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Endotoxin Contamination and Reaction Interfering Substances in the Plant Extract Library

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Endotoxin is an unintentional contaminant that has numerous activities and can affect various biological experiments using cells. In this study, we measured the endotoxin activity of samples from a plant extract library (PEL) and determined their degrees of contamination. Endotoxin was detected in approx. 48% ($n = 139$) and approx. 4% ($n = 5$) of field-collected and crude drug samples, respectively, and in concentrations >5.0 EU/mL in some samples. The concentrations of endotoxin that affect cells *in vitro* vary depending on the target cell type. Although the degree of contamination varied in the present study, it was considered to have little effect on the cell experiments. More than 150 PEL samples had problems with reaction courses or recovery rates of *Limulus* amoebocyte lysate (LAL) tests. In the LAL tests, using three plant extracts [*Sanguisorba officinalis* L. (Rosaceae), *Oenothera biennis* L. (Onagraceae), and *Lythrum salicaria* L. (Lythraceae)], the polyphenolic compounds in the plant extracts affected LAL test and their effects differed depending on the plant species. When the 16 single polyphenol compounds were added to the LAL tests, the compounds with caffeoyl and pyrogallol moieties were found to affect the LAL reaction course and recovery rate. Furthermore, none of the compounds had any effects at concentrations of $1\mu\text{M}$. Because the plant extracts contained analogs of various polyphenolic compounds, they were presumed to actually act synergistically. Our findings demonstrated that attention must be paid to the recovery rate and reaction process of LAL tests with samples containing polyphenolic compounds.

Key words endotoxin; *Limulus* amoebocyte lysate (LAL) assay; plant extract library; polyphenol; polyvinylpyrrolidone (PVPP)

INTRODUCTION

The interest in screening using natural products, such as plants and marine products, has increased. Despite the limitations of combinatorial chemistry, the structural diversity of natural products is of great interest. In Japan alone, there are approx. 8800 species of plants, many of which are presently unused species. In 2013, we started constructing a large plant extract library (PEL) and have since prepared over 13000 samples.¹⁾ Half of this library consists of Japanese wild plants, followed by cultivated plants, crude drugs, and exotic plants. The PEL is recognized for its great importance due to the potential presence of unknown compounds and different behaviors from single compounds. The PEL has already been used in various screenings, including drug development, food chemistry, and cosmetics development.

Plant extracts are mixtures of components, and endotoxin represents one of the unintended contaminants. Endotoxin is a lipopolysaccharide derived from the surface membrane of Gram-negative bacteria. Endotoxins are known to have cellular-level effects (activation of macrophages, B cells, production of various cytokines, and induction of gene expressions) as well as influence the pyrogenic reaction of the living body.^{2–4)} In recent years, endotoxin contamination in the experimental environment has been reduced by the use of media, reagents, and accessories with low endotoxin levels. Therefore, information regarding the extent of endotoxin contamination in samples is very important for accurate experimental results to be obtained. The presence of endotoxin has been reported in

air, soil, and various other environments.^{5–8)} In particular, the underground parts of plants are in contact with the soil, which can lead to contamination by soil microorganisms during the preparation of extracts.

The *Limulus* amoebocyte lysate (LAL) test is a common method used to measure endotoxin activities because of its high sensitivity, specificity, and wide endotoxin detection range. However, the LAL test utilizes serine protease enzymatic reactions,^{9,10)} and various interfering factors, such as organic solvents, proteases, protease inhibitors, chelators, antibiotics, and metal ions, have been reported.^{2,3)} Polyphenol compounds, which are common plant components, are a group of compounds with varied structures and phenolic hydroxyl groups. Among them, some characteristic polyphenols are known as tannins. Polyphenols and tannins are known to affect enzymatic reactions,^{11–13)} however, no reports have assessed their impacts on the LAL test.

The aim of this study was to determine the extent of endotoxin contamination of the PEL using LAL tests. Additionally, since some extract samples were shown to affect the LAL test, we assessed the effects of polyphenolic compounds on the LAL test and the removal of interfering factors from plant extracts.

MATERIALS AND METHODS

Materials

Preparation of PEL and Endotoxin Determination

Chemicals The dimethyl sulfoxide (DMSO) and *Limulus*

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Color KY Test Wako (endotoxin detection kit) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Water for injection (WFI) was purchased from Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan).

Plant Materials Root samples of *Sanguisorba officinalis* L. (Rosaceae, Japanese name: Waremo, MPR-0348) were cultivated in the Tsukuba Division, Research Center for Medicinal Plant Resources (RCMPR), National Institutes of Biomedical Innovation, Health and Nutrition (NIBIOHN) in 2013, and root samples of *Oenothera biennis* L. (Onagraceae, Japanese name: Mematsuyoigusa, MPR-0366) and *Lythrum salicaria* L. (Lythraceae, Japanese name: Ezomisohagi, MPR-0363) were collected in Hokkaido in 2013. For the field-collected plants, information such as the locations and dates of collection, scientific name, amount of extract, and specimens were recorded and managed by Tsukuba Division, RCMPR, NIBIOHN with unique numbers. The commercial crude drugs were provided by the manufacturers and were deposited at the Tsukuba Division, RCMPR, NIBIOHN. Information regarding the samples was recorded and managed with unique numbers, as with the field-collected plants.

Determination of the Total Phenolic Content Folin–Ciocalteu reagent and polyvinylpyrrolidone (PVPP, Polyclar VT) were purchased from FUJIFILM Wako Pure Chemical Corporation. Milli-Q water was used in the assays (Direct-Q, Merck Millipore, MA, U.S.A.).

Polyphenol Compounds Gallic acid (1), methyl gallate (2), ellagic acid (4), chlorogenic acid (8), rosmarinic acid (9), hesperetin (10) were obtained from FUJIFILM Wako Pure Chemical Corporation. Vanillic acid (3), *trans-p*-coumaric acid (5), *trans*-caffeic acid (6), naringenin (11), rutin (13), genistein (14), and puerarin (15) were obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Ferulic acid (7) was obtained from LKT Laboratories (St. Paul, MN, U.S.A.), quercetin (12) was obtained from Cayman Chemical Company (Ann Arbor, MI, U.S.A.), and (+)-catechin hydrate (16) was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Methods

Preparation of PEL Samples The plants collected in the field were washed with water, divided into their different parts, dried at 40°C for 96 h, and then pulverized. After pulverization, the samples were extracted under reflux with 10 mL methanol/g for 2 h. The extracts were then filtered and concentrated under reduced pressure to produce the residues. The residues were then dissolved in DMSO to a concentration of 40 mg/mL, filtered, and stored in cryotubes at −20°C. The crude drug samples were commercial products.

Determination of Endotoxin Activity and Influence on the LAL Test Endotoxins were detected according to the manufacturer's instructions for the reagent. WFI was used to dilute the samples, resulting in the final DMSO concentration in the sample solution being 1 v/v (%), and the final plant extract concentrations being 400 µg/mL. Samples (50 µL) and an equal volume of LAL solution were dispensed into 96-well microplates, and incubated at 37°C. Since plant extracts often contain various interfering factors, recovery tests were performed at the same time. Recovery test samples contained PEL samples, 0.05 EU/mL CSE (EU: endotoxin unit, CSE: Control Standard Endotoxin). Recovery rate of LAL test was calculated as follows: Recovery rate (%) = (spiked sample – non-spiked sample) / 0.05 (EU/mL). Analyses were conducted

Fig. 1. Reaction Courses of Limulus Amoebocyte Lysate Tests in the Presence of Endotoxin

1, negative control; 2, 0.005 EU/mL; 3, 0.05 EU/mL; and 4, 5 EU/mL.

using the Toximaster[®]QC7 MPR (FUJIFILM Wako Pure Chemical Corporation) software and Sunrise Thermo (Tecan, Männedorf, Switzerland) microplate reader. The results were converted to the concentration of PEL in the DMSO solution (EU/mL). The results were judged to be appropriate when the recovery rates reached 50–200% and when the reaction curves showed no deformation (see Fig. 1). Blank samples were prepared using the same protocols described above, without plant samples. The endotoxin concentrations of blank samples were below the detection limit (<0.0005 EU/mL).

Degradation of Endotoxin in PVPP Endotoxin was decomposed according to Niwa *et al.*¹⁴⁾ with slight modification. Briefly, 40 mg PVPP and 1.5 mL 0.1 N NaOH 95% EtOH were combined and mixed together in 2 mL plastic tubes, and then stored at 30°C (with occasional stirring). After 24 h, the PVPP was washed with WFI after the removal of the NaOH solution *via* centrifugation.

Removal of Polyphenols in PEL Samples WFI and PEL samples were added into test tubes with washed PVPP, then stored at 5°C for 30 min. The supernatant was used to determine the endotoxin activity, using the protocols described previously.

Determination of the Total Phenolic Content in PEL Samples The total phenolic contents were measured using microplates and the method described by Fujita *et al.*¹⁵⁾ PEL sample solutions and the 10-fold diluted Folin–Ciocalteu reagent were dispensed into 96-well microplates. After 3 min, 10% aqueous sodium carbonate solution was added. After standing for 1 h, the absorbance was measured at 700 nm using a Synergy H1 microplate reader (BioTek Instruments, Inc., VT, U.S.A.). Gallic acid was used as the standard chemical and the results were converted using the concentration of gallic acid in the DMSO solution (µg-GA/mg-Ext).

Examination of the Effects of Single Polyphenol Compounds Sixteen polyphenol compounds (see Fig. 2) were added at concentrations of 100, 10, and 1 µM and recovery rates were obtained. The recovery rates were calculated in the same formula in the case of PEL samples. To observe the effects of the compounds on the reaction curves, LAL tests with polymyxin B were also performed (final concentration 0.01%).

Statistical Analysis Endotoxin measurements of PEL samples were performed in duplicate, and the other

Fig. 2. Structural Formulae of Polyphenols

Table 1. Plant Extract Samples Subjected to the Limulus Amoebocyte Lysate (LAL) Test for Endotoxin Detection

Endotoxin activity (EU/mL)	Samples	
	Field-collected	Crude drugs
<0.05	149	121 (12) ^{a)}
0.05–0.25	36	3 (3)
0.25–0.50	16	0
0.50–2.50	42	1 (1)
2.50–5.00	15	1 (1)
>5.00	30	0
Total LAL tests that were possible	288	126 (12)
Total samples with endotoxin activity >0.05 EU/mL (endotoxin detected)	139	5
Rate of endotoxin detection (%)	48.3	3.97
Total LAL tests that were not possible	155	(24)

a) Numbers in parentheses indicate the number of kinds of crude drugs.

measurements were performed in triplicate. Statistical analyses were conducted using Microsoft® Excel® 2016 (Microsoft Corp., Redmond, WA, U.S.A.).

RESULTS

Endotoxin Activity in PEL Samples All measured data are presented in Supplementary Materials. Table 1 shows the number of samples tested using the LAL test. Endotoxin was detected in 139 of the 288 samples collected in the field and in only 5 crude drug samples. The endotoxin activity of

samples collected in the field was highly variable, with some exceeding 50 EU/mL. Table 2 shows examples of samples in which endotoxin was detected and Table 3 shows examples of samples for which endotoxin measurement could not be performed. Results that were considered representative from the perspective of sample category (field collected or crude drug), plant species, family name, and endotoxin activity are presented in Tables 2, 3. As shown in Table 2, endotoxin was detected in the plant extracts of various families, and small herbs (e.g. MPR-0269 *Chrysosplenium grayanum*, MPR-0288 *Cymbidium goeringii*) and fern plants with complex under-

Table 2. Examples of Plant Extract Samples for Which the Limulus Amoebocyte Lysate (LAL) Test Was Possible

Sample no.	Scientific name	Family	Plant part ^{a)}	Collection site	Endotoxin activity (EU/mL)
MPR-0022	<i>Achyranthes bidentata</i> Blume var. <i>fauriei</i> (H. Lév. et Vaniot)	Amaranthaceae	R	Ibaraki	<0.05
MPR-0024	<i>Cimicifuga simplex</i> (DC.) Wormsk. ex Turcz.	Ranunculaceae	U	Gunma	<0.05
MPR-0033	<i>Eschscholzia californica</i> Cham.	Papaveraceae	R	Ibaraki	<0.05
MPR-0046	<i>Mercurialis leiocarpa</i> Siebold et Zucc.	Euphorbiaceae	W	Ehime	<0.05
MPR-0058	<i>Lysimachia mauritiana</i> Lam.	Primulaceae	W	Kochi	<0.05
MPR-0079	<i>Justicia procumbens</i> L. var. <i>procumbens</i>	Acanthaceae	R	Kagoshima	<0.05
MPR-0086	<i>Patrinia scabiosifolia</i> Link	Valerianaceae	U	Yamanashi	<0.05
MPR-0096	<i>Adenocaulon himalaicum</i> Edgew.	Compositae	U	Ibaraki	<0.05
MPR-0097	<i>Artemisia japonica</i> Thunb.	Compositae	U	Hokkaido	<0.05
MPR-0119	<i>Disporum sessile</i> D. Don ex Schult. et Schult.f. var. <i>sessile</i>	Liliaceae	W	Yamanashi	<0.05
MPR-0124	<i>Maianthemum japonicum</i> (A. Gray) LaFrankie	Liliaceae	U	Hokkaido	<0.05
MPR-0151	<i>Pteris cretica</i> L.	Pteridaceae	W	Ehime	0.22
MPR-0155	<i>Chloranthus serratus</i> (Thunb.) Roem. et Schult.	Chloranthaceae	W	Ehime	0.12
MPR-0160	<i>Indigofera trifoliata</i> L.	Leguminosae	U	Okinawa	0.13
MPR-0176	<i>Senecio pseudoarnica</i> Less.	Compositae	R	Hokkaido	0.12
MPR-0186	<i>Nephrolepis cordifolia</i> (L.) C. Presl	Davalliaceae	W	Kochi	0.26
MPR-0198	<i>Plantago asiatica</i> L.	Plantaginaceae	U	Hokkaido	0.30
MPR-0204	<i>Pteris cretica</i> L.	Pteridaceae	U	Shizuoka	1.34
MPR-0206	<i>Leptochilus neopothifolius</i> Nakaike	Polypodiaceae	U	Kagoshima	0.72
MPR-0212	<i>Cimicifuga simplex</i> (DC.) Wormsk. ex Turcz.	Ranunculaceae	U	Yamanashi	0.95
MPR-0221	<i>Papaver dubium</i> L.	Papaveraceae	R	Ibaraki	2.17
MPR-0238	<i>Chrysanthemum pacificum</i> Nakai	Compositae	U	Shizuoka	1.07
MPR-0241	<i>Disporum smilacinum</i> A. Gray	Liliaceae	W	Nagano	0.94
MPR-0248	<i>Cryptotaenia canadensis</i> (L.) DC. ssp. <i>japonica</i> (Hassk.) Hand.-Mazz.	Umbelliferae	U	Ibaraki	3.37
MPR-0258	<i>Cyperus flavidus</i> Retz.	Cyperaceae	U	Kagoshima	4.28
MPR-0259	<i>Hymenophyllum barbatum</i> (Bosch) Baker	Hymenophyllaceae	W	Ehime	23.85
MPR-0260	<i>Lemmaphyllum microphyllum</i> C.Presl	Polypodiaceae	U	Chiba	5.96
MPR-0267	<i>Eschscholzia californica</i> Cham.	Papaveraceae	R	Ibaraki	6.21
MPR-0269	<i>Chrysosplenium grayanum</i> Maxim.	Saxifragaceae	W	Nagano	21.50
MPR-0282	<i>Hemisteptia lyrata</i> (Bunge) Fisch. et C. A. Mey.	Compositae	R	Ibaraki	5.96
MPR-0285	<i>Commelina communis</i> L.	Commelinaceae	U	Ibaraki	11.19
MPR-0288	<i>Cymbidium goeringii</i> (Rchb. f.) Rchb. f.	Orchidaceae	W	Nagano	15.74

a) R, root; U, underground part; W, whole plant.

ground structures (e.g. MPR-0259 *Hymenophyllum barbatum*, MPR-0260 *Lemmaphyllum microphyllum*) tended to show high activities. However, more than 150 samples (consisting of both field-collected samples and crude drugs) showed poor recovery or non-sigmoidal response curves (see Tables 1, 3). Some plant extracts (e.g. MPR-0022 and 0317 *Achyranthes bidentata* var. *japonica*, MPR-0221 and 0327 *Papaver dubium*) of the same species were measurable, while some were not. It is possible that changes in the components due to differences in the sampling site and sampling time were involved.

Interfering Factors for PEL Samples The samples that could not be measured were classified as follows: (1) samples with large meandering reaction curves, and (2) samples with similar reaction curves to the sigmoidal type with abnormal recovery. We, therefore, selected polyphenol study samples from each group. *Sanguisorba officinalis* and *L. salicaria* corresponded to (1), *O. biennis* corresponded to (2). In addition, *S. officinalis* was selected because strong inhibitory activities were observed in multiple samples, and *O. biennis* and *L. salicaria* were selected because Onagraceae and Lythraceae

are considered to contain a large amount of polyphenol compounds.^{16–19)}

Figure 3 shows the reaction curves of the LAL test when three PEL plant extracts (root samples of *S. officinalis* L., *O. biennis* L., and *L. salicaria* L.) were added. The normal kinetics response to endotoxin was depicted in a sigmoid form with lagging (Fig. 1). *Sanguisorba officinalis* and *L. salicaria* showed meandering curves (Figs. 3A1, C1). Although the reaction curve of *O. biennis* resembled a sigmoidal form, its recovery was 285%. Dilution of the samples did not remove these interfering reactions. Furthermore, the addition of polymyxin B did not affect the reaction curves (Figs. 3A1, 2, B1, 2, C1, 2). When the extracts treated with PVPP were added, sigmoidal reaction curves were observed (Figs. 3A3, B3, C3). Figure 4 shows the total amount of polyphenols in the three plant extracts. The polyphenol content of the three extracts was reduced by one-tenth after PVPP treatment.

Effects of Polyphenol Components on the LAL Test Table 4 shows the effects of polyphenolic compounds on the LAL test. Because tannin has many structural isomers,

Table 3. Examples of Plant Extract Samples for Which the *Limulus* Amoebocyte Lysate (LAL) Test Was Not Possible

Sample no.	English name or scientific name	Family	Plant part ^{a)}	Collection site	Category
	Rhubarb	Polygonaceae	Rh		Crude drug
	Coptis Rhizome	Ranunculaceae	Rh		
	Astragalus Root	Leguminosae	R		
	Glycyrrhiza	Leguminosae	R		
	Bupleurum Root	Umbelliferae	R		
	Japanese Gentian	Gentianaceae	R		
	Atractylodes Lancea Rhizoma	Compositae	Rh		
	Ginger	Zingiberaceae	Rh		
	Processed Ginger	Zingiberaceae	Rh		
MPR-0290	<i>Lycopodium clavatum</i> L. var. <i>nipponicum</i> Nakai	Lycopodiaceae	U	Hokkaido	Field collected (Ferns)
MPR-0293	<i>Asplenium scolopendrium</i> L. ssp. <i>japonicum</i> (Kom.) Rasbach, Reichst. et Viane	Aspleniaceae	U	Hokkaido	
MPR-0294	<i>Ctenitis subglandulosa</i> (Hance) Ching	Dryopteridaceae	U	Kagoshima	
MPR-0295	<i>Cyrtomium falcatum</i> (L.f.) C.Presl	Dryopteridaceae	U	Kagoshima	
MPR-0305	<i>Persicaria debilis</i> (Meisn.) H. Gross ex W. T. Lee	Polygonaceae	R and S	Nagano	Field collected (Angiosperms)
MPR-0308	<i>Rheum rhabarbatum</i> L.	Polygonaceae	U	Ibaraki	
MPR-0317	<i>Achyranthes bidentata</i> Blume var. <i>japonica</i> Miq.	Amaranthaceae	R	Ibaraki	
MPR-0326	<i>Triadenum japonicum</i> (Blume) Makino	Guttiferae	U	Hokkaido	
MPR-0327	<i>Papaver dubium</i> L.	Papaveraceae	R	Ibaraki	
MPR-0334	<i>Sedum sarmentosum</i> Bunge	Crassulaceae	W	Nagano	
MPR-0343	<i>Agrimonia pilosa</i> Ledeb. var. <i>japonica</i> (Miq.) Nakai	Rosaceae	U	Ibaraki	
MPR-0348	<i>Sanguisorba officinalis</i> L.	Rosaceae	U	Ibaraki	
MPR-0349	<i>Sanguisorba officinalis</i> L.	Rosaceae	U	Ibaraki	
MPR-0354	<i>Lespedeza cuneata</i> (Dum. Cours.) G. Don	Leguminosae	R	Yamanashi	
MPR-0359	<i>Euphorbia lathyris</i> L.	Euphorbiaceae	U	Ibaraki	
MPR-0363	<i>Lythrum salicaria</i> L.	Lythraceae	R	Hokkaido	
MPR-0366	<i>Oenothera biennis</i> L.	Onagraceae	R	Hokkaido	
MPR-0373	<i>Lysimachia clethroides</i> Duby	Primulaceae	U	Ibaraki	
MPR-0400	<i>Aster leiophyllus</i> Franch. et Sav. var. <i>leiophyllus</i>	Compositae	U	Yamanashi	
MPR-0422	<i>Iris ensata</i> Thunb. var. <i>spontanea</i> (Makino) Nakai ex Makino et Nemoto	Iridaceae	U	Hokkaido	
MPR-0423	<i>Iris pseudacorus</i> L.	Iridaceae	U	Ibaraki	

a) Rh, rhizome; R, root; U, underground part; S, stem; W, whole plant.

we first tested the low molecular weight polyphenolic compounds that contain components of tannins. Several flavonoid glycosides were also included in the test, since the solubility of compounds in water is considered to be an important point when studying the effects of enzymes. Deformation of the reaction curves and recovery rates outside of the acceptable range was observed with the addition of 10 of the 16 compounds. Reduced recovery rates were observed for four phenylpropanoids [*trans*-caffeic acid (**6**), ferulic acid (**7**), chlorogenic acid (**8**), and rosmarinic acid (**9**)] at 100 μ M. Chlorogenic acid (**8**) and rosmarinic acid (**9**) showed decreases in absorbance at 405 nm after the addition of LAL, and the reaction solution turned blue. Gallic acid (**1**), methyl gallate (**2**), and rutin (**13**) at 100 μ M also caused deformation of the reaction curves.

(+)-Catechin (**16**), flavanones [hesperetin (**10**) and naringenin (**11**)], vanillic acid (**3**), *trans*-*p*-coumaric acid (**5**), and puerarin (**15**) did not affect the recovery rates and the reaction curves at concentrations of 1–100 μ M. None of the compounds had an effect at the 1 μ M concentration.

DISCUSSION

In this study, we measured the endotoxin activity in PEL and clarified the degree of contamination. In addition, since

the normal measurement could not be performed in more than half of the samples, we examined the influence of polyphenol compounds, which can affect enzyme activity, on the LAL test.

The degree of endotoxin contamination differed between field-collected samples and crude drugs. Although endotoxin was detected in half of the measurable field-collected samples, endotoxin was detected in only five of the crude drugs (Table 1). In the case of the crude drugs, processing, such as peeling and washing, are performed prior to the time of distribution.²⁰⁾ However, because field-collected samples are only washed with water after collection, contamination by endotoxins can occur.

Inagawa *et al.*²¹⁾ detected endotoxin in all 62 crude drugs by extraction with hot water, and Hsu and Fu²²⁾ detected endotoxin in approx. 20% of 35 crude drugs by extraction with cold water. Compared with their results, the PEL samples in the present study showed a lower endotoxin activity. Differences in extraction solvents presumably account for the differences in the results obtained in the present and previous studies. When 0.5 ng/mL lipopolysaccharide (LPS) (O55: B55) was suspended in water and methanol and heated for 2 h, LPS was detected in the water, not in the methanol (data not shown). The concentration of endotoxin that affects cells *in vitro* varies greatly depending on the targeting cell type.

Fig. 3. Reaction Courses of Limulus Amoebocyte Lysate Tests with Plant Extracts

A, 400 µg/mL *Sanguisorba officinalis*; B, 400 µg/mL *Oenothera biennis*; and C, 400 µg/mL *Lythrum salicaria*. Plant extract conditions were as follows: 1, plant extract with no treatment; 2, plant extract and 0.01% polymyxin B; and 3, plant extract treated with PVPP.

Guo *et al.*²³⁾ reported that the viability of MC3T3-E1 cells decreased in the presence of endotoxins at concentrations greater than 10 ng/mL. Zhang *et al.*²⁴⁾ reported that endotoxin, at a concentration of 100000 ng/mL, caused platelet aggregation in the presence of thrombin. Since endotoxins have different activities depending on the type of bacteria, simple comparisons are difficult. When using the conversion 1 ng = 5 to 10 EU,²⁵⁾ the reported value of Guo *et al.*²³⁾ corresponds to 50 to 100 EU/mL, and that of Zhang *et al.*²⁴⁾ corresponds to 500000 to 1000000 EU/mL. In addition, the effects of endotoxins on other cells such as NK cells and fibroblasts²⁶⁻²⁹⁾ have been reported, however, the effects are limited to relatively high doses. These results suggested that most of the PEL samples would not affect experiments with cells. However, some sam-

Fig. 4. Concentration of Total Phenolic Contents of Plant Extracts (*Sanguisorba officinalis*, *Oenothera biennis*, and *Lythrum salicaria*) before and after PVPP Treatment

ples did show high endotoxin activity, therefore, information regarding the endotoxin activity in PEL samples is meaningful.

The PEL samples for which the LAL test could not be performed normally, were derived from specific families such as Polygonaceae and Primulaceae (Table 3). These results suggested that common components in these plants can be involved in the reaction system. These plants are rich in polyphenol components, such as tannins. Polyphenol compounds are known to have several phenolic hydroxyl groups and inhibit the activity of various enzymes.³⁰⁾ Therefore, further experiments on the polyphenols in the plant extracts were conducted.

The interfering factors on the LAL test were divided into those that affect endotoxin and those that affect the LAL reaction system. The former includes surfactants,³¹⁾ metal ions,³²⁾ and antibiotics,³³⁾ and the latter includes proteases and their inhibitors and surfactants.

Similar reaction curves were obtained for all three plants before the addition of polymyxin B, which inactivate endotoxin by binding to the lipid A portion of endotoxin^{34,35)} (Fig. 3A2, B2, C2). If a plant extract affects endotoxin, the addition of polymyxin B would affect the reaction curve and recovery rate. However, such results were not observed in this study.

As endotoxin was measured using the colorimetric method in this study, the changes in absorbance at 405 nm can affect the measured values. Although most plant extract samples were brown to green, the samples diluted 100-fold with water for injection had no problem of coloring. The reaction between endotoxin and LAL reagent is a cascade reaction involving several enzymes³⁾; thus, there is a delay before the increase in absorbance. The extracts of *S. officinalis*, *O. biennis*, and *L. salicaria* showed changes in absorbance at 405 nm immediately after the start of the reaction (Figs. 3A1, B1, C1). Therefore, these initial changes in absorbance are not considered to be due to endotoxin.

Moreover, all reaction curves were sigmoid, and there were no initial changes in absorbance when the extracts were treated with PVPP, a resin that specifically adsorbs polyphenol [see Figs. 3A3, B3, C3. The recovery rates of these reactions were also within the allowable range (data not shown)]. These results suggested that the polyphenol components in the three plant extracts affected the LAL reaction system. Additionally,

Table 4. Influence of Polyphenol Compounds and Concentrations on the Limulus Amoebocyte Lysate (LAL) Test

No.	Chemical name	Compound group	Recovery rate (%) ^{a)}			Effect on reaction curves ^{b)}		
			1 μ M	10 μ M	100 μ M	1 μ M	10 μ M	100 μ M
1	Gallic acid	Benzoic acid derivative	120	102	0	—	m	l,m
2	Methyl gallate		133	150	209	—	—	l,m
3	Vanillic acid		150	141	133	—	—	—
4	Ellagic acid		113	60	11	—	m ^{c)}	m,l ^{c)}
5	<i>trans-p</i> -Coumaric acid	Phenylpropanoid	117	129	139	—	—	—
6	<i>trans</i> -Caffeic acid		130	70	25	—	—	—
7	Ferulic acid		112	93	18	—	—	—
8	Chlorogenic acid		115	112	61	—	d,l	d,l
9	Rosmarinic acid		130	100	59	—	d,l	d,l
10	Hesperetin	Flavanone	128	146	87	—	—	—
11	Naringenin		119	55	152	—	— ^{c)}	— ^{c)}
12	Quercetin	Flavonol	98	—4662	0	—	d ^{c)}	d,l ^{c)}
13	Rutin		152	134	—127	—	—	d,l
14	Genistein	Isoflavone	112	112	67	—	—	m ^{c)}
15	Puerarin		136	102	112	—	—	—
16	(+)-Catechin	Flavanol	131	105	116	—	—	—

a) Spike activity was 0.05 EU/mL. b) Tested in the presence of 0.01% polymyxin B. Letters indicate: m, meandering or deformation; d, decrease in absorbance at 405 nm; l, disappearance of lag. c) Precipitation was observed.

as opposed to *S. officinalis* and *L. salicaria*, the *O. biennis* extract with the polyphenol removed showed a significant decrease in endotoxin activity. It is assumed that the polyphenol compounds contained in *O. biennis* had a LAL reaction-promoting action and thus resulted in higher-than-expected activity levels.

After revealing that polyphenol compounds affect the LAL test, we investigated the effects of single polyphenol compounds on the test. Compounds with caffeic acid derivative structures and pyrogallol structures were shown to affect the reaction of the LAL test (Table 4). Recovery rates outside of acceptable ranges and the deformation of the reaction curves were also observed for ellagic acid (4), quercetin (12), and genistein (14). However, these compounds have low water solubilities and, therefore, precipitation may affect the LAL reaction.

A very noteworthy result was the observation that caffeic acid derivatives had a prominent effect on the LAL test. Phenylpropanoids, such as caffeic acid, are important components of plants, e.g., in Compositae and Labiaceae. Caffeic acid derivatives are even the main polyphenol components of some plants.³⁶⁾ For *Aster leiophyllus* Franch. var. *leiophyllus* (see Table 3, MPR-0400), the color of the test solution turned blue during measurement, thus indicating the involvement of compounds such as chlorogenic acid and rosmarinic acid. Previous reports have revealed that *S. officinalis* contains gallic acid, catechin, and ellagic acid derivatives³⁷⁾; that *O. biennis* contains gallic acid³⁸⁾; and that *L. salicaria* contains caffeic acid derivatives, gallic acid, and chlorogenic acid.³⁹⁾

In this study, none of the compounds affected the LAL test at concentrations of 1 μ M. Polyphenolic compounds have many analogs in plant extracts and are considered to be synergistic in the LAL test. In addition, having a large number of hydroxyl groups per molecule and a strong interaction with proteins, tannin has a higher potential of affecting LAL.

Further investigation is needed to investigate the effects of multiple polyphenolic compounds present at different concentrations, and to investigate the effects of polymers containing more phenolic hydroxyl groups.

CONCLUSION

In this study, we measured the endotoxin activity in PEL samples and determined its degree of contamination. Endotoxin was detected in 139 field-collected samples and 5 crude drug samples. Although the degree of contamination varied, it is suggested that the impacts of PEL samples on experiments with cells would be small. These insights on the activity of endotoxin greatly contribute to the improvement of the quality of our PEL.

More than 150 PEL samples were observed to have problems in their reaction courses or recovery rates in the LAL tests. The LAL tests with the three plant extracts (*S. officinalis*, *O. biennis*, and *L. salicaria*), indicated that the polyphenol compounds affected LAL test and that the effects differed depending on the plant species.

When 16 single polyphenol compounds were added to the LAL tests, the compounds with caffeoyl and pyrogallol moieties were found to affect the LAL reaction courses and recovery rates. In addition, none of the compounds had any effect at concentrations of 1 μ M. Since there are various analogs of polyphenol compounds in plant extracts, it is assumed that they actually act synergistically. It is important to consider the recovery rates and reaction courses of LAL tests with samples containing polyphenol compounds.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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