UNIVERSITI PUTRA MALAYSIA

# *EXTRACTION AND IDENTIFICATION OF DI(2-ETHYLHEXYL) PHTHALATE FROM SAFED MUSLI (CHLOROPHYTUM BORIVILIANUM L.)*

**CHUA BEE LIN**

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**EXTRACTION AND IDENTIFICATION OF DI(2-ETHYLHEXYL) PHTHALATE FROM SAFED MUSLI (*CHLOROPHYTUM BORIVILIANUM* L.)**

**By**

**CHUA BEE LIN**

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy**

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By

**CHUA BEE LIN**

**October 2015**

**Chair: Professor Luqman Chuah Abdullah, PhD**
**Faculty: Engineering**

*Chlorophytum borivilianum* (safed musli) is a medicinally important plant. Its roots are being employed in folk medicine. Presently, the crude extract of *C. borivilianum* has been consumed for the treatment such as anti-diabetic, anti-aging, anti-oxidant, anti-ulcer and anti-inflammatory. Studies have been carried out to further confirm these remarkable bioactivities of *C. borivilianum*. So far, the isolated chemical constituents are mainly saponins. A fructo-oligosaccharide, three fatty acids, one sterol stigmasterol, hecogenin also have been reported to be isolated from the roots of *C. borivilianum*. In this research, di(2-ethylhexyl) phthalate (DEHP) was extracted from the aqueous extract of the roots of *C. borivilianum*. The yield of DEHP was found to be 33.70 mg, which was equivalent to 0.013% with reference to the total weight of root powder (250 g). The structure of DEHP was elucidated based on the spectral data of $^{1}$H-NMR, $^{13}$C-NMR, distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum correlation (HMQC) and also based on the comparison with the previous literature data. This is the first report so far of occurrence and detail spectroscopic description of DEHP from *C. borivilianum*. Single experimental design and response surface methodology (RSM) was implemented to optimize the extraction conditions for obtaining the maximum yield of DEHP from the roots of *C. borivilianum*. DEHP was optimized because it could be a starting point to pave a way to isolate and quantify other pure compounds (minor or rare) from this herbaceous plant in order to use it as a tool for quality control and also for the future development of other therapeutic applications. Furthermore, DEHP was reported to possess some remarkable biological activities such as anti-leukemic, anti-microbial, anti-fungal, anti-tumour and antiviral activity against H1N1 disease. In this study, ultrasound-assisted extraction was applied for the effective extraction of DEHP and DEHP was quantified by high performance liquid chromatography (HPLC) analysis. Herein, three independent variables (extraction time, solid to solvent ratio and extraction temperature) with a five level design were evaluated

using the central composite design (CCD), with the yield of DEHP as the response variable. Second-order polynomial model was found to be satisfactory in describing the experimental data for the total DEHP content. The analysis of variance (ANOVA) indicated that the main effect of solid to solvent ratio and the extraction temperature as well as the quadratic effects of all independent variables had significant effect ("Prob>F"<0.05) on the extraction yield of DEHP. The optimal extraction conditions were established as follow: extraction time of 92 min, solvent to solvent ratio of 1:38 (g/mL) and extraction temperature of 51°C. Using these adjusted optimal conditions, the predicted yield of DEHP by model was 0.44 ppm whereas the actual yield of DEHP was 0.43±0.01 ppm which was in close conformity with the predicted values as the relative error was just 2.72%. The extraction kinetic was studied using equilibrium extraction model (EEM) and diffusion extraction model (DEM). The kinetics results revealed EEM model was more suitable in describing the extraction process and the ethanol extraction of the roots of *C. borivilianum* achieved equilibrium within 70 minutes. Lastly, DEHP extracted from the roots *C. borivilianum* was tested for its anti-inflammatory activity. However, DEHP had shown a low inhibition effect on the anti-inflammatory activity in HYA assay with a percentage of inhibition of 4.02±1.17%. This study implied that DEHP was not active to inhibit the hyaluronidase enzyme and this indicated that the other compounds in the roots of *C. borivilianum* might contribute to the anti-inflammatory activity as the previous researchers had obtained significant anti-inflammatory activity from this plant.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah


**PENGEKSTRAKAN DAN PENGENALPASTIAN DI(2-ETHYLHEXYL) PHTHALATE DARI SAFED MUSLI (*CHLOROPHYTUM BORIVILIANUM* L.)**

Oleh

**CHUA BEE LIN**

**Oktober 2015**

**Pengerusi: Profesor Luqman Chuah Abdullah, PhD**
**Fakulti: Kejuruteraan**

*Chlorophytum borivilianum* (safed musli) merupakan tumbuhan ubatan yang bernilai. Akarnya telah digunakan dalam perubatan rakyat. Baru-baru ini, ekstrak mentah dari *C. borivilianum* telah digunakan untuk rawatan seperti penyakit kencing manis, anti-penuaan, antioksidan, anti-ulser and anti-radang. Kajian telah dijalani untuk mengesahkan bioaktiviti *C. borivilianum*. Setakat ini, konstituen kimia yang diasingkan kebanyakannya merupakan saponins. Satu frukto-oligosakarida, tiga asid lemak, satu stigmasterol sterol dan hekogenin telah dilaporkan diasingkan dari akar *C. borivil ianum*. Dalam penyelidikan ini, di(2-ethylhexyl) phthalate (DEHP) telah diekstrak dari ekstrak akueus akar *C. borivilianum*.Hasil DEHP adalah 33.70 mg, bersamaan 0.013% merujuk kepada jumlah berat serbuk akar (250 g). Struktur DEHP telah dihuraikan berdasarkan data spektrum seperti $^{1}$H-NMR, $^{13}$C-NMR, DEPT, COSY, HMBC dan HMQC dan juga berdasarkan kepada perbandingan dengan data kajian lepas. Ini merupakan laporan pertama setakat ini tentang kehadiran dan huraian spektroskopi yang perinci bagi DEHP yang diasingkan dari *C. borivilianum*. Reka bentuk eksperimen tunggal dan kaedah respons permukaan (RSM) telah digunakan bagi mengoptimumkan syarat-syarat pengekstrakan untuk mendapatkan kadar hasil maksimum DEHP dari akar *C. borivilianum*. DEHP telah dioptimumkan kerana ia boleh digunakan sebagai titik permulaan bagi mengekstrak and mengenalpasti kandungan kompaun-kompaun yang lain (sikit ataupun jarang) dari tumbuhan herba ini supaya kualiti dapat dikawal dalam aplikasi terapeutik pada masa depan. Tambahan pula, DEHP dilaporkan mempunyai beberapa aktiviti biologi seperti anti-leukemia, anti-mikrob, anti-kulat, anti-tumor dan antivirus terhadap H1N1. Dalam kajian ini, pengekstrakan ultrasonik telah dijalani untuk pengekstrakan DEHP secara berkesan dan DEHP telah dikuantitikan oleh analisis kromatografi cecair berprestasi tinggi (HPLC). Dengan ini, tiga pembolehubah bebas (masa pengekstrakan, nisbah pepejal kepada pelarut dan suhu pengekstrakan) dengan reka bentuk berperingkat lima telah dinilai dengan menggunakan reka bentuk komposit tengah dan hasil DEHP telah dipilih sebagai respons pembolehubah. Model polinomial berkuadratik telah dihasilkan dan didapati memuaskan dalam menghuraikan data eksperimen untuk jumlah kandungan DEHP. Analisis varians (ANOVA) menunjukkan bahawa nisbah pepejal kepada pelarut dan suhu pengekstrakan serta kesan-kesan kuadratik semua pembolehubah bebas mempunyai kesan yang ketara ($p<0.05$) terhadap hasil pengekstrakan DEHP. Syarat-syarat pengekstrakan yang optimum telah ditetapkan

seperti yang berikut:  masa pengekstrakan 92 min, nisbah pepejal kepada pelarut 1:38 (g/mL) dan suhu pengekstrakan 51°C. Dengan meggunakan syarat-syarat optimum yang terlaras, hasil DEHP yang diramalkan oleh model ialah 0.44 ppm manakala hasil sebenar DEHP ialah 0.43±0.01 ppm dan nilai sebenar hampir selaras dengan nilai ramalan kerana ralat relatif hanya 2.72%. Keputusan telah menunjukkan bahawa RSM boleh digunakan bagi mengoptimumkan syarat-syarat pengekstrakan DEHP dari akar *C. borivilianum*. Tambahan pula, kinetik DEHP telah dikaji dan dua model (EEM dan DEM) telah dihasilkan. EEM lebih sesuai bagi menghuraikan proses pengekstrakan dan pengekstrakan etanol akar *C. borivilianum* mencapai keseimbangan dalam 70 minit. Akhir sekali, activiti anti-radang HYA DEHP telah dikaji dan DEHP telah menunjukkan kesan yang tidak ketara dengan hanya 4.02±1.17%. Kajian ini mencadangkan bahawa DEHP tidak aktif terhadap antiviti ini dan mungkin adalah kompaun-kompaun lain yang menyumbang kepada activiti anti-radang.  Ini adalan kerana aktiviti anti-radang yang positif telah dilaporkan oleh pengkaji-pengkaji yang sebelumnya.

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University Korea
(External Examiner)

**ZULKARNAIN ZAINAL, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 16 February 2016


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**Luqman Chuah Abdullah, PhD**
Professor
Faculty of Engineering
Universiti Putra Malaysia
(Chairperson)

**Thomas Choong Shean Yaw, PhD**
Professor, Ir
Faculty of Engineering
Universiti Putra Malaysia
(Member)

**Umi Kalsom Yusuf, PhD**
Professor
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Universiti Putra Malaysia
(Member)

**Pin Kar Yong, PhD**
Research Officer
Herbal Technology Center
Forest Research Institute Malaysia
(Member)

**Zunoliza Abdullah, PhD**
Research Officer
Programme Phytochemistry
Forest Research Institute Malaysia
(Member)

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Signature: ______________________
Name of Member of Supervisory Committee: Prof. Dr. Umi Kalsom Yusuf

Signature: ______________________
Name of Member of Supervisory Committee: Dr. Pin Kar Yong

Signature: ______________________
Name of Member of Supervisory Committee: Dr. Zunoliza Abdullah

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 1D, 2D | one-dimensional, two-dimensional |
| ANOVA | analysis of variance |
| CC | column chromatography |
| $CD_3OD$ | deuterated methanol |
| BBD | Box-Behnken |
| CBA | chloroform extract |
| CBC | hexane extract |
| CBH | hexane extract |
| CCD | central composite design |
| C.V. | coefficient variation |
| CH | methine carbon atom |
| $CH_2$ | methylene carbon |
| $CH_3$ | methyl carbon atom |
| COSY | correlation spectroscopy |
| d | doublet |
| DEHP | di(2-ethylhexyl) phthalate |
| DEPT | distortionless enhancement by polarization |
| FRIM | Forest Research Institute Malaysia |
| GRAS | Generally Recognized as Safe |
| HMBC | heteronuclear multiple bond correlation |
| HPLC | high performance liquid chromatography |
| HMQC | heteronuclear multiple quantum correlation |
| i.d. | internal diameter |
| *J* | coupling constant |
| K | number of variable |
| m | multiplet |
| n | total number of experiments |
| NMR | nuclear magnetic resonance |
| MS | mass spectroscopy |
| ODS | octadecylsilanized silica gel |
| PDA | photodiode array |
| PVDF | polyvinylidene difluoride |
| ppm | parts per million |
| q | quartet |
| $R^2$ | correlation coefficient |
| $R_f$ | retention factor |
| RSM | response surface methodology |
| s | singlet |
| SD | standard deviation |
| t | triplet |
| TLC | thin layer chromatography |
| TMS | tetramethylsilane |
| UAE | ultrasound-assisted extraction |

| | |
|---|---|
| UV | ultra violet spectroscopy |
| $W_d$ | weight of the dried extract |
| $W_s$ | weight of solid |
| $x$ | independent variable |
| $x_o$ | number of central points |
| $x_{min}$ | minimum value of the independent variable |
| $x_{max}$ | maximum value of the independent variable |
| X | coded variable |
| $X_1$ | coded variable |
| $X_2$ | extraction time |
| $X_3$ | solid to solvent ratio |
| $X_1^2$ | extraction temperature |
| $X_2^2$ | quadratic effect of extraction time |
| $X_3^2$ | quadratic effect of solid to solvent ratio |
| $X_1X_2$ | quadratic effect of extraction temperature |
| $X_1X_3$ | interactive effect of extraction time and solid to solvent ratio |
| $X_2X_3$ | interactive effect of extraction time and extraction temperature |
| $Y_1$ | interactive effect of solid to solvent ratio and temperature |
| $Y_i$ | yield of the crude extract |
| $\beta_0$ | response variable |
| $\beta_{ii}$ | constant coefficient |
| $\beta_{ij}$ | linear coefficients |

CHAPTER 1

# INTRODUCTION

## 1.1 Introduction to Medicinal Plants

There are a lot of medicinal plants throughout the world with assorted pharmacological activities to treat certain type of diseases. Today, science has isolated the medicinal properties from a large quantity of various types of herbal plants. Most herbal plants remedies are used to treat the health problems and scientific reports have proven success in treating certain chronic conditions. It is crucial to develop a strict standardization procedure and perform pharmacognostical studies of herbal plants in order to avoid accidental herbal medicine misuse due to wrong identification of a medicinal plant and wrong prescription of traditional herbal medicine. Therefore, phytochemistry has become a major branch of pharmacognosy in developing markers for the objective of identification and standardization (Sankh, 2010).

## 1.2 Background of Study

It has been proven in scientific research that the plants have contributed us the active compounds that have potential therapeutic effects. The plant *Chlorophytum borivilianum* (Safed Musli) shown in Figure 1.1 is a medicinal plant belonging the family Liliaceae. *C. borivilianum* holds an important place in the traditional medicinal system due to its therapeutic importance. The economic part of the herb is its roots. Its roots are powdered and widely used in traditional folk medicines over past decades (Thakur et al., 2009a; Deore and Khadabadi, 2010a).

**Figure 1.1: Plant and Roots of *Chlorophytum Borivilianum* (Safed Musli)**

Presently, crude extract of *C. borivilianum* has been utilized for treatment of human diseases especially in the applications such as anti-diabetic (Panda et al., 2007), anti-oxidant (Kenjale et al., 2007), anti-ulcer (Panda et al., 2011a) and anti-inflammatory (Deore and Khadabadi, 2008). *In-vitro* and *in-vivo* studies have confirmed that the crude extract of C. *borivilianum* possesses a wide range of noteworthy pharmacological activities. Interest is increasing to exploit this herbal plant for the development of therapeutics that could be potentially used as sexual stimulant for impotence and to prevent or treat cancers (Thakur et al., 2009b; Kumar et al., 2010).

## 1.3 Problems statement

There are extensive studies reported on the biological activities of the crude extract of the roots of *C. borivilianum* but there were only few studies had been done on the isolation of chemical constituents that responsible for the above reported applications. In addition, there were only few studies regarding the isolation of other chemical compounds since extensive works were carried out in the previous findings were aimed for the isolation of saponins from the roots of the plant. Due to the limited information regarding to the pure compounds other than saponins that have been isolated from this plant, therefore, this research focused on the isolation of any pure compounds other than saponins. At last, DEHP was isolated after few months of isolation procedures. DEHP was reported to possess some remarkable biological activities such as anti-leukemic, anti-microbial, anti-fungal, anti-tumour and antiviral. Therefore, it was selected to be optimized and it could be a starting point to pave a way to isolate and quantify other pure compounds (minor or rare) from this herbaceous plant in order to use it as a tool for quality control and also for the future development of other therapeutic applications. Furthermore, there is a lack of research on the optimization of the extraction parameters in order to maximize the yield of extract isolated from the roots of *C. borivilianum*. Therefore, it could be used as reference to isolate and elucidate a pure compound other than saponins and thereafter to optimize the isolated compound through response surface methodology (RSM).

To the best of our knowledge, no report was available in the literature regarding the optimization of extraction conditions from *C. borivilianum* through response surface methodology (RSM). Therefore, in this present study, there is a need to isolate and identify the chemical constituents that present in this plant and to identify the optimal conditions on the extraction of the roots of *C. borivilianum* using response methodology method in order to obtain the maximum yield of the isolated compound. Considering the residual toxicity of the extraction solvent, ethanol was opted for the extraction procedure. A single factor experimental design with five levels was studied to determine the preliminary experimental range of the extraction parameters on the extraction yield of DEHP, followed by the optimization of the extraction yield of DEHP by the RSM in combination with central composite design experimental design.

DEHP was reported to possess biological activities such as anti-leukemic, anti-microbial, anti-fungal, anti-tumour and antiviral. Considering the medicinal and pharmacological importance of *C. borivilianum* and the reported biological activities of DEHP, it is worth to optimize the extraction yield of DEHP in order to develop a systematic approach for the optimization of the other future isolated active compounds which are responsible for the reported pharmacological activities.

## 1.4 Objectives of the Study

With this respect, the objectives of this study are:

1. Extraction, isolation, purification and structure elucidation of pure compound from the roots of *C. borivilianum* by a combination of various chromatographic methods and different spectroscopic techniques, respectively.
2. Identification and quantification of the isolated compound by the mean of high performance liquid chromatography (HPLC) method.
3. Optimization of the extraction conditions and extraction kinetic of the isolated compound.

## 1.5 Scopes of Study

The work embodied in the present thesis is divided into following stages:

1. Isolation and structure elucidation of the pure compound from the roots of *C. borivilianum*
   The process of extraction and isolation of the pure compound from the roots of the *C. borivilianum* was carried out by mean of chromatography method such as open column chromatography and thin layer chromatography. The structure of the pure compound isolated from the roots of *C. borivilianum* was elucidated by means of nuclear magnetic resonance (NMR) spectroscopy analysis. NMR spectroscopy of the structure elucidation included Proton NMR ($^{1}$H-NMR), Carbon-13 NMR ($^{13}$C-NMR), distortionless enhancement by polarization transfer (DEPT), correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC) and heteronuclear multiple quantum correlation (HMQC). An *in-vitro* anti-inflammatory assay was conducted for the *C. borivilianum* extract using Hyaluronidase (HYA) assay.
2. Identification and quantification of the isolated compound
   A high performance liquid chromatography (HPLC) method was developed and implemented to identify the compound appeared in the standard and in the crude extract. The yield of the compound was quantified based on the calibration curve of the pure isolated compound.
3. Optimization of the extraction conditions and extraction kinetic of the isolated compound
   Four operating parameters including extraction solvent, extraction time, solid to solvent ratio and extraction temperature were studies. Firstly, the selection of suitable solvent system was made and it was evaluated by quantifying the amount of extraction yield. Then, the experimental range of extraction time, solid to solvent ratio and extraction temperature was identified by a single factor experimental design. Also, a polynomial model for the extraction yield of compound from the roots of C. *borivilianum* was developed by mean of response surface methodology (RSM) using a central composite design (CCD) by Design Expert software. The optimum values for each of extraction parameter were established and the model was validated by performing the extraction of the compound under the optimal extraction conditions. Based on the optimization process, the extraction kinetics of the *C. borivilianum* was investigated and mathematical models for solid-liquid extraction of the *C. borivilianum* extract were applied to predict the extraction process.

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