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Cathepsin B inhibitory activities of phthalates isolated from a marine *Pseudomonas* strain

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Abstract—Two cathepsin B inhibitors were isolated from the culture supernatant of a marine *Pseudomonas* sp. PB01 (GenBank Accession No. EU126129). Their structures were elucidated by spectroscopic analyses as dibutyl phthalate and di-(2-ethylhexyl) phthalate. Both dibutyl phthalate and di-(2-ethylhexyl) phthalate showed dose-dependent cathepsin B inhibitions with IC_{50} of 0.42 and 0.38 mM, respectively. It is also observed from kinetic analyses that dibutyl phthalate and di-(2-ethylhexyl) phthalate acted as noncompetitive inhibitors with K_i values of 0.64 and 0.42 mM, respectively. Furthermore, both of them caused inactivation of the pericellular cathepsin B of murine melanoma cell with no acute cytotoxicity. The IC_{50} values were found to be 0.23 mM for dibutyl phthalate and 0.14 mM for di-(2-ethylhexyl) phthalate, respectively, and were 50% compared to that of purified cathepsin B.

The lysosomal cysteine proteases cathepsin B (EC 3.4.22.1) is a unique cysteine member showing dual roles as both endopeptidase and exopeptidase activities, due to the presence of the occluding loop in structure.[1] In normal conditions, cathepsin B plays a role in intracellular protein catabolism; and is also involved in hormone activation and processing of antigens in immune response. Over-expression and mislocation in cell membrane of cathepsin B, however, have contributed to several pathological processes that include cancer and neurogenerative disorders.[2–4] Cathepsin B inhibitors have been reported to cause changes in malignant tumors.[5] The inhibition of cathepsin B has also been proved to decrease the severity of joint inflammation and to reduce the destruction of particular tissues in the rat model of antigen adjuvant-induced arthritis. Cathepsin B inhibitors are therefore expected to be useful for the treatment of inflammatory joint disease, invasion of cancer, and other diseases related to cathepsin B disorder.

Inhibitors of cathepsin B include endogenous inhibitors such as the cystatin superfamily, low molecular weight natural, and chemical synthesis inhibitors, that is, leupeptin, E-64, tokaramide A, CA030, and CA074.[6]. Marine organisms, including microorganisms represent as treasure sources for therapeutic natural products. To explore new cathepsin B inhibitors, culture broths from more than 300 marine microorganisms were investigated by enzyme inhibitory assay. The most interesting microorganism *Pseudomonas* sp. PB01 (GenBank Accession No. EU126129) was chosen. In this study, we described the isolation, structure elucidation, and enzyme inhibitory activities of pure compounds from culture broth of strain PB01. Moreover, pericellular cathepsin B inhibition was studied in order to assess in vivo potential of the isolated compounds.

Cathepsin B and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). *Z*-Arg-Arg-MCA was purchased from Bachem AG (Switzerland). Murine melanoma cell, B16F10 (ATCC CRL-6475) was obtained from American Type of Culture Collection (Manassas, VA, USA). Cell culture medium and all the other materials required for culturing were obtained from Gibco (Grand Is-

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land, NY). Other chemicals and reagents used were of analytical grade.

Pseudomonas sp. PB01 isolated from marine sediment in Daegu (Korea) was identified by 16S rRNA and then submitted on GenBank with Accession No. EU126129 (manuscript in preparation). For production of cathepsin B inhibitors, *Pseudomonas* sp. PB01 was cultured in 2-L flasks containing 800 ml medium and was inoculated with 5% (v/v) overnight grown culture. The medium consisted of 1% glucose, 0.1% beef extract, 0.1% yeast extract, 0.2% peptone, and 60% sea water (pH 6.6). The cultivation was carried out at 28 °C, for 7 days with agitation of 150 rpm rotary shaker. Total 20 L of culture broth was centrifuged at 4000 rpm for 10 min, and was extracted with EtOAc (1:1) to provide a crude extract (2.8 g), which was subjected to silica gel chromatography (Merck 70–230 mesh, 75 g, Ø 2.5×40) and eluted with *n*-hexane to EtOAc. Enzyme inhibitory activity was observed in *n*-hexane/EtOAc fraction (10:1), which was purified by HPLC using a Waters 600 controller equipped with a 2487 dual wavelength detector and Alltima C18 5μm column (10 mm i.d. × 250 mm; Alltech, USA) with a linear gradient of acetonitrile (0–100%), flow rate 2.0 ml/min, and UV detection at 220 nm. Finally, two active compounds dibutyl phthalate (**1**) (15 mg, 0.54% yield based on wet weight, t_R 36.05 min) and di-(2-ethylhexyl) phthalate (**2**) (20 mg, 0.71% yield based on wet weight, t_R 53.44 min) were afforded.

NMR spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer, ^1H and ^{13}C chemical shifts were referenced to the solvent peaks: δ_H 7.23 and δ_C 77.31 for $CDCl_3$. Electron Impact Mass Spectrometric (EIMS) data were measured on a JEOL JMX-700 spectrometer. Optical rotations were determined on a Perkin-Elmer model 341 polarimeter in CD_3OH. IR spectra were recorded on Fourier transform IR spectrophotometer (Bucker FT-IR model IFS-88 spectrometer).

The protease inhibitor activity was carried out in 96-well black plate with method previously described by Barrett et al.[7] Each well contains 12.5 μl of a 0.4 M sodium potassium phosphate buffer (pH 6.0) containing 8 mM dithiothreitol (DTT) and 4 mM EDTA, 12.5 μl of 40 μU/ml cathepsin B solution and 12.5 μl of sample. After 10 min at 37 °C, 12.5 μl of 20 μM *Z*-Arg-Arg-MCA in water was added to start the reaction. The reaction was stopped after 20 min by 50 μl of 100 mM sodium monochloroacetate in 100 mM sodium acetate buffer (pH 4.3). Methanol was used for control value. The fluorescence of 4-Methyl-Coumary-7-Amide released was measured at Ex 360 nm and Em 465 nm with GENios® microplate reader (Tecan Austria GmbH, Austria).

The inhibition modes were determined by Lineweaver–Burk plots. The inhibition constant (K_i) was determined by the secondary plots (intercept on vertical axis of Lineweaver–Burk plots against inhibitor concentrations).[8]

The cytotoxic levels of the inhibitors on B16F10 cells were measured using MTT method as described by Hansen et al.[9] B16F10 cells were cultured in Dulbecco's modification of Eagle's medium-Ham's F-12 (DMEM-F12) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 10% fetal bovine serum (FBS) and maintained at 37 °C under a humidified atmosphere with 5% CO_2. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and were treated with different concentrations of inhibitors. After 48 h of incubation, cells were rewashed and 100 μl of MTT (5 mg/ml) was added and incubated for 4 h. Finally, DMSO (100 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader. Relative cell viability was determined by the amount of MTT converted into formazan salt.

Furthermore, fluorescent microplate assay was employed to measure pericellular cathepsin B activity using the fluorogenic substrate *Z*-Arg-Arg-MCA.[10] B16-F10 cells were plated in 96-well plates and grown a density of 5×10^3 cells/well. The growth medium was then aspirated and the monolayers washed with 0.1 ml Dulbecco's NaCl/P_i. The cells were then incubated with 90 μl of pericellular assay buffer (PAB; Hank's balanced salt solution containing 2 mM L-cysteine, pH 7.0) at 37 °C. After 30 min, 10 μl of 200 μM *Z*-Arg-Arg-MCA was added in the presence or absence of inhibitors. The cells were incubated for an additional 20 min at 37 °C. The fluorescence of the MCA was measured at Ex 360 nm and Em 465 nm by GENios® microplate reader.

Data were presented as means ± standard error of the mean ($n = 3$). Student's *t*-test was used to determine the level of significance. P value < 0.05 was considered significant.

Compound (**1**) was isolated as colorless oil, which had an IR spectrum [aromatic system (1600, 1579, 1465, 740 cm^{-1}) and ester moiety (1724, 1271 cm^{-1})] indicating carbonyl and phenyl functional groups. The molecular formula of (**1**) was provided by LREIMS (positive ion), which gave an $[M+H]^+$ ion peak at 279, which analyzed for $C_{16}H_{22}O_4$ on the basis of ^1H and ^{13}C NMR data as well. Moreover, LREIMS showed phthalate base peak at *m/z* 149 for the characterization of phthalate ester with lateral chains bigger than two carbons.[11] Analysis of the ^1H, ^{13}C, DEPT, and HMQC NMR data of (**1**) indicated the presence of two methyl groups, six methylene units (two O-bearing methylene), four sp^2 aromatic carbons, two sp^2 quaternary carbons, and two carbonyl carbons. The ^1H NMR spectrum showed a characteristic AA′BB′ system at δ 7.47 (2H, dd, $J = 9.22$, 2.19 Hz), 7.25 (2H, dd, $J = 8.78$, 2.63 Hz), as well as ^{13}C NMR data at δ 131.6 (s), 127.9 (d), and 130.0 (d). These data accounted for all required the compound to have di *ortho*-substituted aromatic ring. The partial structure of butane moiety was interpreted mainly by COSY and HMBC NMR data (Table 1). These data described above was suggested compound (**1**) as dibutyl phthalate. The structure and key correlations of compound (**1**) are described in Figure 1.

Table 1. NMR data for dibutyl phthalate (**1**) and di-(2-ethylhexyl) phthalate (**2**)

C#	Dibutyl phthalate		Di-(2-ethylhexyl) phthalate	
	δ_C (mult)	δ_H (mult, *J*)	δ_C (mult)	δ_H (mult, *J*)
1	166.4 (s)		167.5 (s)	
2	131.6 (s)		132.3 (s)	
3	127.9 (d)	7.47 (dd, 9.22, 2.19)	128.5 (d)	7.67 (dd, 9.15, 2.56)
4	130.0 (d)	7.25 (dd, 8.78, 2.63)	130.7 (d)	7.47 (dd, 8.78, 2.19)
1′	64.3 (t)	4.06 (t, 6.59)	67.8 (t)	4.20 (t, 5.85)
2′	29.7 (t)	1.45 (tt, 6.59, 8.34)	38.6 (d)	1.65 (m)
3′	18.3 (t)	1.19 (q, 7.47)	30.2 (t)	1.28 (m)
4′	12.7 (q)	0.70 (t, 7.47)	28.7 (t)	1.28 (m)
5′			22.8 (t)	1.28 (m)
6′			13.8 (q)	0.86 (m)
7′			23.6 (t)	1.39 (m)
8′			10.7 (q)	0.90 (m)

Recorded in $CDCl_3$ at 400 MHz (^1H) and 100 MHz (^{13}C).

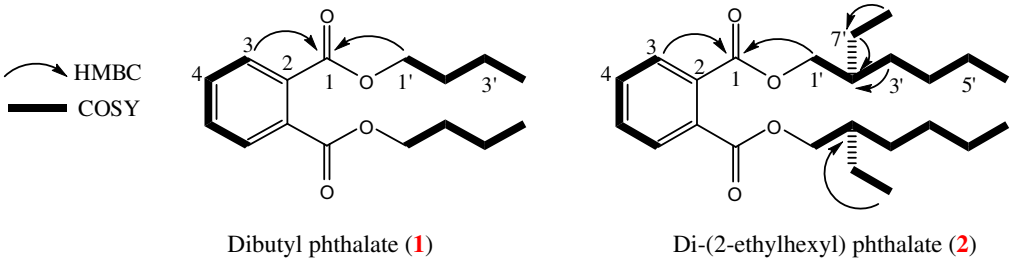

Figure 1. Chemical structures of dibutyl phthalate (**1**) and di-(2-ethylhexyl) phthalate (**2**).

Compound (**2**) was isolated as an optically inactive oil, $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH). Its molecular formula was provided by LREIMS (positive ion), which gave an ion peak $[M+H]^+$ at 391, which analyzed for $C_{24}H_{38}O_4$ on the basis of ^1H and ^{13}C NMR data as well. The IR spectrum revealed the existence of alkane (2924 cm^{-1}), aromatic system (1602, 1575, 1485, 741 cm^{-1}), and ester moiety (1723, 1267 cm^{-1}). The six degrees of unsaturation in this formula could be partially accounted for by one benzene ring and two carbonyl units, which was agreeable to LREIMS ion peak *m/z* 149. Analysis of the ^1H, ^{13}C, DEPT, and HMQC NMR data of (**2**) indicated the presence of four methyl groups, ten methylene units (two O-bearing methylene), four sp^2 aromatic carbons, two sp^2 quaternary carbons, two alkane methane units, and two carbonyl carbons. The ^1H NMR spectrum showed a characteristic AA′BB′ system at δ 7.67 (2H, dd, J = 9.15, 2.56 Hz), 7.47 (2H, dd, J = 8.78, 2.19 Hz), as well as ^{13}C NMR data at δ 132.3 (s), 128.5 (d), 130.7 (d). These data accounted for all required the compound to have di *ortho*-substituted aromatic ring. The connections of di-substituted ester moieties attached to the aromatic quaternary carbons were elucidated by the long-range coupling between δ_H 7.67 and δ_C 167.5 (s). The signals from HMBC and COSY NMR spectra clearly elucidated the partial structure of alkane moieties (Table 1). The HMBC correlation between δ_H 1.65 (m) and δ_C 67.8 (t) finally completed the plane structure of compound (**2**) as di-(2-ethylhexyl) phthalate. The two symmetric chiral carbons (*R*, C-2′, 2″) led to the molecular symmetry, which was supported by the optical rotation data $[\alpha]_D^{20}$ 0° (*c* 0.5, MeOH). The structure and key correlations from HMBC and COSY spectra of compound (**2**) are described in Figure 1.

Dialkyl phthalate, including dibutyl phthalate and di-(2-ethylhexyl) phthalate are used as plasticizers in variety of industrial products and thought to pollute environment. However, both of them were also considered as natural products, which were already mentioned in many papers. For instance some dialkyl phthalate have

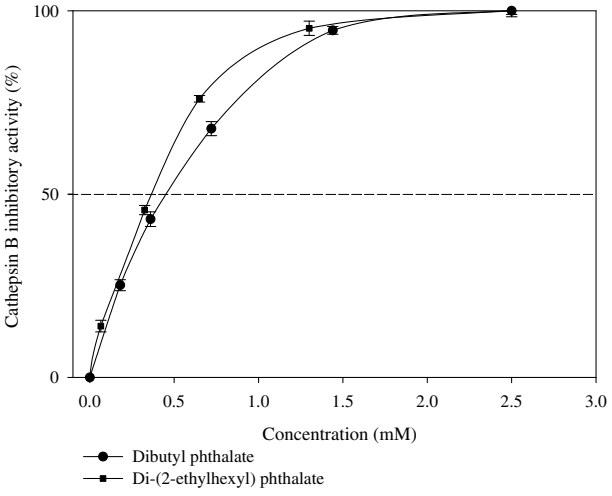

Figure 2. Dose-dependent inhibitions of cathepsin B by dibutyl phthalate (●) and di-(2-ethylhexyl) phthalate (■). The values are means ± SE.

been isolated from terrestrial and marine organisms such as *Aloe vera*, marine algae, and fungal and bacterial culture broths.[12–19] In order to conform that our compounds were from *Pseudomonas* sp. PB01, the culture broth of another marine microorganism, together with pure medium, was investigated (data not showed). As expected, these compounds did not appear in any other culture broths, except *Pseudomonas* sp. PB01 one. As a result of that dibutyl phthalate and di-(2-ethylhexyl) phthalate isolated from *Pseudomonas* sp. PB01 must be biogenetic products of this marine microorganism, because they have not been contaminated from environment. Roles of phthalate esters are unclear. They may be stored in the cell membrane and affect the flexibility of the cell.[13]

Both isolated phthalates showed potently inhibitory activities on cathepsin B. In kinetic studies of the hydrolysis of *Z*-Arg-Arg-MCA into MCA, inhibition of against cathepsin B activity was found to be dose-dependent. Various concentrations of dibutyl phthalate and di-(2-ethylhexyl) phthalate were tested and observed that IC_{50} were 0.42 and 0.38 mM, respectively (Fig. 2). Inhibitory activities of isolated compounds correlated with the carbon number of alkyl groups. A similar correlation has been reported by Ohyama[20] about the inhibitory activities of phthalate on glucose 6-phosphate dehydrogenase. They found that hydrophobic bonding is of major importance in the affinity of these compounds. Several cathepsin B inhibitor peptides have been reported and available as commercial products such as E-64, CA030, leupeptine. However, they generally showed poor pharmacokinetic properties.[21] Moreover, the nonpeptide inhibitors can be more stable because they are less likely to be degraded by peptidases in cell.

To clarify the inhibition mechanism on cathepsin B kinetically, Lineweaver–Burk plots were obtained for dibutyl phthalate and di-(2-ethylhexyl) phthalate (Fig. 3). For each plot, three lines obtained from uninhibited enzyme and two different concentrations of one inhibitor. These plots showed noncompetitive inhibitory mechanisms, with an intercept on the 1/[V] axis. Obviously, di-(2-ethylhexyl) phthalate has showed tighter-binding to cathepsin B than the other. The inhibition constants (K_i) values of dibutyl phthalate and di-(2-ethylhexyl) phthalate were found as 0.64 and 0.42 mM, respectively. Our kinetic analysis showed that dibutyl phthalate and di-(2-ethylhexyl) phthalate inhibited cathepsin B in a noncompetitive fashion. The inhibitors are not binding to the same site as substrate, so the inhibition cannot be overcome by raising substrate concentration. This observation is agreeable with the structural features of our compounds as absence of hydrogen-donors. Cysteine proteases inhibitors reported to date rely on covalent attachment of an electrophilic peptide-derived ligand to the active site thiol to achieve potent enzyme inhibition.[22] Obviously, extracellular matrix degradation activity of cathepsin B is unexpected therefore such kind of mechanism is advantage for cathepsin B inhibition pattern.

The amount of cathepsin B released varies greatly with cell type and culture conditions, it has showed an increased response to malignant progression in melanoma B16 cells. Before doing fluorescent microplate assay, the cytotoxicity effect of dibutyl phthalate and di-(2-ethylhexyl) phthalate was investigated by using B16F10 (Fig. 4). At a concentration of 1000 μM, the low cytotoxicities of dibutyl phthalate and di-(2-ethylhexyl) phthalate on B16F10 were 9.45% and 7.34%, respectively. Since the difference between treated and control group was not too significant, our compounds could be used for pericellular cathepsin B activity assay. Depending on the amount of active cathepsin B present in conditioned medium, fluorescence conjugated substrates are differentially hydrolyzed. Metastatic B16-F10 cells release not only cathepsin B but also other cysteine proteases, such as cathepsin L. In order to prevent other proteases activities, specific substrate for cathepsin B was used. The result suggested that our compounds were able to block pericellular cathepsin B activity with

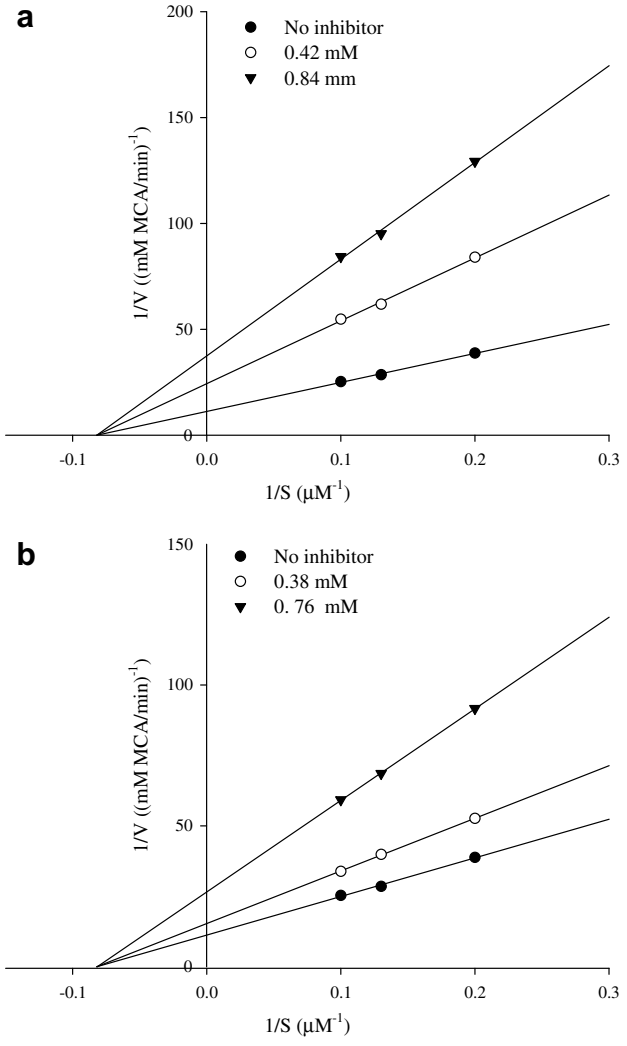

Figure 3. Lineweaver–Burk plots of cathepsin B in the presence of phthalates. (a) Concentration of dibutyl phthalate: (●), 0 mM; (○), 0.42 mM; (▲), 0.84 mM. (b) Concentration of di-(2-ethylhexyl) phthalate: (●), 0 mM; (○), 0.38 mM; (▲), 0.76 mM. S: *Z*-Arg-Arg-MCA concentration; V: MCA release.

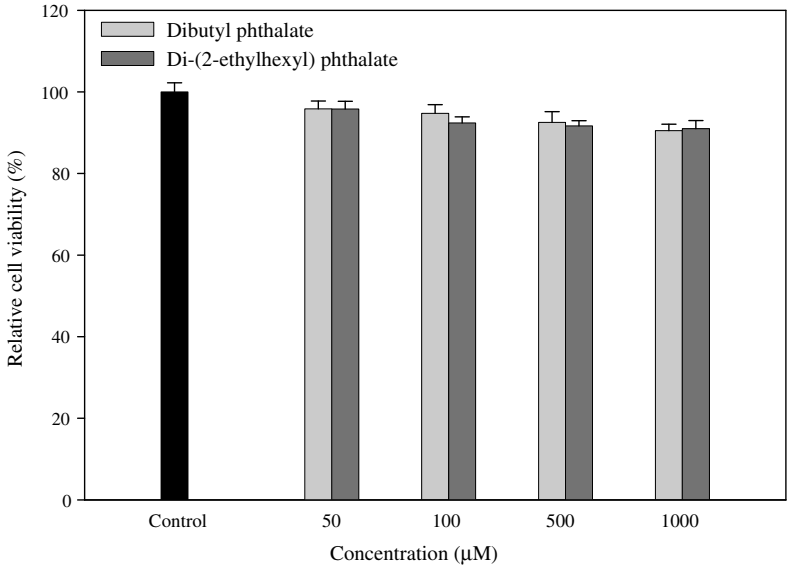

Figure 4. Dose-dependent effects of cathepsin B by dibutyl phthalate and di-(2-ethylhexyl) phthalate on B16F10 cells. The values are means ± SE.

dose-dependent, IC_{50} values of 0.23 and 0.14 mM, respectively. In this case the IC_{50} values of each sample were only 50% in comparison with IC_{50} values of purified cathepsin B (Fig. 5). The difference in inhibition compare to in vitro may depend on the enzyme amount or some unknown other mechanisms related to cell.

In conclusion, two phthalate compounds as cathepsin B inhibitors were isolated from the same marine *Pseudomonas* sp. PB01. To our knowledge, this biological activity is hitherto unreported for these compounds although their activities were moderate. The fluorescent microplate results highlighted the potential to block pericellular cathepsin B of our compounds. Further studies are needed to characterize the interaction of dibutyl phthalate and di-(2-ethylhexyl) phthalate with cathepsin B, as well as to investigate whether dibutyl phthalate and di-(2-ethylhexyl) phthalate are possible to penetrate into the cell and block cathepsin B or not.

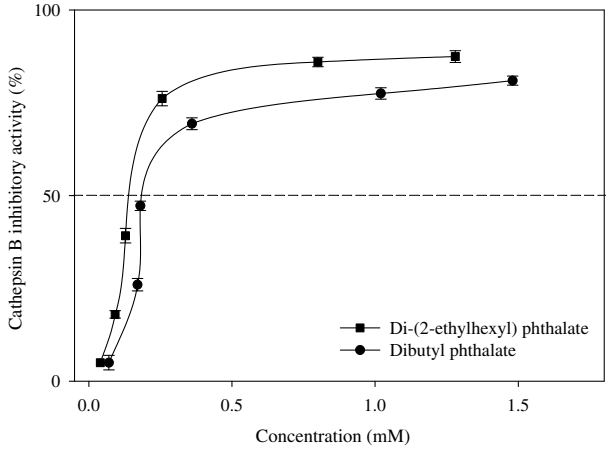

Figure 5. Dose-dependent inhibitions of pericellular cathepsin B by dibutyl phthalate (●) and di-(2-ethylhexyl) phthalate (■). The values are means ± SE.

Acknowledgments

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