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Isolation, Structure Elucidation, Identification and Quantitative Analysis Of Di(2-Ethylhexyl) Phthalate (DEHP) from the Roots of *Chlorophytum Boriviliuanum* (Safed Musli).

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ABSTRACT

Chlorophytum borivilianum (safed musli) is a traditional herbaceous medicinal plant belonging to family Liliaceae. Its roots are being employed in folk medicine. The crude extract of *C. borivilianum* has been consumed due to its versatile therapeutic uses. The scientific studies related to the important pharmacological properties are widely conducted and the remarkable bioactivities of *C. borivilianum* are proven in literatures. So far, the isolated chemical compounds are mainly saponins. In this research, the isolation was focused on compounds other than saponins and bis(2-ethylhexyl) benzene-1,2-dicarboxylate was isolated for the first time from the roots of *C. borivilianum*. The structure was identified based on the spectral data of 1H NMR, 13C NMR, DEPT, COSY, HMBC, HMQC and also based on the comparison with the previous literature data. This is the first report regarding the presence of this compound in *C. borivilianum* as well as its genus. A high performance liquid chromatographic (HPLC) method with photodiode array detection was established to identify and quantify bis(2-ethylhexyl) benzene-1,2-dicarboxylate.

Keywords: *Chlorophytum borivilianum*; Isolation; bis(2-ethylhexyl) benzene-1,2-dicarboxylate; Structure elucidation; Quantification.

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INTRODUCTION

There are a lot of medicinal plants throughout the world with assorted pharmacological activities to treat certain type of human diseases. Herbal plants are more preferable than manufactured drugs as they work gently and less toxic. It is crucial to develop a strict standardization of herbal plants procedures in order to avoid any accidental herbal misuse due to wrong identification of medicinal plant. Therefore, phytochemistry has become a major branch of pharmocognosy in developing markers for the objective of identification and standardization [1].

The plant *Chlorophytum borivilianum* (safed musli) is a medicinal plant belonging to family Liliaceae. *C. borivilianum* is well-known for its therapeutic uses in the traditional medicinal system. The economic part of this plant is root. The roots of *C. borivilianum* are consumed as folk medicine because it is a native Indian plant which is a part of an important class of ayurvedic herbs known as Rasayana. [2-4] and it is a chief ingredient in Ayurvedic, Unani, Homeopathic and Allopathic systems of medicine [3, 5, 6].

Recently, there were studies reported that the crude extract and isolated compounds from the roots of *C. borivilianum* possess some remarkable bioactivities such as hepatoprotective [7], , larvicidal, anti-inflammatory, antioxidant [8], immunomodulatory [9], anti-diabetic [10], anti-ulcer [11], analgesic [10], anti-microbial [12], anti-stress [13], anti-bacterial [14] and anti-arthritic [15] effects. *C. borivilianum* is widely exploited due to its recently reported therapeutic uses to treat cancer [16] and sexual dysfunction [17]. A lot of studies were conducted regarding the isolation of the active chemical constituents from *C. borivilianum* that attributed to the reported bioactivities which are mainly saponins [18-21] and there were only few studies had been done on the isolation of other chemical constituents from the corresponding plant.

The objectives of this study are to isolate, elucidate, identify and quantify for other isolated compounds other than saponins from the roots of *C. borivilianum*. In this study, bis(2-ethylhexyl) benzene-1,2-dicarboxylate was successfully isolated from *C. borivilianum* as well as its genus. Based on the reported literature, bis(2-ethylhexyl) benzene-1,2-dicarboxylate is claimed to possess anti-leukemic, anti-mutagenic [22], antimicrobial, cytotoxic [23], antitumour [23] and antiviral activities. Therefore, the isolation method of this compound was established and an efficient HPLC method was developed for the quantitative determination of bis(2-ethylhexyl) benzene-1,2-dicarboxylate in the crude extract using the isolated bis(2-ethylhexyl) benzene-1,2-dicarboxylate as standard.

MATERIALS AND METHODS

Plant Material

Fresh roots of *C. borivilianum* were collected from Professor Luqman Chuah Abdullah, Universiti Putra Malaysia, Selangor Darul Ehsan, Malaysia. The roots were washed and dehydrated in a drying oven (Model UFE-800, Memmert, Germany) at 40℃. The dried roots were ground into coarse powder with a cutting mill (Model SM-100, Retsch, Germany) and sieved to pass through 60-mesh. The powder was subsequently sealed in a plastic container and stored at room temperature until needed for further extraction and analysis. The roots were kept in Raw Material Storage Room of Herbal Technology Centre, FRIM.

Chemicals

Methanol, hexane and chloroform used for thin layer chromatography (TLC analysis), solid-liquid extraction and isolation were of analytical grade. Methanol, acetonitrile and formic acid used for HPLC analysis were of HPLC grade. Deuterated methanol was used for NMR experiments. Water was deionized (M) using a Milli-Q water purification system (Millipore, Bedford, MA). All solvents were purchased from Merck, Germany.

Isolation of bis(2-ethylhexyl) benzene-1,2-dicarboxylate

250 g of coarsely ground roots of *C. borivilianum* was extracted with 2.5 L of fresh methanol and left to stand at room temperature for of three days. Thereafter, the resulting extract was filtered and the filtrate was dried in a rotary evaporator at 40°C. The residue was subjected to the same procedure thrice. The yield of the methanol extract was found to be 24 g. The methanol extract was partitioned sequentially with water, then with hexane and finally with chloroform. The solvents were removed and this afforded three partition

fractions and encoded as CBA, CBH, CBC from the layers of aqueous, hexane and chloroform, respectively. CBA was further chromatographed over MCI gel CHP 20P (75-150 μm, Supelco, Bellefonte, PA, USA), octadecylsilanized silica gel (ODS) (100-200 mesh, Fuji Silysia Chemical Ltd. Japan) and silica silica gel (200-400 mesh, Merck, Germany). Bis(2-ethylhexyl) benzene-1,2-dicarboxylate utilizing hexane and ethyl acetate was purified by repeated column chromatography over silica gel and final purification of this compound was performed with an isocratic separation using a mixture of hexane and ethyl acetate (10:0.5) to afford 33.7 mg of bis(2-ethylhexyl) benzene-1,2-dicarboxylate.

TLC Analysis

The whole isolation procedure was monitored by using thin-layer chromatography (TLC) analysis and was performed on the TLC precoated silica gel F254 plates (0.2 mm thick, Merck, Germany) 5cm x 5 cm. The TLC chromatograms were developed using a solvent system composed of hexane and ethyl acetate (10:0.5). The pure single compound on the TLC plate was spotted in UV light at 254 nm, after spraying with 10% sulphuric acid reagent followed by gradual heating.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The chemical structure of bis(2-ethylhexyl) benzene-1,2-dicarboxylate was determined on the basis of 1D and 2D spectroscopic analysis (^{1}H NMR, ^{13}C NMR, DEPT, COSY, HMBC and HMQC). NMR spectroscopy was performed using a JEOL (Japan Electronic Optics Laboratory Co. Ltd., Tokyo, Japan) ECX 500MHz Fourier transform NMR spectrometer system (500 MHz) operating at 500MHz at Chemistry Department, Faculty of Science, Universiti Putra Malaysia. Isolated compound was dissolved in deuterated methanol (CD_3OD) and TMS was used as internal standard.

HPLC Analysis

HPLC analysis was performed with a system of HPLC equipped with Waters 600E system controller, Waters 996 sintered glass Büchner filter funnel, Waters online degasser, Waters 717 plus auto-sampler, column oven and the chemical compounds that pass through Phenomenex Luna C_{18} 100A column (250 mm x 4.6 mm, 5 µm particle size, USA) was detected by Water 996 photodiode array (PDA). Stationary phase was developed as 0.1% formic acid and acetonitrile in a ratio of 40:60 (v/v). The flow rate and injection volume were 1 mL/min and 10 µL, respectively. The detection wavelength was 200 nm.

All samples of HPLC analysis were filtered through a 0.45 µm Millipore membrane syringe filter (diameter: 17 mm, porosity: 0.45 µm, PVDF membrane, Whatman, USA). The previously isolated bis(2-ethylhexyl) benzene-1,2-dicarboxylate from the roots of *C. borivilianum* was used as the standard. The stock solution of standard was prepared in methanol at a concentration of 10 mg/mL. Samples used for calibration curve was prepared by a series of dilutions from the stock solution with methanol at a final volume of 1 mL in the concentration range of 1-7 ppm (0.001-0.007 mg/mL). A seven-points standard calibration curve of standard bis(2-ethylhexyl) benzene-1,2-dicarboxylate with linear relationship between the peak area at the Y-axis and the concentration of standard bis(2-ethylhexyl) benzene-1,2-dicarboxylate injected (ppm) at the X-axis was generated.

RESULT AND DISCUSSION

The roots of *C. borivilianum* were extracted with methanol and partitioned sequentially with water, hexane and chloroform. The extracts of aqueous, hexane and chloroform layers were encoded as CBA, CBH and CBC, respectively. CBA was further fractionated and isolated using column chromatography and finally yielded a pure compound. The structural elucidation was carried out by detailed interpretation of 1D and 2D NMR spectroscopic data. The structure of the isolated compound was elucidated as bis(2-ethylhexyl) benzene-1,2-dicarboxylate (Fig.1). The spectroscopic data was compared with those reported in the literature review for this compound and was found to be in agreement with those literatures [19-21]. The spectroscopic data (1H NMR, 13C NMR, COSY, DEPT, HMQC, HMBC) are shown in Table 1.

The isolated bis(2-ethylhexyl) benzene-1,2-dicarboxylate was used as a standard marker. The separation of the crude methanol extract was completed within 20 min and bis(2-ethylhexyl) benzene-1,2-

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dicarboxylate in the sample had a retention time of 7.166 min. The HPLC chromatogram of the crude extract was recorded at 200 nm (Fig. 2) whereas the purified bis(2-ethylhexyl) benzene-1,2-dicarboxylate extract was recorded at the same wavelength and the retention time was 7.171 (Fig. 3). Fig. 4 presents the calibration curve of standard bis(2-ethylhexyl) benzene-1,2-dicarboxylatein the concentration of 1 to 7 ppm (0.001-0.007 mg/mL). The regression equation of the calibration curve was linear and developed as *Y = 309071 X – 110258* while the correlation coefficient (r^2) was found to be 0.9914.

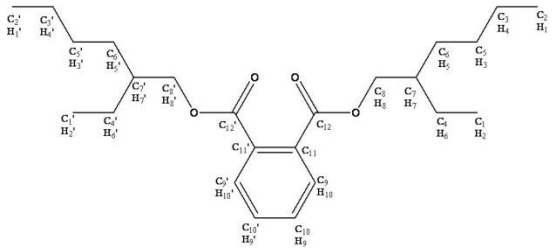

Figure 1: Structure of bis(2-ethylhexyl) benzene-1,2-dicarboxylate

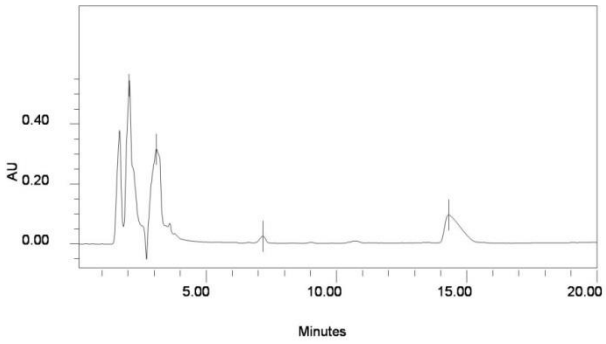

Figure 2: HPLC chromatogram of methanolic extract of *C. borivilianum*

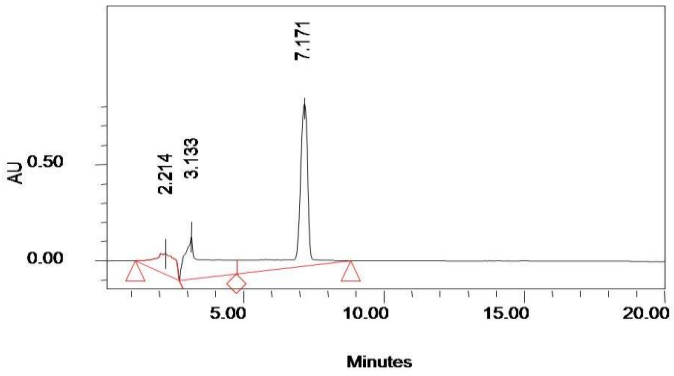

Figure 3: HPLC chromatogram of standard bis(2-ethylhexyl) benzene-1,2-dicarboxylate

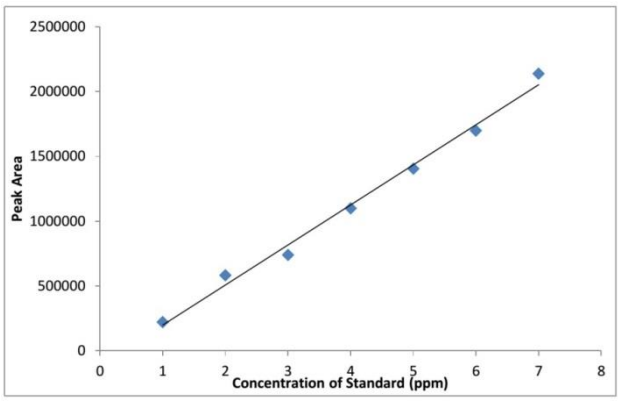

Figure 4: Calibration curve of bis(2-ethylhexyl) benzene-1,2-dicarboxylate

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Table 1: 1D and 2D Spectral Data Corellations for bis(2-ethylhexyl) benzene-1,2-dicarboxylate Recorded at 126 and 500 MHz.

No.	δ_H (mult, *J* in Hz)	δ_C, DEPT		HMQC	^1H-^1H COSY	HMBC
H_1/H_1'	0.81 (t)	C_2/ C_2'	14.6 CH_3	C_2/ C_2'-H_1/H_1'	H_1/H_1'-H_4/H_4'	$H_1/H_1' \rightarrow C_3/ C_3', C_5/ C_5'$
H_2/H_2'	0.84 (t)	C_1/ C_1'	11.5 CH_3	C_1/ C_1'-H_2/H_2'	H_2/H_2'-H_6/H_6'	$H_2/H_2' \rightarrow C_4/ C_4', C_7/ C_7'$
H_3/H_3'	1.23 (m)	C_5/ C_5'	30.2 CH_2	C_5/ C_5'-H_3/H_3'		$H_3/H_3' \rightarrow C_3/ C_3'$
H_4/H_4'	1.24 (m)	C_3/ C_3'	24.1 CH_2	C_3/ C_3'-H_4/H_4'		
H_5/H_5'	1.27 (m)	C_6/ C_6'	31.7 CH_2	C_6/ C_6'-H_5/H_5'	H_5/H_5'-H_7/H_7'	$H_5/H_5' \rightarrow C_7/ C_7', C_4/ C_4'$
H_6/H_6'	1.33 (m)	C_4/ C_4'	25.0 CH_2	C_4/ C_4'-H_6/H_6'	H_6/H_6'-H_7/H_7'	$H_6/H_6' \rightarrow C_6/ C_6'$
H_7/H_7'	1.58 (m)	C_7/ C_7'	40.2 CH	C_7/ C_7'-H_7/H_7'	H_7/H_7'-H_8/H_8'	$H_7/H_7' \rightarrow C_4/ C_4', C_1/ C_1'$
H_8/H_8'	4.11 (m)	C_8/ C_8'	69.2 CH_2	C_8/ C_8'-H_8/H_8'		$H_8/H_8' \rightarrow C_4/ C_4', C_{12}/ C_{12}'$
H_9/H_9'	7.51 (dd)	C_{10}/ C_{10}'	132.5 CH	C_{10}/ C_{10}'-H_9/H_9'		$H_9/H_9' \rightarrow C_{11}/ C_{11}', C_9/ C_9'$
H_{10}/H_{10}'	7.61 (dd)	C_9/ C_9'	130.0 C	C_9/ C_9'-H_{10}/H_{10}'		$H_{10}/H_{10}' \rightarrow C_{12}/ C_{12}', C_{11}/ C_{11}'$
		C_{11}/ C_{11}'	133.7 C=O			
		C_{12}/ C_{12}'	169.4 C=O			

CONCLUSIONS

A simple solid-solvent extraction, partition and isolation using the column chromatography was reported to be efficient on a laboratory scale for the isolation of bis(2-ethylhexyl) benzene-1,2-dicarboxylate. An isocratic HPLC method was done for identification and quantitative analysis of bis(2-ethylhexyl) benzene-1,2-dicarboxylate presented in the roots of *C. borivilianum*. It could be a good attempt to pave a way for isolation and quantification of more isolated active compounds other than saponins from this herbaceous plant for the future development of other potential therapeutic applications.

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