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Bis(2-Ethylhexyl) Terephthalate from *Olax Imbricata*, Natural Substance or Artifact?

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ABSTRACT

The medicinal plant *Olax imbricata* is well-known for its antioxidant, anti-inflammatory, antibacterial, and diabetes-treating properties. It is essential to investigate the chemical composition and biological activity of *Olax imbricata*. During our investigation of this plant, a terephthalate derivative, bis(2-ethylhexyl)terephthalate, was isolated from methanol extract using chromatographic techniques and elucidated using nuclear magnetic resonance spectroscopy. Terephthalate's presence in *Olax imbricata* has raised concerns regarding whether or not it is a natural substance or an artifact. It is challenging to persuade others that bis(2-ethylhexyl)terephthalate isolated from *Olax imbricata* is the biosynthetic compound produced by this medicinal plant. Sequestration of bis(2-ethylhexyl)terephthalate from *Olax imbricata* may be the result of laboratory equipment, adsorbents, and solvents used during extraction or isolation. This finding cautions laboratory staff when using plastic utensils exposed to organic solvents. A sign identifying bis(2-ethylhexyl)terephthalate is silica gel thin layer-chromatography performance eluting with *n*-hexane : ethyl acetate (96:4). A dark spot appears at R_f 0.46 under ultra-violet light at 254 nm or as a steel blue spot when stained with a solution containing 1% vanillin and 10% H_2SO_4 in ethanol.

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1. Introduction

Species in the genus *Olax* (Olacaceae) have been used for centuries as traditional medicines all over the world. The Duong-dau tree is scientifically known as *Olax imbricata* and is a member of the Olacaceae family [1]. *Olax imbricata* is a well-known medicinal plant with antioxidant, anti-inflammatory, antibacterial, and diabetes-treating properties. According to the medicinal plant data set of the Pako-Van Kieu ethnic group related to anti-cancer effects, the Pako ethnic group experimentally treats breast tumors and stomach distress with *Olax imbricata* [2]. *Olax imbricata* is endemic to tropical regions with sandy soils and sunny, arid climates, such as the central region of Vietnam. From the *Olax imbricata* roots, 16 compounds were isolated. The chemical structures of these compounds were determined by the interpretation of spectroscopic data (MS, 1D and 2D NMR, X-rays and ECD). The chemical structures of these compound were classified into some groups: phenolic compounds [3], [4], triterpenoid glycoside compounds [5], the tropolone derivatives, the 1,2,3,4-tetrahydronaphthalene derivatives [6]. and acetylenic fatty compounds [7]. Seven compounds from *Olax imbricata* were evaluated the inhibitory activity against α-glucosidase and analysed the structure-activity relationship. A triterpenoid glycoside, oleanolic acid 28-*O*-β-D-glucopyranoside, exhibited greater activity (IC_{50} = 34.75 μg/mL) than acarbose, the positive control (IC_{50} = 187.50 μg/mL). Due to the formation of a stable ligand-α-glucosidase complex via hydrogen bonds and hydrophobic interactions, this compound strongly inhibits α-glucosidase, according to the docking results [8]. In *in vitro* experiments, the *n*-hexane extract from the roots of *Olax imbricata* was more resistant to α-glucosidase than acarbose. The *n*-hexane extract from the roots of *Olax imbricata* has been shown to be highly biologically active in preventing and supporting the treatment of diseases related to overweight and obesity, aiding in the

regulation of blood glucose and blood glucose levels, and thereby lowering the risk of type 2 diabetes [9].

It is essential to investigate *Olax imbricata*'s chemical composition and biological activities. Consequently, we continue our research on this plant. On our journey, we discovered a terephthalate derivative, bis(2-ethylhexyl)terephthalate or di(2-ethylhexyl)terephthalate (DEHT) (**1**) (Figure 1), which raised suspicions. This paper will elucidate whether the terephthalate found in *Olax imbricata* is a natural substance or an artifact.

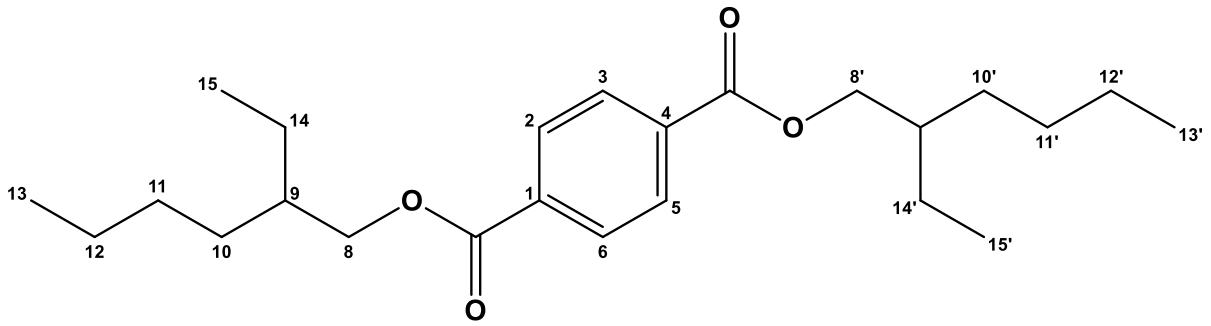

Figure 1. *Bis(2-ethylhexyl)terephthalate (**1**) isolated from Olax imbricata*

2. Materials and Methods

2.1. Materials

The roots of *Olax imbricata* were gathered in August 2020 in the province of Phu Yen. Mr. Hoang Xuan Lam from the Middle Vietnam Research and Manufacturing Organic Medicinal Herb Centre in Phu Yen province authenticated the scientific name of the plant. A voucher specimen (UTE-A002) was deposited in the herbarium of the Ho Chi Minh City University of Technology and Education, Department of Chemical Technology.

2.2. Chemicals

Diaion HP-20 (Mitsubishi), Silica gel 60 (0.040–0.063 mm) (Himedia, India), and Sephadex LH-20 (GE Healthcare, USA) are adsorbents for column chromatography (CC). For CC monitoring, precoated thin layer-chromatography (TLC) Silica gel 60 F254 (Merck, Germany) has been utilized. The spots on TLC plates are visualized with a 1% vanillin and 10% H_2SO_4 solution in ethanol. Solvents for extraction and isolation include *n*-hexane, dichloromethane, chloroform, acetone, methanol, and ethyl acetate (Chemsol, Vietnam). For preparative HPLC, *n*-hexane and ethyl acetate (Merck, Germany) were used.

2.3. Apparatus

Purification was carried out using silica gel column (150 mm × 10 mm, 10 µm) equipped with preparative HPLC Pure C-835 Prep Advance, Buchi (Switzerland) with PDA (200 – 800 nm) as a detector. NMR spectra were measured on a Bruker Avance NEO spectrometer, at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C-NMR, using residual solvent signal as internal reference.

2.4. Extraction and isolation

The dried powdered roots of *Olax imbricata* (50 kg) were exhaustedly extracted with ethanol by maceration, and the ethanolic filtrate was concentrated *in vacuo* to yield a residue of 1,500 g. This crude residue was soaked in *n*-hexane and stirred thoroughly. The solvent has been removed from the *n*-hexane-soluble portion under low pressure to obtain *n*-hexane extract (H, 600 g). The same procedure was applied for the *n*-hexane-insoluble portion, using methanol instead of *n*-hexane to afford methanol extract (M, 750 g).

The methanol extract (M, 750 g) was applied on a diaion chromatographic column, eluting with ethanol : water (20:80, 40:60, 60:40, 80:20, 100:0), then acetone to give 6 fractions (M1-6). The fraction M5 (45.47 g) was separated on a silica gel column eluting with *n*-hexane : dichloromethane in a ratio of 8:2 to 5:5 to obtain 6 subfractions (M5.1–6). Subfraction M5.1 (4.33 g) was subjected to a silica gel column with the eluent *n*-hexane : ethyl acetate 100:0 to 95:5 to give 6 fractions (M5.1.1–6). Subfraction

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M5.1.3 (0.125 g) was separated on a sephadex LH-20 column with the eluent chloroform : methanol 1:4 to collect 3 fractions (M5.1.3.1–3). Subfraction M5.1.3.2 (97.7 mg) was separated on a silica gel column by a preparative HPLC system eluting with *n*-hexane : ethyl acetate 97:3, pressure as 350 psi, flow speed of the measured liquid as 3 mL/minute, and a PDA detector with four wavelengths (210 nm, 254 nm, 280 nm, and 340 nm) to obtain **1** (2 mg).

Bis(2-ethylhexyl)terephthalate (**1**): transparent oil, TLC performance with eluent *n*-hexane : ethyl acetate 96:4 appears as a dark spot at R_f 0.46 under UV light at 254 nm or a steel blue spot visualized by staining in a 1% vanillin and 10% H_2SO_4 solution in ethanol, then heating (Figure 2). ^{1}H-NMR ($CDCl_3$, 600 MHz) δ_H (ppm, Hz) 8.09 (4H, s, H-2, H-3, H-5, H-6), 4.27 (4H, m, H-8/8'), 1.32-1.48 (16H, m), 0.95 (6H, t, 7.2, H-15/15'), 0.91 (6H, t, 7.2, H-13/13'); ^{13}C-NMR ($CDCl_3$, 150 MHz), δ_C (ppm) 166.0 (C-7/7'), δ_C 134.3 ppm (C-1, C-4), δ_C 129.5 ppm (C-2, C-3, C-5, C-6), δ_C 67.8 ppm (C-8/8'), δ_C 14.0 ppm (C-13/13'), δ_C 11.1 ppm (C-15/15'), and δ_C 23.0 – 39.0 (aliphatic carbons).

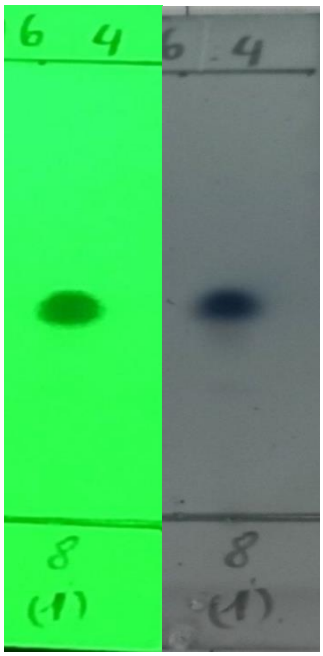

Figure 2. *TLC performance of compound 1; Eluent as n-hexane: ethyl acetate 96:4; Visualization under UV light at 254 nm (left side) and stainationin a 1% vanillin and 10% H_2SO_4 solution in ethanol (right side)*

3. Results and Discussion

3.1. Structural elucidation of compound 1

Compound **1** was isolated as a colorless oil. The ^{1}H-NMR spectrum revealed proton signals in downfield with two protons attached in a benzene ring at δ_H 8.09 (4H, s, H-2, H-3, H-5, H-6), oxymethylene protons at δ_H 4.27 (4H, m, H-8/8'), aliphatic protons at δH 1.32-1.48, and terminal methyls at δ_H 0.95 (6H, t, 7.2, H-15/15'), 0.91 (6H, t, 7.2, H-13/13').

The ^{13}C spectra exhibited a carboxyl signal at δ_C 166.0 ppm (C-7/7'), aromatic carbons at δ_C 134.3 ppm (C-1, C-4), δ_C 129.5 ppm (C-2, C-3, C-5, C-6), oxygenated carbons at δ_C 67.8 ppm (C-8/8'), ten methylene carbons at δ_C 23.0–39.0, and terminal methyls at δ_C 14.0 ppm (C-13/13') and δ_C 11.1 ppm (C-15/5').

In the COSY spectrum, the correlations are displayed in Figure 3, supporting continuous carbon chains. The attachment of the fatty chain was determined at C-9 by the HMBC spectrum. (Figure 3 and 4)

The HMBC correlations (Figure 4) from signal δ_H 8.09 (4H, s, H-2, H-3, H-5, H-6) with carbons at δ_C 166.0 (C-7/7'), δ_C 134.3 (C-1, C-4), δ_C 129.5 (C-2, C-3, C-5, C-6) allow the determination of the carboxyl carbon attached to the benzene ring. The δ_H 4.27 (4H, m, H-8/8') signal correlated with δ_C

166.0 (C-7/7'), indicating that **1** is an ester. The HMBC correlation between H-10/10', H-11/11', and H-12/12' with surrounding carbons allows to determine the straight chain from C-10/10' to C-13/13'.

Based on this information and the comparison of the published data [10], the compound **1** was determined as bis(2-ethylhexyl)benzene-1,4-dicarboxylate or bis(2-ethylhexyl)terephthalate (DEHT).

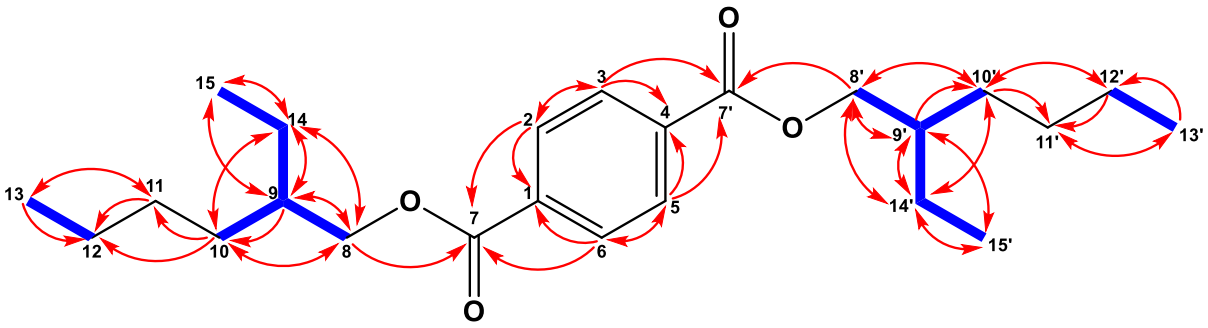

Figure 3. *Some key HMBC and COSY correlations in compound 1*

3.2. Origin of terphthalate from Olax imbricata

Phthalate esters are colorless, oily liquid chemicals used as plasticizers to enhance materials' flexibility, adhesion, and solubility. They have a high boiling point, poor solubility in water, and satisfactory solubility in the majority of organic solvents. These compounds are primarily utilized in pigments, varnishes, cosmetics, and personal care products, including shampoo, body deodorant, pomade, and gels [11]. Depending on their structures, the toxicity of phthalates can vary significantly. Di-(2-ethylhexyl) phthalate (DEHP), the most popular phthalate ester, has been investigated as an endocrine disruptor and a potential carcinogen. The other ones have been identified causes of attention-deficit hyperactivity disorder, obesity, type II diabetes, neurodevelopmental problems, behavioral problems, autism spectrum disorders, altered reproductive development, and male fertility problems. Less toxic compounds have been studied to replace phthalates, which are terephthalates, with a typical representative being di-(2-ethylhexyl) terephthalate [10], synonymized as bis(2-ethylhexyl)terephthalate. Phthalates are released from industrial products, and their photodegradation under natural conditions is extremely sluggish, depending on the length of aliphatic chains. Phthalate substances could migrate into the environment and enter the human body via water, air, food, and medical apparatus. Due to the hydrological cycle, rainfall transfers phthalate esters from the atmosphere to water on land, resulting in their widespread distribution in rivers, lakes, and sediments. Due to its limited solubility in water, DEHP concentrates from water into soil and sediments. Numerous reports indicate that diverse phthalates are present in agricultural soils and are absorbed by crops and vegetables [12]. Phthalates have been extracted from numerous plant species, including algae, bacteria, and fungi [10]. Regarding the prevalence of phthalates in nature, there are two perspectives. The first viewpoint is that they are derived from natural compounds [12], [13]. The second opinion is that the investigated plant material contains phthalates from industry [11]. Thies Thiemann reviewed the published materials to figure out whether phthalates are natural substances or whether they are all pollutants of artificial origin, as following [10]. Phthalates may arise from infected laboratory equipment, but the majority of phthalates found in natural sources come from fertilizers, other agricultural chemicals, farming water, or atmospheric import. A few naturally occurring phthalates are not produced industrially. However, it is necessary to consider in more detail whether esterification or selective transesterification by enzymes is responsible for this. On another hand, tt has been demonstrated that the di-*n*-butyl phthalate (DnBP) biosynthetic pathway is the shikimic acid pathway [14]. Nonetheless, it must be derived from phthalic acid. The natural biosynthetic pathway of phthalic acid is still poorly understood. Nature makes it challenging to synthesize an aromatic ring with two electron-withdrawing groups. Natural compounds containing aromatic rings frequently contain electron-donating groups such as hydroxyl, methoxyl, amino, and so on. This is evident in the natural products such as flavonoids, phenylethanoids, phenylpropanoids, lignans,...

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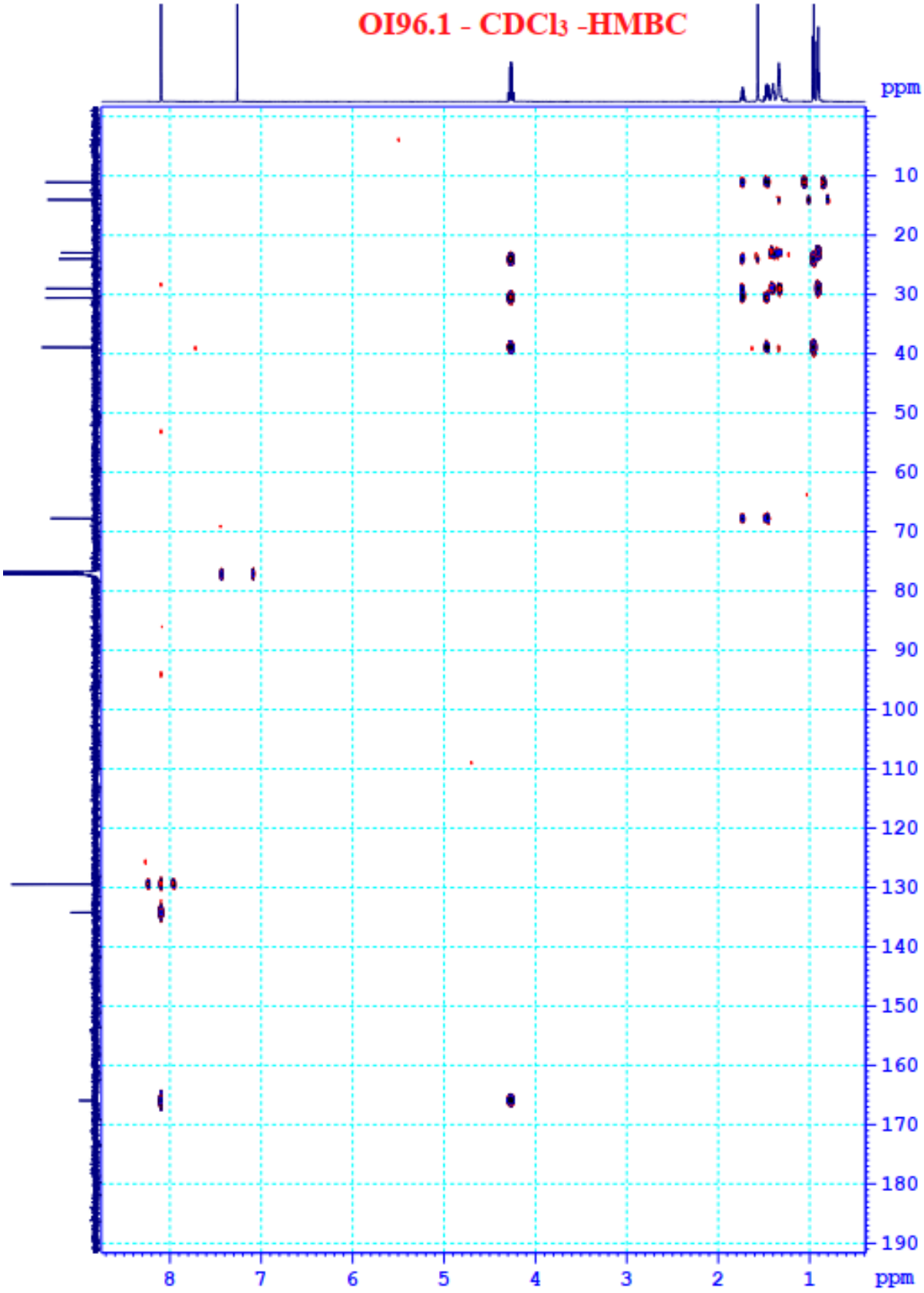

Figure 4. *HMBC spectrum of compound 1*

In the case of DEHT isolated from *Olax imbricata*, it is challenging to persuade others that this is the substance biosynthesized by this medicinal plant. Aromatic compounds isolated from this plant possess aromatic rings containing hydroxyl, methoxyl, glycopyranosyl, or only one carboxyl group. *Olax imbricata*-isolated fatty compounds contain double and triple bonds. In contrast, DEHT isolated from *Olax imbricata* lacked these characteristics.

The investigation by Nguyen measured the concentration of DEHP released from regular laboratory materials. The concentration of DEHP released by three prevalent solvents, including methanol, *n*-hexane, and chloroform, is 0.06-0.07 mg/L. Normal phase silica gel resin and RP18 silica gel resin discharge DEHP at concentrations of 0.4 and 0.3 mg/kg, respectively. Different kinds of tubing have various DEHP amounts, with Teflon tubing releasing 0.04 to 0.16 mg/g, silicone tubing releasing 0.14 mg/g, and PVC tubing releasing 100 mg/g [15]. The separation of DEHT from *Olax imbricata* involves extraction, fractionation, and column chromatography. The extraction process employs a significant amount of methanol solvent, which is contained in PE plastic cans. Different resins, such as normal phase silica gel, diaion resin, and LH20, are utilized for fractional separation and column chromatography. In addition, widespread solvents such as *n*-hexane, chloroform, ethyl acetate, and methanol are used in the laboratory. Therefore, DEHT sequestration from *Olax imbricata* may be due to release from laboratory equipment and chemicals used in the extraction and isolation stages.

A sign to identify DEHT is deploying a silica gel TLC performance eluting with *n*-hexane : ethyl acetate 96:4. A dark spot at R_f 0.46 appears under UV light at 254 nm or a steel blue spot visualized by staining in a 1% vanillin and 10% H_2SO_4 solution in ethanol. This discovery cautions laboratory workers to be careful when using plastic tools that touch organic solvents.

4. Conclusions

Through chromatographic techniques, bis(2-ethylhexyl)terephthalate was isolated from the *Olax imbricata* root methanol extract. Utilizing 1D and 2D NMR spectroscopic techniques, its chemical structure was revealed. It is challenging to persuade others that the DEHT isolated from *Olax imbricata* is the biosynthetic compound produced by this medicinal plant. Sequestration of DEHT from *Olax imbricata* may be the consequence of laboratory apparatus and compounds used for isolation or extraction. This discovery cautions laboratory personnel against using plastic instruments in contact with organic solvents. A silica gel TLC performance eluting with a 96:4 mixture of *n*-hexane and ethyl acetate identifies DEHT. A dark spot appears at Rf 0.46 under UV light at 254 nm or as a steel blue spot when stained in a solution containing 1% vanillin and 10% H_2SO_4 in ethanol, following by heating.

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