screen microorganisms from different sources that have the ability to transform artonin E from *Artocarpus sp*.
2. To identify the potential microorganisms using gram staining and further analyzed by 16S rRNA analaysis and Internal Transcribed Spacer Region for fungi identification.
3. To optimize the biotransformation reaction and identify the transformation products using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC).
4. To identify the biotransformed products by Nuclear Magnetic Resonance (NMR) Spectroscopy and evaluate their antioxidant property using free radical scavenging (DPPH) assay.

REFERENCES

Abdel-Rahim Sayed Ibrahim. 2005. Biotransformation of Chrysin and Apigenin by Cunninghamella elegans. *Chem Pharm Bull (Tokyo)*. 53 (6): 671-673

Akinwunmi Olumide Latunde-Dada, Francisco Cabello-Hurtado, Nikola Czittrich, Luc Didierjean, Christel Schopfer, Norbert Hertkorni, Danie` le Werck-Reichhart and Ju ¨ rgen Ebel. 2000. Flavonoid 6-Hydroxylase from Soybean (Glycine max L.), a Novel Plant P-450 Monooxygenase. *The Journal Of Biological Chemistry*. 276(3): 1688 – 1695

Alex Ciegler. Microbial Transformation of Flavanoids. 1971. *Applied microbiology*. Dec.1971. 22 (6) :974-979

Anming Wang, Fangkai Zhang, Lifeng Huang, Xiaopu Yin, Haifeng Li, Qiuyan Wang, Zhaowu Zeng and Tian Xie. 2010. New progress in biocatalysis and biotransformation of flavonoids. *Journal of Medicinal Plants Research*. 4(10): 847-856

Becker, J. V. W., G. O. Armstrong, M. J. Van der Merwe, M. G. Lambrechts, M. A. Vivier and I. S. Pretorius. 2003. Metabolic engineering of *Saccharomyces cerevisiae* for the synthesis of the wine-related antioxidant resveratrol. *FEMS Yeast Research*. 4(1): 79–85

Beecher, GR. 2003. Overview of dietary flavonoids: nomenclature, occurrence and intake. *Journal of Nutrition*.133:3248S–54S

Bera, D., Lahiri, D., Antonella De Leonardis, K.B.De and A. Nag . 2006. A Novel

Azeotropic Mixture for Solvent Extraction of Edible Oils. *Agricultural Engineering International.* (8): 1-8

Birt, D. F., Hendrich, S. and Wang W. 2001. Dietary agents in cancer prevention: flavonoids and isoflavonoids. *Pharmacol Therapeutics*. 90(2–3): 157–177

Block, G., Patterson, B.and Subar, A. 1992. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutrition and . Cancer* 18(1): 1–29

Bong-Gyu, Kim, Bo-Ra, Jung. 2006. Regiospecific Flavonoid 7 O-Methylation with *Streptomyces avermitilis*-Methyltransferase Expressed in *E.coli*. *Journal of Agricultural and Food Chemistry*.54:823-828

Chemler, Joseph A., Leonard, Effendi. and Koffas, Mattheos A.G. 2009. Flavonoid Biotransformations in Microorganisms. In: Winefield, Chris ed. *Anthocyanins*. New York: Springer New York. 191-255

Demetzosa, C., Magiatisa P., Typasb M. A., Dimasc K., Sotiriadouc R., Perezc S. and Kokkinopoulosc D. 1997. Biotransformation of the flavonoid tiliroside to 7-methylether tiliroside: bioactivity of this metabolite and of its acetylated derivative. *Cellular and Molecular Life Sciences*. 53:587–592

Duarte Silva, I., Gaspar, J., Rodrigues, A., Gomes da Costa, B., Laires, A. and Rueff, J. 1996. Mechanisms of myricetin mutagenicity in V79 cells: involvement of radicalar species. *Teratogenesis. Carcinogenesis and. Mutagenesis.*.16, 5,253-268

Elliot, A.J., Scheiber, S.A., Thomas. C. and Pardini, R.S. 1992. Inhibition of glutathione reductase activity by flavonoids: a structure activity study. *Biochemistry Pharmac-ology*. 44: 1603-1608

Gaspar, J., Rodrigues, A., Laires, A., Silva, F., Costa, S., Monteiro, MJ., Monteiro.C. and Rueff, J. 1994. On the mechanisms of genotoxicity and metabolism of quercetin. *Mutagenesis.* 9: 445-449

Harbone, B. Jeffrey. The Flavonoids: Advances in Research since 1986. 1994. *CRC Press*. 624

Harborne, J. B. and Williams, C. A. 2000. Advances in flavonoid research since 1992. *Phytochemistry*. 55(6): 481–504

Havsteen, B. 1983. Flavonoids, a Class of Natural Products of High Pharmacological Potency. *Biochemistry and. Pharmacology*. 32: 1141-1148

Hung, L.B., Parcher, J.F., Shores, J.C. and Ward, E.H. 1988. Theoretical and experimental foundation for surface-coverage programming in gas-solid chromatography with an adsorbable carrier gas. Analytical Chemistry. 60 (11): 1090–1096

Hodnick, W.F., Kung. F.S., Roettger. W.J., Bohmont. C.W. and Pardini. R.S. 1986. Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids. *Biochemistry and. Pharmacology*. 35: 2345-2357

Howitz, K. T., K. J. Bitterman, H. Y. Cohen, D. W. Lamming, S. Lavu, J. G. Wood, R. E. Zipkin, P. Chung, A. Kisielewski, L. L. Zhang, B. Scherer and D. A. Sinclair. 2003. Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* 425(6954): 191–196

Hyo-Kon, Chun, Yasuo Ohnishi, Kazutoshi Shindo, Norihiko Misawa, Kensuke Furukawa, Sueharu Horinouchi. 2003. *Journal of Molecular Catalysis B: Enzymatic*. 21:113–121

Jagtap, U.B., Bapat, V.A. 2010. Artocarpus: A review of its traditional uses, phytochemistry and pharmacology. *Journal of Ethnopharmacology*. 129:142–166

Jarrett, F. M. 1959. Studies in Artocarpus and allied genera, I. General Considerations. *Journal of the Arnold Arboretum* 40: 1–29

Jagtap, U.B. and Bapat, V.A. 2010. Artocarpus: A Review of its Traditional Uses, Phytochemistry and Pharmacology. *Journal of Ethnopharmacology*. 129: 142– 166

Joseph, A.C., Effendi, L., Mattheos, A.G. and Koffas. 2009. Flavonoid Biotransformations in Microorganisms. Chemical and Biological Engineering, State University of New York at Buffalo

Kochummen, K. M. 2000. Artocarpus J. R. & G. Forster, nom. conserv. in Tree flora of Sabah and Sarawak, eds. E. Soepadmo, and L. G. Saw. Kuala Lumpur: Sabah Forestry Department, Forest Research Institute Malaysia, and Sarawak Forestry Department. 187–212

Laughton, MJ., Halliwell, B., Evans, P.J. and Hoult, J.R. 1989. Antioxidant and prooxidant actions of plant phenolics quercetin, gossypol and myricetin. Effects on lipid peroxidation, hydroxyl radical generation and bleomycin dependent DNA damage. *Biochemistry and. Pharmacology.* 38: 2859-2865

Nomura, T., Hano, Y., and Aida, M. 1998. Isoprenoid-Substituted Flavonoids from *Artocarpus* Plants (Moraceae), *HeteroCycles*, 47: 1179-1205,.

Nomura, T. 1999. The Chemistry and Biosynthesis of Isoprenylated Flavonoids from Moraceous Plants. *Pure and Applied. Chemistry*. 71: 1115-1118

Noorarifah. R. 2009. *Optimization of Biotransformation of Artonin E by Using Locally Isolated Microorganisms*. Degree Thesis, UTM,

Okuno, Y., Miyazawa, M. 2004. Biotransformation of isoflavones by *Aspergillus niger*, as biocatalyst. *Journal of Natural Products*. 27: 91-95

Patel, Jay M. (2008) A Review of Potential Health Benefits of Flavonoids . *Lethbridge Undergraduate Research Journal*. 3-2(ISSN 1718-8482). Institute of Chemical Technology, University of Mumbai

Rathbone, D. A. and Bruce, N. C. 2002. Microbial transformation of alkaloids. *Current Opinion in Microbiology*. 5:274–281

Robak, J. and Gryglewski, R.J. 1988. Flavonoids are Scavengers of Superoxide Anions. *Biochemistry. Pharmacology.* 37: 837-841

Sahu, S.C. and Gray, G.C. 1991. Effect of Antioxidants on Quercetin Induced Nuclear DNA Damage and Lipid Peroxidation. *Cancer Letters.* 60: 259-264

Sanches, M., and Rosazza, J.P.N. 2004. Microbial Transformation of Chalcones; Hydroxylation, O-demethylation, and cyclization to flavonones, *Journal of. Natural. Products*.67: 553-558

Seeger, M., Gonza´lez, M., Ca´mara, B., Mun˜oz, L., Ponce, E., Mej ́as, L., Mascayano, C., Va ́squez, Y., and lveda-Boza, S. S. 2003. Biotransformation of Natural and Synthetic Isoflavonoids by Two Recombinant Microbial Enzymes. *Applied and Environmental Microbiology*. 69 (9): 5045-5050

Shajarahtunnur, J. Phytochemical and Bioactivities of Malaysian Artocarpus lowii King, A. scortechhinii king and A. teysmanii Miq. Doctor of Philosophy. UTM. 2006

Shuvendu, Das. and John P.N Rosazza. 2006. Microbial and Enzymatic Transformation of Flavonoids. *Journal of Natural Products.* 69:499-508

Simmering, Rainer, Pforte, Holger. 2002. The growth of the flavonoid-degrading intestinal bacterium, *Eubacterium ramulus*, is stimulated by dietary flavonoids in vivo. *FEMS Microbiology Ecology.* 40(3): 243-248

Sritularak, B., Tantituvanont, A., Chanvorachote, P., Meksawan, K., Miyamoto. T., Kohno, Y., and Likhitwitayawuid, K. 2010. Flavonoids with free radical scavenging activity and nitric oxide inhibitory effect from the stem bark of *Artocarpus gomezianus. Journal of Medicinal Plants Research*. 4(5): 387-392

Tang Siew Hon. Biotransformation of Flavonoids. Degree Thesis. UTM. 2008

Ueno, I., Kohno, M., Haraikawa, K. and Hirono, I. 1984. Interaction Between Quercetin and Superoxide Radicals. Reduction of Quercetin Mutagenicity. *Journal of Pharmaceutical Science. Dyn.* 7: 798-803

Wai Yan Cheah. Isolation and screening of detergent-degrading microbes. Degree Thesis. UTM.2008

Watts, K. T., Lee, P. C. and Schmidt-Dannert, C. 2006. Biosynthesis of plant-specific stilbene polyketides in metabolically engineered *Escherichia coli*. BMC Biotechnology. 6: 22.