CHEMICAL PROFILING AND OPTIMIZATION OF RHOIFOLIN EXTRACTION IN RELATION TO ANTIOXIDANT PROPERTIES FROM *Fortunella polyandra*

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ABSTRACT

The chemical profile and optimization of extraction process towards antioxidant response of *Fortunella polyandra* were studied. Essential oils from the leaves, peels, and twigs of *F. polyandra* were obtained by hydrodistillation and analyzed using gas chromatographymass spectrometry (GC-MS). α-Eudesmol (31.0%), hedycrayol (20.1%) and γ-eudesmol (19.7%) were the major components in the leaves oil. The most abundant components of the peels oil consisted of limonene (71.4%) and γ-terpinene (15.2%), while α-gurjunene (26.4%), hedycrayol (23.5%), and γ-eudesmol (22.1%) were the main constituents in the twigs oil. Chromatographic separation on the hexane and methanol extract has yielded lupeol and a mixture of γ-sitosterol, stigmasterol and campesterol, respectively. The chemical composition of the leaves methanolic extract was determined using ultra performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-QToF-MS/MS). Six flavonoid glycosides were identified and tentatively characterized as 3',5'-di-*C*-β-glucopyranosylphloretin, apigenin 8-*C*-neohesperidoside, acacetin 6-*C*-neohesperidoside, acacetin 8-*C*-neohesperidoside, apigenin 6,8-di-*C*-glucoside and apigenin 7-*O*-neohesperidoside (rhoifolin). The influence of the extraction parameters, namely temperatures (30–70°C), times (5–15 minutes), and solvent ratios (30:70–70:30) of ethanol:water were investigated using response surface methodology (RSM) in order to determine the optimum extraction conditions that could produce maximum antioxidant activity from *F. polyandra* extract. Optimal extraction conditions for 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging activity were obtained at extraction time of 5 minutes, temperature of 30°C and solvent ratio of 70:30, ethanol:water. The obtained IC_{50} value in the DPPH assay was 0.126±0.004 mg/mL, which is close to the predicted value of 0.137 mg/mL. The optimum parameters for ferric reducing antioxidant potential (FRAP) assay were extraction time of 5 min, temperature of 30°C, and solvent ratio of 30:70, ethanol:water. Under these conditions, rhoifolin content obtained was 67.83±0.37 ppm, which is very close to the predicted value of 67.58 ppm, whereas FRAP equivalent value of 0.22±0.01 mg/mL obtained is equal to the predicted value from the model. All three responses from the model achieved 95% confidence level. Correlation study conducted shows that there is no relationship between the rhoifolin content and the antioxidant activities of *F. polyandra* extract.

ABSTRAK

Profil kimia dan pengotimuman proses pengekstrakan terhadap gerak balas antioksidan *Fortunella poliandra* telah dikaji. Minyak pati daripada daun, kulit buah, dan ranting *F. poliandra* telah diperoleh daripada penyulingan hidro dan dianalisis menggunakan kromatografi gas-spektrometri jisim (GC-MS). α-Eudesmol (31.0%), hedikrayol (20.1%) dan γ-eudesmol (19.7%) adalah komponen utama di dalam minyak daun. Komponen yang paling banyak di dalam minyak kulit buah terdiri daripada limonena (71.4%) dan γ-terpinena (15.2%), manakala α-gurjunena (26.4%), hedicrayol (23.5%) dan γ-eudesmol (22.1%) adalah juzuk utama di dalam minyak ranting. Pemisahan kromatografi ke atas ekstrak heksana dan metanol, masing-masing menghasilkan lupeol dan satu campuran γ-sitosterol, stigmasterol dan kampesterol. Komposisi kimia ekstrak metanol daun *F. poliandra* ditentukan menggunakan kromatografi cecair prestasi ultra yang digandingkan dengan spektrometri jisim masa-penerbangan kuadrupol (UPLC-QToF-MS/MS). Enam flavonoid glikosida telah dikenal pasti dan dicirikan secara tentatif sebagai 3',5'-di-*C*-β-glukopiranosilfloretin, apigenin 8-*C*-neohesperidosida, acacetin 6-*C*-neohesperidosida, acacetin 8-*C*-neohesperidosida, apigenin 6,8-di-*C*-glukosida dan apigenin 7-*O*-neohesperidosida (rhoifolin). Pengaruh ke atas parameter pengekstrakan, iaitu suhu (30–70°C), masa (5–15 minit), dan nisbah pelarut (30:70–70:30) etanol:air telah dikaji menggunakan kaedah permukaan gerak balas untuk menentukan keadaan pengekstrakan optimum yang dapat menghasilkan aktiviti antioksidan maksimum daripada ekstrak *F. poliandra*. Keadaan pengekstrakan optimum bagi aktiviti pemerangkapan 2,2- difenil-1-pikrilhidrazil (DFPH) diperoleh pada masa pengekstrakan 5 minit, suhu 30°C dan nisbah pelarut 70:30, etanol:air. Nilai IC_{50} yang diperoleh dalam cerakin DPPH adalah 0.126±0.004 mg/mL, yang hampir dengan nilai ramalan 0.137 mg/mL. Parameter optimum bagi cerakin keupayaan antioksidan penurunan ferik (FRAP) adalah masa pengekstrakan 5 minit, suhu pengekstrakan 30°C, dan nisbah pelarut 30:70, etanol:air. Di bawah keadaan ini, kandungan rhoifolin yang diperoleh adalah 67.83±0.37 ppm, yang sangat hampir dengan nilai ramalan 67.58 ppm, sementara nilai setara FRAP 0.22±0.01 mg/mL yang diperoleh adalah sama dengan nilai ramalan daripada model. Ketiga-tiga gerak balas daripada model mencapai aras keyakinan 95%. Kajian korelasi yang dijalankan menunjukkan bahawa tiada hubungan antara kandungan rhoifolin dan aktiviti antioksidan ekstrak *F. poliandra*.

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LIST OF ABBREVIATIONS

RSM	-	Response Surface Methodology
CCRD	-	Central Composite Rotatable Design
UTM	-	Universiti Teknologi Malaysia
UKM	-	Universiti Kebangsaan Malaysia
DOE	-	Design of Experiments
LCMS	-	Liquid Chromatography Mass Spectrometer
NMR	-	Nuclear Magnetic Resonance
FTIR	-	Fourier Transform Infra-Red
GC-MS	-	Gas Chromatography Mass Spectrometer
HPLC	-	High Performance Liquid Chromatography
UAE	-	Ultrasound-Assisted Extraction
ANOVA	-	Analysis of Variance
SD	-	Standard Deviation
Ofat	-	One Factor Analysis at a time
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
FRAP	-	Ferric reducing antioxidant potential

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Modern chemistry has opened a new era of the uses of natural products. The plant diversity in Malaysia provides many opportunities for natural product research. Herbal products and traditional medicine are convenient, obtainable with least side effects to mankind. Natural products have been the basis of treatment of human diseases (Lahlou, 2013).

The uses of plant material as medicines can be found as earlier as the middle paleolithic age some 60,000 years ago. History, documents and archaeological find archives has recorded that ancient uses plant as their medicine. It have been reported that 80,000 of 250,000 species of flowering plant used by people for medicinal purpose, and this figure is believed to be higher as some of the medicinal folks and traditional medicines from plants has mostly stayed unregistered. Some of the information on the benefit mainly passed on verbally from one generation to another (Jantan *et al*., 2015).

Biotechnology industry in Malaysia is developing and can potentially become one of the alternative resources to treat disease. Traditional medicine from plants, animals and micro-organisms are still use as a basis for health care in the rural area communities. A part of commercial drugs in the market are base from plants and numbers of supplements are also derived from plants (Ministry of Natural Resources and Environment, 2006).

Phytochemical study in plant has been an interest to the research world for a long time. Secondary metabolites such as flavonoids and phenolic compound have shown wide range of benefit to human health attributed by its biological activity. The potential of natural sources from plant needs to be fully utilized to maximize the uses of its bioactive compounds (Ministry of Natural Resources and Environment, 2006). Design of experiments (DOE) is commonly used as an optimizing tool to optimize the extraction of natural products.

1.2 Problem Statement

In the past few years, interest on investigation of biological active component in a plant had been significantly increasing. Nutraceutical, polyphenolic and more specifically flavonoids have become the focus for researchers. Flavonoid are the most common component found in plant and it can be used as a main dietary source for human, and have shown to have antioxidant activity, to inhibit angiogenesis, and to slow down cancer cell migration and proliferation (Peng *et al*., 2013).

Phytochemical and bioactivities studies on *Fortunella* plants had discovered the biological activity such as antioxidant, antimicrobial, tyrosinase inhibitory, antimetabolic disorder, anticancer and antitumor. However, there is still inadequate literature on the quantification and optimization of its bioactive compounds. Furthermore, there is still lack of information on the phytochemical study of *F. polyandra*. Hence the present study will provide the optimum condition to produce high yield of bioactive compounds, chemical profiling study and as well as the relationship on the major compound towards the contribution of its bioactivities (si Tan *et al.*, 2016).

1.3 Research Objectives

The purposes of this study are stated as below:

(a) To profile the chemical constituents of: (a) essential oils from peel, leaves, and twigs by hydrodistillation extraction using GC-MS; (b) methanolic leaves extracts by Soxhlet extraction using UPLC-QToF-MS/MS.

(b) To isolate and elucidate the structure of phytochemicals from the crude leaves from Soxhlet extraction of *Fortunella polyandra.*

(c) To verify the optimum condition of leaves extract in *Fortunella polyandra* by ultrasound-assisted extraction for antioxidant activities and apigenin 7-*O*-neohesperidoside (rhoifolin) contents using response surface methodology.

(d) To correlate the antioxidant activities with the content of apigenin 7-*O*-neohesperidoside (rhoifolin) in *Fortunella polyandra* leaves extracts.

1.4 Scope of Study

This research was focused on the study of the phytochemical analysis of *Fortunella polyandra*. The chemical composition of essential oils including the leaf, twigs and peels of *F. polyandra* was extracted by hydrodistillation and investigated using GC-MS. Furthermore, The dried sample of *F. polyandra* leaves was extracted using Soxhlet extraction with *n*-hexane and methanol, a small portion from the methanolic leaves extracts was then analysed using UPLC-QToF-MS/MS to screen its chemical composition, while the other remaining extracts was subjected under different chromatographic techniques for isolation of compounds. The isolated compounds was elucidated using gas chromatography mas spectrometry (GCMS), nuclear magnetic resonance spectroscopy (NMR) and Fourier transform infrared spectroscopy (FTIR)

This research also consists of an experimental study on extracts optimization using ultrasound-assisted extraction method by response surface methodology based central composite rotatable design (CCRD) approach with three factors and five levels (α, 1, 0, -1, and –α). The three factors were solvent concentration, extraction time and extraction temperature, while the response analyses performed involved free radical scavenging capacity by DPPH assay, ferric reducing antioxidant power assay (FRAP) and quantification of rhoifolin using high performance liquid chromatography (HPLC). Lastly, the selected major compound tested by UPLC-QToF-MS/MS from the methanolic leaves extracts was then studied to evaluate its contribution and correlation toward the antioxidant activity of the extracts.

1.5 Hypothesis of Study

In this study, the hypothesis that can be formulated is *F. polyandra* oils are rich in terpenoids while the crude extracts are rich in flavonoid composition. The optimization prosess will provide the high antioxidant yield from the optimization condition in aspect of its solvent concentration, extraction time and extraction temperature. The major compound of *F. polyandra* will gives a positive contribution towards the bioactivity by its correlation study with the crude extracts of the leaves.

1.6 Significance of Study

Fortunella fruits and juices have several beneficial effects on health and nutritive properties. Based on their used for medicinal purposes among the local folks, needed now are the scientifically biological and chemical studies to find the promising chemical constituents and establish the major compounds and its bioactivity. To date, there is no study on chemical constituent from the *F. polyandra* as well as their optimum condition for the extraction of crude extract and its antioxidant activity.

The phytochemical and antioxidant property of *Fortunella japonica* and *Fortunella margarita* has been extensively studied, but no significant finding for *F. polyandra*. Based on the knowledge of operating parameters for the extraction of *F. polyandra*, the extraction yield will increase by monitoring the involved parameters so that the crude can be used effectively.

This research will fill the gap on genus *Fortunella* research as well as contribute to the collection on the database of its phytochemical. The optimization result will be beneficial for further research on the production of bioactive compounds on a larger scale.

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