Isolation of Bis(2-Ethylhexyl) Terephthalate and Bis(2-Ethylhexyl) Phthalate from Capparis spinosa L. Leaves

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Abstract: Capparis spinosa L., commonly known as the caper bush, is a spiny shrub known for its edible flower buds and its use as a medicinal plant in traditional medicine. While performing bio-guided isolation of active compounds from Capparis spinosa L. leaves and buds, large amounts of bis(2-ethylhexyl) terephthalate (DEHT, a.k.a. dioctyl terephthalate or DOTP) and bis(2-ethylhexyl) phthalate (DEHP) were isolated from a fraction from the leaf extract that showed antifungal activity against Cryptococcus neoformans. The structures of these two compounds were confirmed by NMR and mass spectroscopic data, which matched with those from the standards that were purchased from Sigma-Aldrich. DEHT and DEHP are phthalic and terephthalic acid esters, the main plasticizers that are used to confer elasticity and flexibility to various fiber and plastic products. This is the first time DEHT and DEHP have been isolated from the leaves of a plant that is as commonly used as Capparis spinosa L. This study adds to the increase in the detection of plasticizers in our food and medicine sources and to the alarming concern about the potential effects of these compounds on human health.

Keywords: Capparis spinosa L., bis(2-ethylhexyl) terephthalate, dioctyl terephthalate, bis(2-ethylhexyl) phthalate, plasticizers in medicinal plants

INTRODUCTION

Capparis spinosa L., commonly known as the caper bush, is an aromatic and medicinal plant that grows in most of the Mediterranean basin and some parts of Western Asia.1–5 This plant grows naturally in mountainous regions, but has been cultivated in flat areas as an important source of food and medicine. Capparis spinosa L. is best known for its edible flower buds (called capers) and fruits, which are usually salted, pickled, and used as ingredients, seasonings, condiments, or garnishes.1 Capparis spinosa L. is also one of the few medicinal plants that are widely used in traditional medicine in many different civilizations because it possesses many therapeutic properties.1,6,15,7–14

As part of our research on bio-guided isolation of active compounds from medicinal plants, we collected C. spinosa L. leaves and buds from two different regions of Morocco, Fez-Meknès and Marrakesh-Safi, dried, ground, and extracted with ethanol (95%), performed fractionation via silica column chromatography, and tested each fraction in various antibacterial and antifungal assays. One fraction from the leaf extract showed activity against Cryptococcus neoformans, a human
pathogen fungus that causes an infectious disease known as cryptococcosis by damaging host cells and tissues at the molecular level. Further fractionation via silica column chromatography and semi-preparative HPLC on this fraction resulted in large amounts of bis(2-ethylhexyl) terephthalate (DEHT, a.k.a. dioctyl terephthalate or DOTP) and bis(2-ethylhexyl) phthalate (DEHP) isolated. Notably, the corresponding fraction from the bud extract also showed activity against Cryptococcus neoformans, but it did not contain DEHT or DEHP.

DEHT and DEHP are widely used in the petrochemical and polymer industries and belong to a chemical class of phthalic and terephthalic acid esters, the main plasticizers that are used to confer elasticity and flexibility to various fiber and plastic products. DEHT has been found and isolated from several plant species, including Grewia lasiocarpa E. Mey. ex Harv, Uncaria rhynchophylla, and Alnus nitida, and the marine fungus Penicillium griseofulvum. Meanwhile, DEHP has been found and isolated from several other plant species, including Ligusticum porteri, Ficus carica, Alchornea cordifolia, Phyllanthus muellerianus, and Aloe vera, and several microorganisms, such as Streptomyces sp. and Nocardia levis. Herein, we report for the first time the isolation of DEHT and DEHP from the leaves of a plant that is as commonly used as Capparis spinosa L. Our study adds to the increase detection of plasticizers in our food and medicine sources and to the alarming concern about the potential effects of these compounds on human health. Other heavily used phthalic and terephthalic acid esters have also been found in many different plants, including tris(2-ethylhexyl) trimellitate isolated from Moringa oleifera, diethyl terephthalate isolated from Mangifera indica, and dimethyl terephthalate from Goniothalamus tapis Miq.

MATERIALS AND METHODS

General Experimental Procedures

\[^{1}\text{H}\text{ and }^{13}\text{C} \text{ NMR spectra were recorded on a Bruker AscendTM (400MHz) using methanol-}d_4\text{ as the solvent. Chemical shifts (\(\delta\)) were recorded in parts per million (ppm, \(\delta\) units) and referenced to methanol-}d_4\text{ (3.35 and 4.78 ppm for }^{1}\text{H NMR, and 49.3 ppm for }^{13}\text{C NMR). Coupling constants (\(J\)) are given in Hz. The following abbreviations were used to designate the multiplicities: }s=\text{ singlet, }t=\text{ triplet, hept}=\text{ heptet, }d=\text{ doublet of doublets, }ddt=\text{ doublet of doublet of triplets, }m=\text{ multiplet. The solvent peaks were determined as internal standards. Exact mass was measured with a JEOL AccuToF 4G LCplus atmospheric pressure ionization time-of-flight mass spectrometer (Jeol, Tokyo, Japan) equipped with direct analysis in real time (DART) ion source (IonSense DART controller, Sangus, MA, USA.). The atmospheric pressure ionization source operated in positive ion mode with the following operating conditions: orifice 1 potential: –20 V; orifice 1 potential: –5 V; ring lens potential: –5 V; temperature of orifice 1: 120 °C; radio frequency (RF) ion guide potential: 1000 V; helium gas flow: 4.0 L/min; temperature of gas heater: 350 °C; and the source grid (350 V). DART operated in the range m/z 50-1000 with 1 mg/mL concentration samples. Polyethylene glycol (PEG 600) was used for the exact mass calibration, and the elemental composition could be determined based on the selected peaks using Mass Mountaineer_4. Sonication was carried out by using an ultrasonic bath sonicator (Branson 2510 60Hz/40KHz). Flash column chromatography (CC) was performed using silica gel, pore size: 60 Å 0.036-0.071 mm (215-400 mesh - VWR). Size exclusion chromatography was performed using Sephadex gel LH-20 (GE Healthcare). Analytical TLC was performed on silica gel plates (MilliporeSigma TLC Silica Gel 60 F254: 25 glass plates, 20 cm × 20 cm). The detection of UV-active compounds was performed with 254 nm UV light (Spectroline CM-10A portable fluorescence analysis cabinet). Spots were visualized by staining with vanillin (Sigma) (15 g of vanillin, 250 mL of 95% EtOH, and 2.5 mL of conc. H_2SO_4) followed by heating.\]
Analytical grade solvents (Fisher Chemicals) were used for extraction and purification. Chromatographic analysis and separation by high-performance liquid chromatography (HPLC) were performed with an Agilent 1260 infinity instrument (Palo Alto, CA, U.S.A) equipped with a diode array detector (DAD) and an RP-C18 column (Zorbax SB-C18, 9.4 mm × 250 mm; 5 µm). A step gradient of elution with acetonitrile:water (v/v, 2:98 to 98:2 for 50 min, 3 mL/min flow rate) was used for the semi-preparative HPLC.

**Plant Materials**

Plant samples were collected in July 2020 from Fez-Meknè and Marrakesh-Safi, two main production regions of capers in Morocco. In Fez, the samples were collected from the Messassa commune 34°16'57.6"N 4°32'58.0"W and Lamtalsa El Bsabsa commune 34°21'37.7"N 4°41'23.1"W in the Taounate Province. In Safi, the samples were collected from the Esh-Shayf area 32°20'51.1"N 9°16'07.0"W and Saadla commune 32°19'32.5"N 9°06'00.2"W. From each region, caper leaves and buds were harvested and stored separately. The leaves were carefully picked without the thorns, and the buds were picked without the stems. Dirt, dust, and little bugs or worms were removed by hand without washing with water. The leaves and buds from each region were dried on a net elevated from the ground to avoid any kind of moisture in a dark room at room temperature. Measurement of the sample weights was conducted every three days until no further decrease in weights was observed. The decrease in weight is a sign of water loss. After the leave and bud samples from each region were dried, they were ground separately with a grinder machine (Moulinex) to fine powders and stored in separate, sealed glass containers, which were stored in the dark at room temperature until use.

**Extraction and Isolation**

Small samples of the leaf and bud powders from each region were extracted with 95% ethanol, and thin-layer chromatography (TLC) was performed on each sample. While the TLCs of the leaf and bud extracts look different from each other, the TLCs of the leaf extracts or the TLCs of the bud extracts from different areas looked similar. Therefore, the leaf powders from the Fez-Meknè and Marrakesh-Safi regions were combined, and the same thing was done for the bud powders. The *C. spinosa* L. leaf powders (140 g) and bud powders (100 g) were soaked separately in 600 mL of 95% ethanol, sonicated for 1 hour at a controlled temperature (25–40 °C), and stored overnight in the dark at room temperature. A glass Buchner filter funnel was used with vacuum to separate the filtrates from the solids. Small amounts of 95% ethanol were used to wash the solids and added to the filtrate. The solids were again soaked with 400 mL of 95 % ethanol, sonicated, and stored for the second day under the same conditions as the first day. The whole extraction process was repeated for the third and fourth days. The filtrates from four days were combined, concentrated using a rotatory evaporator at 40 °C, and lyophilized using a freeze dryer. Sticky solids (20.8 g of leaf extract and 24.0 g of bud extract) were obtained. 10 g of each extract was dry-loaded with 50 g of silica gel onto a column (152 mm × 55 mm (inside diameter)) of silica gel (250 g). Six different solvent systems (1.0 L each) were run through the column (50% CH$_2$Cl$_2$ in hexanes, 100% CH$_2$Cl$_2$, 5% methanol in CH$_2$Cl$_2$, 10% methanol in CH$_2$Cl$_2$, 15% methanol in CH$_2$Cl$_2$, and 20% methanol in CH$_2$Cl$_2$). 12 fractions (500 mL per fraction) were collected for the leaf extract (L1–12), and 12 fractions (500 mL per fraction) were collected for the bud extract (B1–12). All fractions were concentrated, lyophilized using a freeze dryer, and submitted to the National Center for Natural Products Research at the University of Mississippi School of Pharmacy to screen for biological activity. Two fractions from the leaf extract (L1 and L2) and two fractions from the bud extract (B1 and B11) showed activity against *C. neoformans*. Further fractionation via silica column chromatography [152 mm × 30 mm (inside diameter)] column of silica gel, eluted with a gradient of CH$_2$Cl$_2$ from...
40% to 60% in hexanes] was performed separately on fractions L1, L2, and B1 (nonpolar fractions) to isolate the main compounds that appeared in thin-layer chromatography (TLC) under UV and vanillin staining method. Fractionation via size exclusion chromatography [800 mm × 35 mm (inside diameter) column of Sephadex gel LH-20 (GE Healthcare), eluted with methanol] was performed on fraction B11 (a polar fraction) to isolate the main compounds that appeared in TLC under UV and vanillin staining method. For each of L1, L2, B1, and B11, subfractions that contained the same main compounds were combined and further purified via semi-preparative HPLC [RP-C18 column (Zorbax SB-C18, 9.4 mm × 250 mm, 5 μm) with acetonitrile:water elution (v/v, gradient from 2:98 to 98:2 in 50 min, 3 mL/min flow rate)]. Compounds 1 (20 mg) and 2 (3.2 mg) were isolated from L1. Compounds 1 and 2 were not observed to be present in L2, B1, or B11.

Characterization of Compounds 1 and 2

Compound 1

Colorless oily liquid (20 mg). Light blue spot on TLC plate under vanillin staining method. $^1$H NMR (400 MHz, methanol- $d_4$) δ 8.09 (s, 4H), 4.28 (dd, $J = 5.7, 1.2$ Hz, 4H), 1.73 (hept, $J = 6.1$ Hz, 2H), 1.53–1.45 (m, 4H), 1.42 (ddt, $J = 7.7, 6.0, 1.4$ Hz, 4H), 1.39–1.32 (m, 8H), 0.97 (t, $J = 7.5$ Hz, 6H), 0.94–0.89 (m, 6H). $^{13}$C NMR (101 MHz, methanol- $d_4$) δ 167.1, 135.6, 130.6, 68.7, 40.3, 31.7, 30.1, 25.1, 24.0, 14.4, 11.5. The $^1$H and $^{13}$C NMR spectra matched those of bis(2-ethyhexyl) terephthalate (purchased from Sigma-Aldrich). HRMS m/z calc for C_{24}H_{36}O_{4}: 391.2854 [M+H]$^+$, found 391.2857.

Compound 2

Colorless oily liquid (3.2 mg). Dark blue spot on TLC plate under vanillin staining method. $^1$H NMR (400 MHz, methanol- $d_4$) δ 7.76–7.71 (m, 2H), 7.66–7.61 (m, 2H), 4.28–4.18 (m, 4H), 1.69 (hept, $J = 6.0$ Hz, 2H), 1.51–1.42 (m, 4H), 1.42–1.31 (m, 12H), 1.00–0.90 (m, 12H). $^{13}$C NMR (101 MHz, methanol- $d_4$) δ 169.3, 133.6, 132.4, 129.9, 69.1, 40.2, 31.6, 30.1, 25.0, 24.0, 14.4, 11.4. The $^1$H and $^{13}$C NMR spectra matched those of bis(2-ethyhexyl) phthalate (purchased from Sigma-Aldrich). HRMS m/z calc for C_{24}H_{36}O_{4}: 391.2854 [M+H]$^+$, found 391.2841.

Anti-Cryptococcus neoformans Activity Assay

Cryptococcus neoformans ATCC 90113 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Susceptibility testing was performed using a modified version of the CLSI method. \(^{24}\) Samples were serially diluted in 20% DMSO/saline and transferred in duplicate to 384-well flat-bottomed microplates, maintaining final DMSO conc. 1% in the assay. Inocula were prepared in Sabouraud Dextrose for C. neoformans to afford recommended inocula as per CLSI. Amphotericin B was used as a drug control. The optical density (530nm) was taken using a Bio-Tek plate reader prior to and after incubation (at 35 °C for 72 h). The concentration of the fraction responsible for 50% growth inhibition (IC$_{50}$) was calculated using XLfit 4.2 software (IDBS, Alameda, CA) and fit model 201.

RESULTS

C. spinosa L. leaves and buds were collected from the regions of Fez-Meknès and Marrakesh-Safi in Morocco and dried in the open air in a dark room at room temperature for 2 weeks until no further decrease in weight was observed. The samples (140 g of leaf powders and 100 g of bud powders) were extracted with 95% ethanol [total 1.8 L, soaked and sonicated (1 hour/day) over 4 days]. The solvent was removed using a rotary evaporator and a freeze drier. Sticky solids of the crude extracts (20.8 g of leaf extract and 24.0 g of bud extract) were obtained. Fractionation via silica column chromatography (300 g of silica gel, 152 mm × 55 mm column) was performed on 10 g of the crude leaf extract and 10 g of the crude bud extract separately. Six different solvent systems (1.0 L each) were run through the column (50% CH$_2$Cl$_2$ in hexanes, 100% CH$_2$Cl$_2$, 5% methanol in
CH₂Cl₂, 10% methanol in CH₂Cl₂, 15% methanol in CH₂Cl₂, and 20% methanol in CH₂Cl₂). 12 fractions (500 mL per fraction) were collected for the leaf extract (L1–12), and 12 fractions (500 mL per fraction) were collected for the bud extract (B1–12). All fractions were concentrated, lyophilized using a freeze dryer, and screened for biological activity. Two fractions from the leaf extract (L1 and L2) and two fractions from the bud extract (B1 and B11) showed activity against *C. neoformans*. The full results of the bioassay screening will be reported in a later report.

Further fractionation via silica column chromatography (152 mm × 30 mm column, eluted with a gradient of CH₂Cl₂ from 40% to 60% in hexanes) for L1, L2, and B1, size exclusion chromatography (800 mm × 35 mm column of Sephadex gel LH-20, eluted with methanol) for B11, and semi-preparative HPLC (RP-C18 column (Zorbax SB-C18, 9.4 mm × 250 mm, 5 µm) with acetonitrile:water elution (v/v, gradient from 2:98 to 98:2 in 50 min, 3 mL/min flow rate)] were performed to isolate the main compounds that appeared in thin-layer chromatography (TLC) under UV and vanillin staining method. Compound 1 (colorless oily liquid, 20 mg, Figure 1) and compound 2 (colorless oily liquid, 3.2 mg, Figure 1) were isolated from fraction L1 (from the leaf extract). Both were UV-active and stained under vanillin staining method. The structures of compounds 1 and 2 were elucidated to be bis(2-ethylhexyl) terephthalate and bis(2-ethylhexyl) phthalate, respectively, based on ¹H and ¹³C NMR spectroscopy data (Supporting Information, Figures S1–4) and high-resolution mass spectrometry data (Supporting Information, Figure S5). Furthermore, bis(2-ethylhexyl) terephthalate and bis(2-ethylhexyl) phthalate were purchased from Sigma-Aldrich and their ¹H and ¹³C NMR spectroscopy and HRMS were measured, which matched those of compounds 1 and 2, respectively. The comparison of ¹H and ¹³C NMR data of compound 1 and bis(2-ethylhexyl) terephthalate is shown in Table 1, and the comparison of ¹H and ¹³C NMR data of compound 2 and bis(2-ethylhexyl) phthalate is shown in Table 2. Notably, compounds 1 and 2 were not observed to be present in the corresponding fraction B1 (from the bud extract).

Figure 1. Elucidated structures of compounds 1 and 2 that were isolated from *Capparis spinosa* L. leaves.

Table 1. Comparison of ¹H and ¹³C NMR data in methanol-d₄ of compound 1 and bis(2-ethylhexyl) terephthalate

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Superscript a: Bis(2-ethylhexyl) terephthalate was purchased from Sigma-Aldrich, catalog number 49234.

**Table 2.** Comparison of $^1$H and $^{13}$C NMR data in methanol-$d_4$ of compound 2 and bis(2-ethylhexyl) phthalate

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Superscript b: Bis(2-ethylhexyl) phthalate was purchased from Sigma-Aldrich, catalog number 36735.
DISCUSSION

Plasticizers have been found in many different plants and microorganisms, which has raised an alarm about the contamination of plasticizers in the environment, including water sources, soil, and atmosphere, and the accumulation of plasticizers in plants and microorganisms. Our study reports the first time that plasticizers have been found in a plant that is as commonly used as the caper bush for food seasonings and medicines. While it is a relief that DEHT and DEHP were found in the leaves of *C. spinosa* L. and not in the buds (which are pickled and consumed), it is still very concerning that such large amounts of plasticizers are isolated from the plant (20.8 g of crude material was extracted from 140 g of dried and ground leaves; 20 mg of DEHT and 3.2 mg of DEHP were isolated from 10 g of the crude material). Because these amounts are obtained after extensive purification via column chromatography and semi-preparative HPLC, the real amounts of DEHT and DEHP present in the plant can be estimated to be twice or three times as much. We have double-checked our sample handling and experimental protocols to make sure that DEHT and DEHP did not come from human errors or laboratory contamination. All samples and solvents were stored in glass containers and never in contact with plastic materials. Also, the fact that DEHT and DEHP were not present in the corresponding fraction B1 from the buds eliminated the possibility of laboratory contamination. Furthermore, the isolated DEHT and DEHP from L1 were subjected to the anti-*C. neoformans* activity assay and did not show activity against the fungus, confirming that the anti-*C. neoformans* activities of L1 and B1 must have come from other compounds in these two fractions. DEHP, as well as diisobutyl phthalate and bis(2-ethylhexyl) isophthalate (3 and 4, Figure 2, respectively), were previously detected in *C. spinosa* L. seed oil via GC/MS analysis.\(^\text{35}\) DEHP was also detected in *Buchholzia coriacea* Engler seed oil, another plant in the *Capparaceae* family, via GC/MS analysis.\(^\text{36}\)

There are a couple of other phthalate compounds that have been found in the *Capparaceae* family (5–6, Figure 2), but no terephthalate compounds have been reported until our study. 5 mg of bis(7-methyloctyl) phthalate (5) was isolated from 20 g of fruit extract of *Capparis ovata*.\(^\text{37}\) DIBUTYL phthalate (6) was detected in a water-distilled essential oil from the aerial parts of *Capparis spinosa* var. *aegyptia* (Lam.) Boiss. via GC/MS analysis.\(^\text{38}\)

![Figure 2. Structures of phthalate compounds previously found in the Capparaceae family.](image)

Although there is still some discussion about whether DEHP is a natural product or a pollutant,\(^\text{39}\) there has been little evidence to support that it is a natural product. The only worth-noting study is the detection and evaluation of DEHP and di-*n*-butyl phthalate (DBP) contents in red alga *Bangia atropurpurea* cultured in an artificial seawater medium and a natural seawater medium, reported by Chen in 2004.\(^\text{40}\) No similar experiments have been reported since then. Meanwhile, extensive industrial use of DEHP and numerous reports on its persistent presence in the environment have strongly supported the idea that DEHP is a pollutant.\(^\text{41–43}\) It has been found in wastewaters (0.716–122 µg/L), surface waters (0.013–18.5 µg/L), landfill leachate (88–460 µg/L), sludge (12–1250 mg/kg), and soil (2–10 mg/kg).\(^\text{41}\) DEHP and its by-products are known to be toxic.\(^\text{41}\)
Cucumis sativus. A study on the uptake of DEHP by 10 vegetable plants cultivated in a field of plastic mulch film showed the accumulation of this plasticizer in all plants. Wax gourd (Benincasa hispida), cucumber (Cucumis sativus), and pumpkin (Cucurbita moschata) showed high levels of uptake and accumulation of DEHP. In another study investigating the uptake, accumulation, and enhanced dissipation of DEHP spiked in soil (with a concentration of 117.4 ± 5.2 mg/kg) by 11 plants, the results showed a significantly enhanced dissipation of DEHP in soil by the plants. The removal rates of DEHP ranged from 66.8% (for the control) to 87.5% (for the maize cultivar of Huanong-1) after 40 days of treatment. In a study from fast-growing regions in China, high levels of phthalate esters, including DEHP, were detected in soils and vegetables and were found to pose a similar risk to that of organochlorine pesticides.

DEHP is well-known for its reprotoxicity and endocrine-disrupting activity and must be replaced by alternative compounds. Chronic exposure to phthalates, including DEHP, adversely influences the endocrine system and has negative long-term impacts on pregnancy, child growth and development, and reproductive systems in both young children and adolescents. DEHT could be an alternative to DEHP. In an in vitro and in silico study on the effects of DEHP and DEHT metabolites on thyroid receptors, 5-hydroxy-mono-(ethylhexyl)phthalate (5-OH-MEHP), an oxidized metabolite of DEHP, showed agonistic activity on thyroid receptors, while oxidized metabolites of DEHT had no effects. More studies on the effects of DEHT on human health are needed. DEHT has been found and isolated from several plant species, including Grewia lasiocarpa E. Mey. ex Harv, Uncaria rhynchophylla, and Alnus nitida, and the marine fungus Penicillium griseofulvum. Our report on the isolation of large amounts of DEHT and DEHP from the leaves of a plant that is as commonly used as C. spinosa L. adds to the increase in the detection of plasticizers in our food and medicine sources and to the alarming concern about the potential effects of these compounds on human health.

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**Conflict of Interest**

The authors declare no competing financial interest.

**Acknowledgment**

Work was supported by Fulbright Morocco and Moroccan American Commission for Educational and Cultural Exchange (MACECE) (Fulbright Joint-Supervision Program to A. K.) and National Institute of General Medical Sciences (P30GM122733 pilot project award to H. V. L.) and funds from Mohammed V University in Rabat and the Department of BioMolecular Sciences at the University of Mississippi, School of Pharmacy. The antimicrobial screening was performed at NCNPR, School of Pharmacy, University of Mississippi, and was supported by USDA-ARS grant # 58-6060- 6015. The content is solely the responsibility of the authors and does not necessarily represent the official views of these funders.

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Capparis spinosa L., a.k.a. the caper bush

Bio-guided isolation

Capparis spinosa L., a.k.a. the caper bush