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# Effects of di-(2-ethylhexyl) phthalate (DEHP) released from laboratory equipments

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**Abstract**−Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in polyvinyl chloride (PVC) manufacturing and is an endocrine disrupter. DEHP was released from laboratory tubing and resins during solvent extraction of natural plants to isolate skin depigmenting compounds. Contamination of DEHP significantly interfered with the purification of depigmenting compounds, since DEHP showed high depigmenting activity ($IC_{50}$=24 μM) and did not show cell toxicity up to 20 μM. Release of DEHP depended on the composition of tubing materials and solvents used in the extraction process. This result provides practical information for the proper selection of laboratory materials and solvents especially in the extraction and isolation of skin depigmenting compounds for cosmetic ingredients.

Key words: Di-(2-ethylhexyl) Phthalate, Melanogenesis, Solvent Extraction

## INTRODUCTION

Di (2-ethylhexyl) phthalate (DEHP) is produced by reacting 2-ethylhexanol with phthalic anhydride (Fig. 1) [1]. It is used as a plasticizer of polyvinyl chloride (PVC) in the manufacture of a wide variety of consumer products [2]. DEHP does not bind with the plastic; it leaches with time from vinyl products, thus becoming a ubiquitous environmental contaminant and poses a potential risk for human exposure through several pathways [3,4]. DEHP produces a spectrum of toxic effects in developing and adult animals and in multiple organ systems including the liver, reproductive tract (testes, ovaries, secondary sex organs), the kidneys, lungs, and heart [5-9]. DEHP is also a common laboratory contaminant from analytical equipment due to the use of flexible vinyl in laboratory equipment and tubing. Plastics, glassware, aluminum foil, cork, rubber, glass wool, Teflon sheets, and solvents have all been found to be contaminated with DEHP [10,11]. In experiments that address the isolation of active compounds from natural products, many kinds of solvents, resin, and tubing are used. However, DEHP contamination during this process has not yet been studied.

Melanin is a ubiquitous class of biological pigments that are responsible for the color of human skin, eyes and hair [12]. It is produced by specialized pigment cells known as melanocytes and are deposited within discrete membrane-bound organelles called melanosomes. Melanin plays an important role in preventing ultraviolet (UV) light-induced skin damage [13,14]. However, increased levels of epidermal melanin synthesis can darken the skin. Various dermatologic disorders result in the accumulation of excessive levels of epidermal pigmentation. These include melasma, lentigines, and post-inflammatory hyperpigmentation. Because of the visible nature of dermatologic diseases, they have a considerable psychological effect on affected patients [15]. Therefore, a number of whitening compounds have been screened for their effectiveness in reducing melanogenesis [16-18].

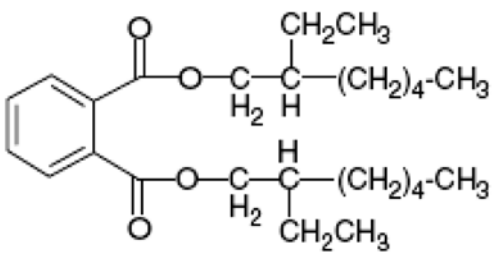

**Fig. 1. Structure of DEHP.**

In this study, the content of DEHP in organic solvent, resin and flexible vinyl tubing in laboratory equipment was measured. The effects of DEHP on melanogenesis were also investigated. The results showed that the amount of DEHP significantly interfered with the interpretation of experimental results. From these results, we suggest that researchers should consider the DEHP contamination from laboratory materials especially when skin whitening agents are isolated.

## MATERIAL AND METHODS

### 1. Materials and Reagents

Five commercially available tubing materials, frequently used in isolation procedures, were examined in this study. Three were polytetrafluoroethylene (TEFE) based, one that was silicone based and flexible polyvinyl chloride (PVC) based tubing. The TEFE tubing materials were referred to as materials 1 through 3 and purchased from Shimadzu Co. (Japan), Waters Co. (USA) and Omnifit Co (USA), respectively. The silicone tubing materials were designated as material 4 and obtained from O.M.G Co. (South Korea), while PVC tubing was designated as material 5 and purchased from Korea Vaccine Co. (South Korea). DEHP (99% purity) was purchased from Sigma Chemical Co. (St. Louis, U.S.A). Methanol, Hexane, Chloroform (99.5% purity) was purchased from Samchun Chemical Co. (South Korea). Methanol of high purity (99.9%) was purchased from J. T. Barker (USA). Silica gel resin and $C_{18}$ resin were purchased from Water Co. (MA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin-EDTA, Phos-

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phate buffered saline (PBS), penicillin/streptomycin were purchased from Invitrogen Corp. (CA, U.S.A). Mushroom tyrosinase, L-DOPA (3,4-dihydroxy-L-phenylalanine), arbutin, DMSO (dimethyl sulfoxide) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, U.S.A).

B16F10 murine melanoma cells were acquired from ATCC (American Type Culture Collection). B16F10 melanoma cells were cultured in DMEM that was supplemented with 10% (v/v) FBS, 100 units/ml of penicillin and 100 units/ml of streptomycin at 37 °C in a humidified, $CO_2$-controlled (5%) incubator. The cells were subcultured every three days until a maximal passage number of 30 were achieved.

**2. Assays**

The DEHP contents in three kinds of solvents (Hexanes, Chloroform, Methanol), five kinds of tubing lines and two kinds of resin were identified. One liter of solvent was completely evaporated at 40 °C under vacuum. Five laboratory tubing lines and two resins were immersed into methanol for 24 hrs at room temperature. The methanol extract was then completely evaporated at 40 °C under vacuum. The concentration of DEHP was calculated based on a standard curve of DEHP concentration (0.004, 0.04, 0.4, 2.0, 4.0 mg/ml). Determinant of DEHP in all materials was performed in duplicate.

The concentration of DEHP in all materials was measured by HPLC. Analytical HPLC (Binary HPLC pump - Waters 1525) with a C18 column (Nova Pak 3.9×150 mm, 4 μm) was used. Mixtures of water (A) and methanol (B) were used as a mobile phase. The HPLC condition was 50% B (0-10 min); 50-80% B (10-20 min); 80% B (20-30 min); 80-100% B (30-40 min); 100 B (40-50 min) and 100-50% B (50-60 min). The flow rate was 1 ml/min. A dual $\lambda$ absorbance detector (Waters 2478) was used for UV detection at 254 nm.

For measuring melamin content, B16F10 cells were cultured at $6\times10^4$ cells in 6-well plates. After 24 hrs, the cells were treated with various concentrations of samples for 48 hrs. The cells were then detached by the addition of 0.05% trypsin-EDTA. After transferring to test tubes and washing twice with PBS, samples were dissolved in 200 ml of 1 N NaOH containing 10% DMSO. The samples were then heated at 80 °C for 1 hr. The amount of melanin was determined spectrophotometrically based on absorbance at 405 nm [19]. Cell viability was determined by using MTT based colorimetric [20].

For measuring cellular tryrosinase, B16F10 cells were cultured at $6\times10^4$ cells in 6-well plates. After 24 hrs, the cells were treated with various concentrations of sample. The cells were harvested by trypsinization (0.5% EDTA-trypsin) and washed three times with ice-cold phosphate-buffered saline (PBS) by centrifugation at 5,000 rpm for 5 min. The cells were lysed in a 0.1 M sodium phosphate buffer (pH 7.0) containing 1% Triton ×100. The cells were then disrupted by sonicating for one hour at 4 °C, and the lysates were then clarified by centrifugation at 13,000 rpm for 20 minutes. After protein content was quantified by using a protein assay kit (Bio-Rad, U.S.A.), the cell lysates were adjusted to the same amount of protein with a lysis buffer. The reaction mixtures, consisting of 40 μg of protein, 40 μl of 5 mM L-DOPA and 0.1 M PBS (pH 6.8), were assayed on a 96-well plate at 37 °C. After 1 hr, absorbance was measured at 475 nm with an ELISA reader at 1 hr [19]. Each determination was made in triplicate and data shown are means± S.D.

## RESULTS AND DISCUSSIONS

A calibration curve was made to determine the concentration of DEHP in laboratory materials. The curve was obtained by series of DEHP concentration corresponding to up to 4.0 mg/ml. The obtained calibration graph illustrated ranges of linearity (data not shown).

To investigate the contamination of DEHP in laboratory tools, three kinds of solvents, two kinds of resins and five kinds of tubing lines were tested. The results are summarized in Table 1. The results show that the concentration of DEHP released from two kinds of resin is 0.3-0.4 mg/kg. The concentration of DEHP in 3 kinds of solvent is 0.06-0.07 mg/*l*. However, in different type of tubing, the DEHP concentration is different. The Teflon tubing released 0.04-0.16 mg/g, the silicone tubing released 0.14 mg/g and the PVC tub-

**Table 1. Concentration of DEHP in various solvents, resins and tubings**

| | | |
|---|---|---|
| Solvent | Methanol | 0.06±0.005(mg/*l*) |
| | Hexane | 0.07±0.005(mg/*l*) |
| | Chloroform | 0.06±0.005(mg/*l*) |
| Resin | Silica gel F254 resin | 0.4±0.01(mg/kg)† |
| | C18 resin | 0.3±0.01(mg/kg) |
| Tubing | PTFE*(1)** | 0.06±0.004(mg/g)† |
| | PTFE(2) | 0.04±0.001(mg/g) |
| | PTFE(3) | 0.16±0.002(mg/g) |
| | Silicone(4) | 0.15±0.004(mg/g) |
| | Polyvinyl chloride (5) | 100±10.0(mg/g) |

*PTFE: Polytetrafluoroethylene.

**Manufacturer of tubing (1-5) is described in the Materials and Methods.

†: Note that unit is different.

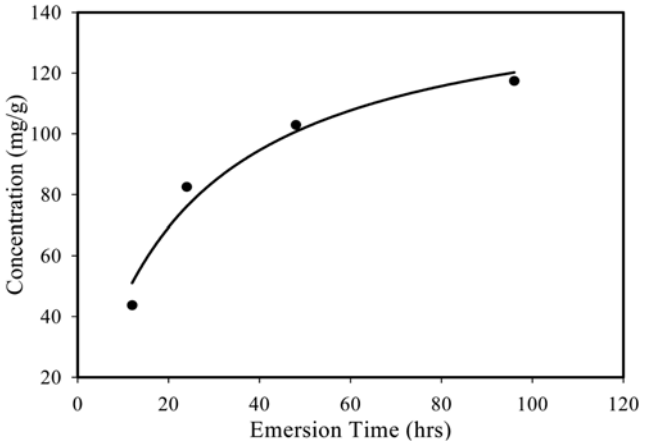


**Fig. 2. Time course release of DEHP.**
The PVC tubing was immersed in methanol for various times (18 hrs to 96 hrs). The methanol extracts were completely evaporated under vacuum. The concentrations of DEHP were calculated based on a standard curve using DEHP concentration.

ing released 100 mg/g.

From all laboratory materials investigated, the PVC tubing showed the highest concentration of DEHP (100 mg/g). When operating open column chromatography, this line is usually used to connect between different parts of system. For further understanding of the leaching of DEHP during specific time periods, the concentration of DEHP at different times was examined (Fig. 2). The results showed that the release of DEHP increases in a time-dependent manner. After 4 days immersed in methanol, the PVC tubing released DEHP up to 120 mg/g material.

To investigate the effect of DEHP in melanin synthesis, melanin content and tyrosinase activity in B16F10 melanoma cells were measured after being treated with different concentrations of DEHP. The results showed that DEHP can significantly inhibit melanogenesis in a dose-dependent manner with $IC_{50}$=24 μM (Fig. 3). DEHP also inhibited 25.8% activity of cellular tyrosinase of treated-B16F10 melanoma cells at a concentration of 10 μM (Table 2). To confirm that the melanin inhibition activity is not caused by cell toxicity, an MTT assay was performed (Fig. 3). The results showed that DEHP did not show any toxicity up to 20 μM.

DEHP is used as a plasticizer and poses a potential risk for human exposure through several pathways. During the isolation of the active compounds from natural products procedure, many kinds of solvents, resin, and tubing are used. To investigate the leaching effects of DEHP from these materials, different kinds of materials such as organic solvents, resins and plastic tubing were examined. The results showed that all of the materials became contaminated with DEHP at different concentrations (0.06 mg/kg or 1 liter up to 100 mg/g materials after 1 day immersion in methanol). Among these materials, PVC tubing released the highest amount of DEHP. The release of DEHP in PVC tubing increased in a time-dependent manner. Furthermore, DEHP has been found in many vegetables such as Chinese tomato fruit, cabbage, *Benincasa hispida* [21,22]. These data suggest that during the isolation and analysis process, DEHP may accumulate and contaminate laboratory materials as well as samples. DEHP also exhibited a high potential inhibitory effect on melanogenesis of B16F10 melanoma cells with $IC_{50}$=24 μM. DEHP inhibited activity of murine tyrosinase of B16F10 melanoma cells by 25.8% at a concentration of 10 μM and did not show cell toxicity at effective concentration. Recently, many functional cosmetic products have been sold in the market without confirming their exact components. Our study suggests that plant extracts used in the study of depigmenting agents should be evaluated for possible contamination by DEHP, because such chemicals are prohibited as components of cosmetics.

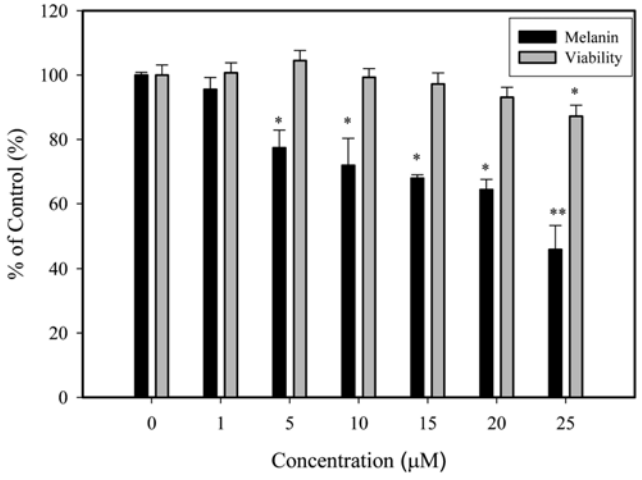


**Fig. 3. Effect of DEHP on melanogenesis of cultured B16F10 melanoma cells.**
B16F10 cells were treated with DEHP (0 to 25 μM) for 2 days and the cells were then harvested. Melanin contents and cell viability were measured as described in Materials and Methods. Data were expressed as the change of the melanin content level and cell viability relative to untreated control. Each determination was made in triplicate and data shown are means± S.D. *p<0.05, **p<0.01: statistically significant vs. the value of the control group.

**Table 2. Inhibition of DEHP on B16F10 tyrosinase activity**

| Sample | Concentration (μM) | Tyrosinase inhibition (%) |
|---|---|---|
| PBS (control) | 0 | 0 |
| Arbutin | 730 | 20.4±2.4 |
| DEHP | 10 | 25.8±1.2 |

## CONCLUSION

In this study, we have shown that DEHP is released from common materials used in the laboratory. In PVC tubing, DEHP is released in concentrations up to 90 mg/g after one day immersion in methanol. DEHP also significantly caused melanin inhibition with $IC_{50}$=24 μM and inhibited 25.8% tyrosinase activity at 10 μM but did not show cell toxicity up to 20 μM. Because of the high concentration of DEHP released and high inhibition in melanin synthesis, researchers should consider DEHP contamination from laboratory materials during analytical and isolation procedures.

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