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PURIFICATION OF MIRACULIN FROM MIRACLE FRUIT [SYNSEPALUM DULCIFICUM (SCHUMACH. & THONN.) DANIELL]

HE ZUXING

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By

HE ZUXING

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in fulfillment of the requirements for the Master of Science

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PURIFICATION OF MIRACULIN FROM MIRACLE FRUIT [*SYNSEPALUM DULCIFICUM* (SCHUMACH. & THONN.) DANIELL]

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April 2015

Chairman : Arbakariya B. Ariff, Ph.D
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Synsepalum dulcificum, is a kind of berries, when eaten causes sour foods subsequently consumed to taste sweet. An efficient and low cost purification method to extract this protein needs to be developed. The pure miraculin is needed for characterization and identification of its function prior to commercialization. The potential use of the various parts of miracle fruit as well as the useful components could be extracted also needs to be explored to provide data of the importance of this fruit in economic point of view.

The physico-chemical properties of miracle fruit including percentage weight and nutritional elements (ash, crude protein, fat, total dietary fiber, carbohydrate, vitamin A and vitamin C) of miracle fruit were determined to give a full understanding of miracle fruit. Content of total anthocyanin in skin was determined using the pH-differential method. Content of phenolic in seed, skin and pulp was determined by using Folin-Ciocalteau colorimetric method. Antioxidant activity in seed, skin and pulp was analysed by DPPH free radical scavenging method. Study found that vitamin C content (40.1 mg/100 g FW) and total phenolic content (625.57 mg GAE/100 g FW) in miracle fruit flesh are very high and they together contribute to the high antioxidant activity of miracle fruit. At the same time, the sugar content is relatively low (5.6 g/100 g FW) in miracle fruit, making it become a healthy food, especially for the patients of diabetes and obesity, when considering its potential pharmaceutical benefits.

Seed oil of miracle fruit was extracted from fine powder seed of miracle fruit using Soxhlet Extractor with petroleum ether. Triacylglycerol profile was determined by HPLC, fatty acid composition was determined by GC analysis after methyl

esterification, thermal behavior was determined by Differential Scanning Calorimetry. Triacylglycerol profile and free fatty acid composition showed that the fatty acid in seed oil of miracle fruit was similar to the one of palm oil.

Miraculin is a sweet-inducing active protein that comes from miracle fruit and shows many benefits to human. However, optimization of efficient purification method of miraculin from miracle fruit has not been reported in the literature. Immobilized metal ion affinity chromatography (IMAC) with nickel-NTA was employed for miraculin purification from the extract of the pulp with optimization. The effect of extraction buffer on the amount of the extracted total protein was evaluated. This study demonstrated IMAC could be applied as one step process for purification of miraculin. The preferred conditions for high performance of IMAC with nickel-NTA were obtained with the use of crude extract at pH 7, Tri-HCl buffer at pH 7, and 300 mM imidazole with pH 8 used as elution buffer upon. The effect of optimizing crude extract was more important than optimizing the binding buffer. In elution stage, the effect of imidazole was more important than acetic acid. The IMAC charging with nickel was successfully used to purify miraculin from *S. dulcificum* with the yield and purity of 80.3% and 97.5%, respectively.

To reduce the purification cost, the possibility of using reverse micelle extraction (RME) for miraculin purification was also explored. Results from this study have demonstrated that reverse micelle formed by AOT/isooctane system can be applied as simple, convenient and low-cost process for the purification of miraculin from miracle fruit. Different effects for forward extraction and backward stripping were examined. Crude at pH 8 as the aqueous phase and 100 mM AOT/isooctane as the solvent phase during forward extraction; 0.5 M NaCl solution at pH 11, without isopropanol, as the aqueous phase during backward stripping were the optimal conditions to purify miraculin by the RME system. The maximum purification factor, purity and total purified miraculin obtained by RME were 1.63, 94.78% and 41.52 µg/mL, respectively.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

PENULENAN "MIRACULIN" DARIPADA BUAH AJAIB [*SYNSEPALUM DULCIFICUM* (SCHUMACH. & THONN.) DANIELL]

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Synsepalum dulcificum, merupakan sejenis buah beri yang diketahui apabila dimakan akan menyebabkan makanan yang masam bertukar menjadi rasa manis. Kesan ini adalah disebabkan oleh glikoprotein, miraculin. Pada masa kini, pengkomersialan miraculin sedang dijalankan. Satu kaedah penulenan yang cepat, mudah, cekap dan kos rendah untuk mengekstrak protein miraculin amat diperlukan. Selain itu, penggunaan keseluruhan buah perlu diterokai untuk mencari potensi kegunaan buah ajaib. Kajian ini bertujuan untuk menulenkan miraculin secara terus daripada buah ajaib, mencirikan minyak daripada biji buah ajaib dan menentukan bioaktiviti seperti aktiviti antioksidan daripada bahagian-bahagian lain buah ajaib.

Bagi memberikan kefahaman yang lebih jelas berkenaan buah ajaib yang tidak boleh ditemui dalam kajian bertulis, beberapa sifat-sifat fizik-kimia termasuk peratus berat dan unsur-unsur nutrisi (abu, protein kasar, lemak, jumlah serat dietari, karbohidrat, vitamin A dan vitamin C) buah ajaib telah ditentukan. Jumlah kandungan antosianin dalam kulit ditentukan dengan menggunakan kaedah pembezaan pH. Kandungan fenol di dalam benih, kulit dan pulpa ditentukan dengan menggunakan kaedah kolorimetrik Folin-Ciocalteau. Aktiviti antioksidan di dalam benih, kulit dan pulpa dianalisis dengan menggunakan kaedah pemerangkapan radikal bebas DPPH. Kajian mendapati bahawa kandungan vitamin C dan kandungan fenolik dalam isi buah ajaib adalah sangat tinggi yang menyumbangkan kepada aktiviti antioksidan yang tinggi. Di samping itu, kandungan gula yang agak rendah dalam buah ajaib menjadikannya penting

sebagai makanan yang sihat, terutama bagi pesakit diabetes dan obesiti dengan mempertimbangkan potensinya sebagai bahan farmaseutik yang bermanfaat.

Minyak biji buah ajaib diekstrak daripada serbuk halus biji buah ajaib dengan menggunakan pemerah Soxhlet bersama eter petroleum. Profil trigliserida ditentukan oleh HPLC, komposisi asid lemak ditentukan oleh analisis GC selepas pengesteran metil, sifat haba ditentukan dengan kalorimeter pengimbasan perbezaan. Profil trigliserida dan komposisi asid lemak bebas menunjukkan bahawa asid lemak dalam minyak biji buah ajaib adalah sama dengan minyak sawit.

Miraculin adalah protein aktif yang merangsangkan rasa manis buah ajaib dan menunjukkan banyak faedah kepada manusia. Walau bagaimanapun, kaedah penulenan miraculin dari buah ajaib yang cekap tidak pernah dilaporkan. Kromatografi afiniti logam ion terikat (IMAC) dengan nikel-NTA digunakan untuk penulenan miraculin daripada ekstrak pulpa dengan pengoptimuman. Kesan penggunaan penimbal dalam pengekstrakan keatas jumlah protein yang diekstrak juga dianalisis. Kajian ini menunjukkan IMAC boleh digunakan sebagai salah satu kaedah untuk proses penulenan miraculin. Keputusan kajian menunjukkan pengaruh pH penimbal ikatan, pH ekstrak mentah dan kepekatan imidazole dalam penimbal elusi pada prestasi IMAC dengan nikel-NTA. Kesan pengoptimuman ekstrak mentah adalah lebih penting daripada pengoptimuman penimbal ikatan. Pada peringkat elusi, kesan imidazole adalah lebih penting daripada asid asetik.

Demi mengurangkan kos penulenan, penggunaan "reverse micelle" untuk penulenan miraculin juga diterokai. Hasil daripada kajian ini menunjukkan bahawa "reverse micelle" yang dibina daripada sistem AOT/isooktana boleh digunakan sebagai proses yang mudah, cekap dan kos rendah untuk penulenan miraculin daripada buah ajaib. Kesan yang berbeza untuk pengekstrakan hadapan dan pengupasan belakang juga telah dikaji. Hasil kajian menunjukkan bahawa pH merupakan faktor yang penting bagi kaedah penulenan ini, manakala kepekatan AOT dan kepekatan NaCl juga menjejaskan angkali hasil dan peratus ketulenan.

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

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LIST OF ABBREVIATIONS

ABTS	2, 2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid
AOT	Aerosol-OT
Ag-RP-HPLC	RP-HPLC combined with silver chromatography
ATPS	Aqueous two-phase systems
BSA	Bovine serum albumin
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	Differential scanning colourmetric
EDTA	Ethylenediaminetetra acetic acid
ELSD	Evaporative light scattering detector
ESR	Electron spin resonance
FA	Fatty acids
FAMEs	Fatty acid methyl ester
FDA	Food and Drug Administration
FID	Flame ionization detector
FRAP	Ferric reducing ability of plasma
FW	Fresh fruit weight
GAE	Gallic acid equivalents
GC	Gas chromatography
GLC	Gas-liquid chromatography
HPLC	High-performance liquid chromatography
IDA	Iminodiacetic acid
IMAC	Immobilized-Metal Affinity Chromatography
LDL	Low-density lipoprotein
MFSO	Miracle fruit seed oil
MPP	Dipalmitic-myristic acid
NTA	Nitrilotriacetic acid
NUS	Neglected and underutilized species
OLL	Dilinoleic-oleic acid
OOL	Dioleic-linoleic acid
OOO	Oleic acid
ORAC	Oxygen radical absorbance capacity
pI	Isoelectric point value
PLL	Dilinoleic-palmitic acid
PLP	Dipalmitic-linoleic acid
POL	Palmitic-oleic-linoleic acid
POO	Dioleic-palmitic acid
POP	Dipalmitic-oleic acid
POS	Palmitic-oleic-stearic acid
PPP	Palmitic acid
PPS	Dipalmitic-stearic acid
PSS	Distearic-palmitic acid
RME	Reverse micelle extraction
RP-HPLC	Reversed-phase high-performance liquid chromatography
SOO	Dioleic-stearic acid
TAG	Triacylglycerols

TLC	Thin-layer chromatography
Trolox	(S)-(-)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

CHAPTER 1

INTRODUCTION

1.1 Background

The miracle fruit, *Synsepalum dulcificum*, is an evergreen shrub which belongs to the *Sapotaceae* family and *Synsepalum* genus. Among the 53 species identified in this genus (Anderberg and Swenson, 2003; Ayensu, 1972), *Synsepalum dulcificum* is the most widely known species. The plant was first discovered in West Africa, from Ghana to Congo (Wang et al., 2011), where the native diet revolved around a few basic foods, mostly of sour taste (Sun et al., 2006b). The sweet inducing active ingredient in *S. dulcificum*, known as miraculin, is a glycoprotein which can make the sour taste substances such as citric acid, ascorbic acid, acetic acid and hydrochloric acid to be tasted as sweet after consumption (Gibbs et al., 1996). Thus, miraculin may be used as low-calorie sweeteners because it has such distinctive ability but has almost no calories (Kant, 2005).

Human has used plant oil for hundreds of years. Plant oil is widely used as cooking oils salad oils, liquid and solid shortenings, ingredients in bakery products and fried foods (Cunha and Oliveira, 2006). Plant oil is also used in soap making, emulsions, lubricants, polyurethanes, insulation and also substrate such as *Jatropha curcas* oil for biodiesel production (Foidl et al., 1996). Plant oil is also the essential oil that fulfill the requirement of potential pharmaceutics and therapeutics. Essential oils have shown cancer inhibition activity to many kinds of human cancer cell lines including human liver tumor, glioma, gastric cancer and colon cancer (Edris, 2007). The oil can be extracted from the plant seed which may have the potential to be applied as edible oil and essential oil.

Most free radicals in chemicals, food, and even in living systems are produced from the processes of oxidation. Although free radicals are important in food and chemical material degradation, more than one hundred disorders or diseases in humans are associated with free radicals (Gorghiu et al., 2004; Jalil and Ismail, 2008; Ye and Song, 2008). Nucleic acids, proteins and lipids are easily oxidized by highly reactive free radical and oxygen species presence in biological systems, resulting in degenerative disease (Bourgeois, 2003). Antioxidants such as tocopherols, polyphenols, glutathione, and carotenoids significantly prevent or delay the oxidation of substrates that easily oxidable (Pisoschi et al., 2009).

Phenolics and their functional derivatives are the substances possessing an aromatic ring bearing one or more hydroxyl group. Phenolics play an important role as free radical scavengers and chelators of pro-oxidant metals in plant

secondary metabolites and thus preventing low-densiy lipoprotein oxidation and DNA strand scission or enhancing immune function (Shahidi and Naczk, 2003).
Fruits and vegetables naturally contain abundant of anthocyanins. According to Hertog et al. (1993), People intake anthocyanins about 200 mg per day in the United States while the other dietary flavonoids daily intake is only 20 to 25 mg. Moreover, anthocyanin content of foods has many possible health benefits and thus leads to an increasing concern. For example, a study led by Wang has shown that anthocyanins exhibited anti-carcinogenic activity against tumor types *in vivo* and multiple cancer cell types *in vitro* (Wang and Stoner, 2008).

The purification of miraculin has been studied by several researchers (Duhita et al., 2009; Duhita et al., 2011; Inglett et al., 1965; Theerasilp and Kurihara, 1988). Immobilized metal affinity chromatography (IMAC) was the most recent method used for the purification of miraculin from biological sources (Duhita et al., 2009). IMAC has been utilized for protein purification for decades. It is one of the powerful tools to purify target protein from the crude with a large amount of impurities in single step purification.

In the past few decade, reverse micelle has successfully been applied as a novel method for separating and purifying many biological products, thus attracting a lot of attention (Leser and Luisi, 1990; Ono et al., 1996). As reverse micelle provides a special microenvironment in a bulk organic medium that retain the structure of biomolecules (Ono et al., 1996), a biotechnology application of reverse micelle as an alternative for solvent extraction methods has been developed.

1.2 Objectives

The main objectives of the present study were as follows:

1. To characterize miracle fruit seed oil and physico-chemical properties of miracle fruit
2. To purify miraculin from miracle fruit extract using immobilized metal ion affinity chromatography (IMAC)
3. To purify miraculin from miracle fruit extract using reverse micelle extraction (RME) method

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