

1 **Lysophosphatidic acid in medicinal herbs enhances prostaglandin E<sub>2</sub>**  
2 **and protects against indomethacin-induced gastric cell damage *in***  
3 ***vivo* and *in vitro***

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26

27 **Abstract**

28 Lysophosphatidic acid (LPA) is a bioactive phospholipid that induces diverse biological  
29 responses. Recently, we found that LPA ameliorates NSAIDs-induced gastric ulcer in mice. Here,  
30 we quantified LPA in 21 medicinal herbs used for treatment of gastrointestinal (GI) disorders.  
31 We found that half of them contained LPA at relatively high levels (40–240 µg/g) compared to  
32 soybean seed powder (4.6 µg/g), which we previously identified as an LPA-rich food. The LPA  
33 in peony (*Paeonia lactiflora*) root powder is highly concentrated in the lipid fraction that  
34 ameliorates indomethacin-induced gastric ulcer in mice. Synthetic 18:1 LPA, peony root LPA  
35 and peony root lipid enhanced prostaglandin E<sub>2</sub> production in a gastric cancer cell line, MKN74  
36 cells that express LPA<sub>2</sub> abundantly. These materials also prevented indomethacin-induced cell  
37 death and stimulated the proliferation of MKN74 cells. We found that LPA was present in  
38 stomach fluids at 2.4 µM, which is an effective LPA concentration for inducing a cellular  
39 response *in vitro*. These results indicated that LPA is one of the active components of medicinal  
40 herbs for the treatment of GI disorder and that orally administered LPA-rich herbs may augment  
41 the protective actions of endogenous LPA on gastric mucosa.

42 **Keywords:** Lysophosphatidic acid; Medicinal herbs; Indomethacin; Prostaglandin E<sub>2</sub>; Cell  
43 death; Cell proliferation

44 **Abbreviations:** GI, gastrointestinal; LPA, lysophosphatidic acid; PA, phosphatidic acid; TLC,  
45 thin-layer chromatography; PL, phospholipid; MALDI-TOF MS, matrix-assisted laser desorption  
46 ionization time-of-flight mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass

47 spectrometry; CMC, carboxymethylcellulose; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>;  
48 NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase.

## 49 **1. Introduction**

50 Lysophosphatidic acid (LPA) is a bioactive phospholipid that induces diverse cellular  
51 responses including proliferation, protection of cells from apoptosis, and migration of cells [1].  
52 These cellular responses are mediated through six LPA-specific G-protein coupled receptors,  
53 LPA<sub>1-6</sub> [2]. Recent studies have revealed important actions of LPA in the mammalian  
54 gastrointestinal (GI) tract [3, 4]. These include inhibition of diarrhea, regulation of intestinal  
55 electrolyte transport, protection of intestinal cells from apoptosis, and wound healing [5-9].

56 Peptic ulcer is a major GI disorder that occurs due to an imbalance in mucosal offensive  
57 (gastric acid secretion) and defensive (gastric mucosal integrity) factors [10, 11]. Infection with  
58 *Helicobacter pylori*, smoking, drinking alcohol, and chronic ingestion of drugs are major causes  
59 of peptic ulcer. Recently, the number of patients with non-steroidal anti-inflammatory drug  
60 (NSAID)-induced gastric ulcer is increasing due to increased use of NSAIDs for pain treatment  
61 and prevention of thrombosis [12]. NSAIDs cause gastric ulcer by inhibition of cyclooxygenase  
62 (COX)-1 and COX-2, which produce a predominant mucosal defense factor, prostaglandin E<sub>2</sub>  
63 (PGE<sub>2</sub>) [13]. Our recent study showed that orally administered synthetic LPA ameliorates  
64 aspirin- and indomethacin-induced gastric ulcer in mice [14, 15]. We also showed that LPA up-  
65 regulates COX-2 and enhances production of PGE<sub>2</sub> via activation of LPA<sub>2</sub> receptors, which are  
66 located on the apical side of gastric mucosal cells [16]. However, further uncharacterized  
67 mechanisms other than COX-2 induction are considered to function in the protective action of  
68 LPA, because LPA protects the gastric mucosa from the acute toxicity of NSAIDs.

69 Medicinal herbs have been traditionally used for the treatment of many diseases, including  
70 gastric ulcer [17]. Considering that some medicinal herbs and vegetables contain LPA  
71 abundantly [18, 19], it is rational to postulate the existence of anti-ulcer medicinal herbs that  
72 contains LPA as an active component. To examine this possibility, we determined the LPA  
73 content of 21 herbs that are traditionally used for the treatment of GI disorders. We also aimed to  
74 examine the effects of LPA and herbal lipids on NSAID-induced gastric ulcer. The results  
75 showed that peony root lipid, which contain highly concentrated LPA, had an ameliorative effect  
76 on NSAID-induced gastric ulcer and enhanced PGE<sub>2</sub> production in gastric cells. We also showed  
77 evidence that LPA/LPA<sub>2</sub> signaling protects against acute cytotoxicity of NSAIDs in cultured  
78 gastric cells.

## 79 **2. Materials and Methods**

### 80 **2.1 Materials**

81 Herbs used for the treatment of various digestive disorders were selected based on the  
82 descriptions in the oldest Chinese traditional herbal medicine book, the Shennong Ben Cao Jing.  
83 Coptis rhizome (*Coptis japonica*), moutan cortex (*Paeonia suffruticosa*), atractylodes rhizome  
84 (*Atractylodes japonica*), atractylodes lancea rhizome (*Atractylodes lancea*), amomum seed  
85 (*Amomum xanthioides*), peony root (*Paeonia lactiflora*), poria sclerotium (*Poria cocos*), and  
86 phellodendron bark (*Phellodendron amurense*) were obtained from Yoshimi Seiyaku Co. Ltd.  
87 (Osaka, Japan). Licorice root (*Glycyrrhiza glabra*), platycodon root (*Platycodon grandiflorum*),  
88 bupleurum root (*Bupleurum falcatum*), zedoary rhizome (*Curcuma zedoaria*), fennel fruit  
89 (*Foeniculum vulgare*), dried ginger rhizome (*Zingiber officinale*), and stripped, steamed, and  
90 dried ginger rhizome (*Zingiber officinale*) were purchased from Nakaya Hikojuro Co. Ltd.

91 (Ishikawa, Japan). Sophora root (*Sophora flavescens*) and schisandra fruit (*Schisandra chinensis*)  
92 were obtained from Kojima Kampo Co. Ltd. (Osaka, Japan). Pinellia tuber (*Pinellia ternata*),  
93 cimicifuga rhizome (*Cimicifuga simplex*), panax rhizome (*Panax japonicus*), and corydalis tuber  
94 (*Corydalis turtschaninovii*) were purchased from a local drug store.

## 95 **2.2 Reagents**

96 One-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (18:1 LPA), 1-heptadecanoyl-2-hydroxy-*sn*-  
97 glycero-3-phosphate (17:0 LPA), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (16:0  
98 LPC), and arachidonic acid were purchased from Avanti Polar Lipids (Alabaster, AL, USA).  
99 Peony root LPA was freshly prepared from peony root powder as described below. Pertussis  
100 toxin (PTX) and calcium ionophore A23187 were purchased from Sigma-Aldrich (St. Louis, MO,  
101 USA). Phos-tag was obtained from Wako Pure Chemical Industries (Osaka, Japan).  
102 Carboxymethylcellulose (CMC), aspirin, and indomethacin were obtained from Kanto Chemical  
103 Co. (Tokyo, Japan), Wako Pure Chemical Industries (Osaka, Japan), and Nacalai Tesque Inc.  
104 (Kyoto, Japan), respectively. A prostaglandin E<sub>2</sub> EIA kit was obtained from Cayman Chemical  
105 Co. (Ann Arbor, MI). A lactate dehydrogenase (LDH) assay kit was obtained from Dojindo  
106 Laboratories Co. Ltd. (Kumamoto, Japan). A bromo-2-deoxy-uridine (BrdU) cell proliferation  
107 ELISA kit was purchased from Roche (Mannheim, Germany).

## 108 **2.3 Animals**

109 Five-week-old male ICR mice (35g body weight) were obtained from Charles River  
110 Laboratories Japan, Inc. (Kanagawa, Japan). The animals were adapted to an animal room  
111 maintained at 24 ± 2 °C and housed in a 12 h light/dark cycle. The care and handling of mice

112 were in accordance with the National Institute of Health guidelines. All experimental procedures  
113 were approved by the Tokushima University Animal Care and Use Committee.

#### 114 **2.4 Extraction of lipid and isolation of LPA from herbs**

115 Lipids were extracted from the medicinal herbs by the Bligh and Dyer method [20] with  
116 acidification of the water/methanol phase, as described previously [19]. In brief, 1 g of herb  
117 powder was mixed with 15.2 ml of a solvent consisting of chloroform/methanol/water in the  
118 ratio of 1:2:0.8 (v/v/v) and centrifuged to collect the supernatant. The pellet was added to the  
119 same amount of the mixed solvent consisting of chloroform/methanol/water and centrifuged. The  
120 combined supernatant fraction was mixed with an appropriate volume of chloroform and water  
121 to make solvent system consisting of chloroform/methanol/water in the ratio of 1:1:0.9 (v/v/v).  
122 The resulting two-layer solution was mixed with 0.15 ml of 5 N HCl and centrifuged. Lipids  
123 were obtained from the lower phase (chloroform phase). The LPA in the lipid extract was  
124 isolated by TLC. The solvent system of the chromatography was chloroform/methanol/28%  
125 aqueous ammonia (60:35:8, v/v/v). After development, the plate was dried for a few minutes  
126 with blowing air and sprayed with primulin for visualization under UV light. LPA was identified,  
127 extracted from the silica gel by the Bligh and Dyer method [20], and quantified by the  
128 colorimetric method based on phospho-molybdenum-malachite green formation [21]. The weight  
129 of phospholipids ( $\mu\text{g/g}$ ) was determined from a weight of lipid phosphorus ( $\mu\text{g}$  inorganic  
130 phosphorus/g) in a way recommended by American Oil Chemists' Society [22].

#### 131 **2.5 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-** 132 **TOF MS)**

133 Molecular species of LPA in herbs were determined by MALDI-TOF MS as described  
134 previously [15]. An aliquot of LPA was dissolved in 100  $\mu$ l of methanol containing 0.1%  
135 aqueous ammonia. This solution (10  $\mu$ l) was mixed with 5  $\mu$ l 0.1 mM  $^{68}\text{Zn}$  Phos-tag solution. A  
136 small portion (0.5  $\mu$ l) of this mixture was spotted on a sample plate. Immediately, 0.5  $\mu$ l of 2, 4,  
137 6-trihydroxyacetophenone (THAP) solution (10 mg/ml in acetonitrile) was layered onto the  
138 mixture as a matrix solution. The sample plate was dried for a few minutes, and the  
139 matrix/analyte co-crystal that formed was subjected to MALDI-TOF MS. MALDI-TOF mass  
140 spectra were acquired using a Bruker Microflex mass spectrometer (Bruker Daltonics, Bremen,  
141 Germany) in positive ion detection mode. The wavelength of the nitrogen-emitting laser and the  
142 accelerating voltage were 337 nm and 20 kV, respectively. To enhance the reproducibility, 300  
143 laser shots were averaged for each mass spectrum.

## 144 **2.6 NSAIDs-induced gastric ulcer**

145 Mouse models of aspirin- and indomethacin-induced gastric ulcer were developed as described  
146 previously [14, 15]. In brief, aspirin (300 mg/kg body weight), indomethacin (22.9 mg/kg body  
147 weight), or peony root lipid was suspended in 3% (w/v) CMC and sonicated for 1 min. Peony  
148 root powder was suspended in water. Fasted mice were intragastrically administered peony root  
149 lipid or powder suspension in a volume of 0.2 ml. After 0.5 h, 0.2 ml of the aspirin or  
150 indomethacin suspension was administered intragastrically. The mice were anesthetized with  
151 diethyl ether and sacrificed 3 or 5 h after the aspirin or indomethacin administration, respectively.  
152 The isolated stomach was ligated at both ends, filled with 1.5 ml of 2% formalin, and immersed  
153 in 2% formalin for 15 min. Then, the stomach was cut along the greater curvature, and the  
154 lengths of lesions on the stomach wall were measured using a millimeter scale with a magnifying  
155 glass. The total length of lesions was used as a lesion index.

## 156 **2.7 Cell culture and reverse transcription-PCR (RT-PCR)**

157 MKN74 cells, a human gastric cancer cell line, were obtained from the RIKEN Cell Bank  
158 (Tsukuba, Japan). The MKN74 cells were grown in RPMI-1640 medium containing 10% fetal  
159 bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin in a humidified  
160 atmosphere of 5% CO<sub>2</sub> and 95% air at 37 °C. Total RNA from MKN74 cells was prepared by  
161 using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's  
162 instructions. The first-strand complementary DNA (cDNA) was synthesized from the purified  
163 total cellular RNA with random hexamer primers using the SuperScript™ III synthesis system  
164 for RT-PCR kit (Invitrogen, Carlsbad, CA). The cDNA was then subjected to PCR amplification  
165 with primer sets and condition as described in supplementary Table 1. The PCR products were  
166 examined by electrophoresis on 2% agarose gel, stained with Gelred (Biotium, Hayward, CA)  
167 and visualized with UV light. Images of the fluorescent band on the gel were captured by a Fuji  
168 LAS-4000 imaging system (FujiFilm, Tokyo, Japan), and the digitized image data were analyzed  
169 by NIH image.

## 170 **2.8 PGE<sub>2</sub> production assay**

171 PGE<sub>2</sub> production in MKN74 cells was performed as described previously [16]. In brief,  
172 MKN74 cells were seeded in 35-mm dishes and added 10 µM arachidonic acid (AA) for AA-  
173 enrichment in the cells. After 24 h, the medium was changed to serum-free medium, and further  
174 incubated for 24 h. Then, the cells were treated with 10 µM acetylsalicylic acid for 30 min to  
175 minimize the effect of the preexisting COX activity. After replacement of the medium, cells were  
176 incubated with 18:1 LPA, peony root lipid extract or purified peony root LPA in the presence or  
177 absence of pertussis toxin (PTX). After 5 h, the cells were stimulated with 1 µM A23187 for 10

178 min. The supernatant were collected and PGE<sub>2</sub> was evaluated by EIA kit according to the  
179 manufacturer's instructions.

## 180 **2.9 Indomethacin-induced cell damage of MKN74 cells**

181 MKN74 cells were seeded at a density of  $1 \times 10^5$  in 35-mm polystyrene dishes and serum starved  
182 for 24 h. LPA dissolved in 0.3% BSA in PBS were added 2 h before addition of indomethacin  
183 solution. The final concentration of indomethacin was fixed at 0.8 mM. After 30 h, the cells were  
184 gently washed with PBS and stained with propidium iodide (PI) for 30 min. The extent of cell  
185 death was determined by observation with a fluorescent microscope Axiovert 200 M (Zeiss,  
186 Oberkochen, Germany). For the LDH assay, MKN74 cells were treated in the same manner as  
187 described above. At the end of incubation, 100  $\mu$ l of the culture medium supernatant was added  
188 per well of a 96-well microplate. The LDH activity was measured according to the instructions  
189 of the LDH assay kit. The activity was expressed as % of maximum release of LDH that can be  
190 obtained in a sample of the lysis buffer-treated cells. The extent of cell death was also  
191 determined by flow cytometric analysis. The PI-stained cells were subjected to a flow cytometer  
192 (Becton Dickinson) that was operated using Cell Quest software, and at least 10,000 cells were  
193 analyzed for each sample.

## 194 **2.10 Cell proliferation assay**

195 MKN74 cells seeded in 35-mm polystyrene dishes were serum-starved for 24 h. Then they  
196 were cultured with or without synthetic or peony root-derived LPA in the presence or absence of  
197 PTX. After 24 h, cells were harvested by trypsinization, mixed with trypan blue dye, and the  
198 number of living cells was counted using a hemocytometer. The proliferation of MKN74 cells  
199 was also confirmed by a BrdU cell proliferation assay. In brief,  $2 \times 10^3$  cells were seeded in 96-

200 well microplates in 100  $\mu$ l/well culture medium and kept in serum-starved condition for 48 h.  
201 The synthetic or peony root-derived LPA was added to the cells and further incubated for 24 h.  
202 BrdU was added to the cell culture 4 h before termination of incubation. The incorporated BrdU  
203 was determined as described by the manufacturer's protocol.

## 204 **2.11 Determination of LPA in a mouse stomach fluid**

205 Stomachs of overnight-fasted mice were isolated and gently washed with PBS. The stomach  
206 was cut along the greater curvature. The stomach inner surface was carefully washed with a  
207 small amount of PBS. After addition of 17:0 LPA (0.5 nmol) as an internal standard, lipids were  
208 extracted from the stomach washing solution by using an acidified Bligh and Dyer method as  
209 described above. Extracted lipids were dissolved in 0.8 ml of methanol and filtered through 0.2  
210  $\mu$ m nylon filter. After filtration, methanol was evaporated and reconstituted in 0.1 ml of  
211 methanol/water mixture (95:5, v/v) containing 5 mM ammonium formate for LC/MS/MS.  
212 LC/MS/MS was performed as described previously [23] using a quadrupole-linear iontrap hybrid  
213 mass spectrometry system, 4000 Q TRAP™ (Applied Biosystems/MDS Sciex, Concord, Ontario,  
214 Canada) with an Agilent 1100 liquid chromatography system (Agilent Technologies,  
215 Wilmington, DE, USA). In the negative ion mode of operation with multiple reactions  
216 monitoring, Q1 was set to the deprotonated molecular ion of each class of LPA as the precursor  
217 ion. The fragment ions, [deprotonated cyclic glycerophosphate]<sup>-</sup> at m/z 153 were selected for Q3.  
218 The ratios of the negative ion peak areas of the endogenous LPA to that of the corresponding  
219 internal standard were calculated.

## 220 **2.12 Statistical analysis**

221 Statistical analyses of the difference between two means were performed by Student's *t*-test.

222

### 223 **3. Results**

#### 224 **3.1 Abundance of LPA in medicinal herbs**

225 Our previous study [19] revealed that LPA is abundant in cruciferous plants, such as cabbage  
226 leaves and radish roots (9.2 and 2.3  $\mu\text{g/g}$  wet weight, respectively). Soybean seed powder was  
227 also found to be rich in LPA (4.6  $\mu\text{g/g}$ ). In this study, we determined LPA content in 21 dried  
228 medicinal herbs used for the treatment of GI disorders (Fig. 1A). Compared to LPA-rich foods,  
229 about half of the medicinal herbs contained LPA at high levels (40–240  $\mu\text{g/g}$ ). Among them,  
230 peony root powder (240  $\mu\text{g/g}$ ) contained the highest level of LPA. It was 52 times that of  
231 soybean seed powder. We previously revealed that phosphatidic acid (PA), a diacyl derivative of  
232 LPA, serves as a source of LPA in the digestive tract [14]. The amounts of PA in these medicinal  
233 herbs were comparable (Fig. 1B) to those in cabbage and soybean, which were characterized as  
234 PA-rich foods previously [24]. Surprisingly, the percentage of LPA in total phospholipids (PLs)  
235 in peony root was 11%, which is 30 and 400 times of those in cabbage leaves and soybean seed  
236 powder, respectively (Table 1). These results indicated that peony root powder contains abundant  
237 LPA with a high concentration in its lipid fraction. This is evident from the relative intensity of  
238 TLC bands of its lipid extract (Supplementary Fig. 1).

239 MALDI-TOF MS of PA and LPA in medicinal herbs showed that the predominant PA species  
240 were 16:0/18:2 and 18:2/18:2 (or 18:1/18:3) PA (Supplementary Table 2) and the predominant  
241 LPA species were 16:0, 18:2 LPA (Supplementary Table 3). This is also the case in peony root  
242 as shown in Fig. 2A, B.

#### 243 **3.2 Anti-ulcer effect of peony root lipid and powder**

244 As shown previously, orally administered aspirin (300 mg/kg body weight) produces 15–20  
245 mucosal lesions in the gastric corpus of mice [14]. The lesions were linear and extended from the  
246 fundic area to the pyloric area as erosion. Similar morphological lesions were observed in the  
247 experiments with indomethacin (22.9 mg/kg body weight) (Fig. 3A). We used the total length of  
248 lesions as the lesion index.

249 Orally administered peony root lipid reduced indomethacin-induced lesion formation in a dose-  
250 dependent fashion (Fig. 3A, B). The maximum reduction was observed when mice were  
251 administered 1 mM of peony root lipid (Fig. 3A, B), which corresponds 4.4 mg PLs/kg (animal  
252 body weight). We confirmed that synthetic LPA at 1 mM has protective effect against  
253 indomethacin-induced lesion formation (Fig. 3B). We also examined the gastro protective effect  
254 of peony root powder on an aspirin-induced acute gastric ulcer mouse model. The peony root  
255 powder at 2 g/kg body weight effectively reduced gastric mucosal lesion formation (Fig. 3C).  
256 This dose of the powder corresponds to the administration of 1 mM peony root lipid.

### 257 **3.3 Enhancement of PGE<sub>2</sub> production by LPA-rich herbal lipids in gastric cells**

258 According to the Human Protein Atlas database (<http://www.proteinatlas.org/>), LPA<sub>2</sub>, LPA<sub>5</sub>,  
259 and LPA<sub>6</sub> are expressed abundantly in human stomach epithelia. On the other hand, expression  
260 levels of LPA<sub>1</sub>, LPA<sub>3</sub>, and LPA<sub>4</sub> are very low. Firstly, we examined LPAR<sub>1-6</sub> mRNA expression  
261 in human gastric cancer cell line, MKN74 cell. Results showed that order of abundance of LPAR  
262 mRNA was LPA<sub>2</sub>=LPA<sub>5</sub>>LPA<sub>6</sub>. Levels of mRNA of LPA<sub>1</sub>, LPA<sub>3</sub>, and LPA<sub>4</sub> were under  
263 detectable (Fig. 4A). The relative abundance of mRNA of LPARs in MKN74 cells is good  
264 agreement with the expression profile of LPARs in human stomach tissue. We used this cell line  
265 as a representative mucosal cell model of the human stomach. We confirmed that synthetic 18:1

266 LPA can enhance PGE<sub>2</sub> production in MKN74 cells (Fig. 4B). This is consistent with our  
267 previous observation that LPA enhances PGE<sub>2</sub> production via up-regulation of COX-2 [16].  
268 This effect was also observed with LPA prepared from peony root. Peony root lipid, which has  
269 concentrated LPA, enhanced PGE<sub>2</sub> production at high efficacy. Enhancements of PGE<sub>2</sub>  
270 production induced by LPA and peony root lipid were completely abolished by PTX (Fig. 4C),  
271 suggesting the involvement of Gi-coupled receptor in their actions. **It should be mentioned that**  
272 **high concentration of the peony root lipid (10 μM) did not induce PGE<sub>2</sub> production (Fig. 4B).**  
273 **This is due to cytotoxicity of the lipid extract which contain various components other than LPA.**  
274 **In fact, we observed that most of the cells were floating at the end of the incubation with peony**  
275 **root lipid at 10 μM.**

### 276 **3.4 LPA protects indomethacin-induced cell injury of MKN74 cells**

277 Effect of LPA on indomethacin-induced cell injury was examined in MKN74 cells. As shown  
278 in Fig 5A, non-treated MKN74 cells were tightly attached each other. We found that treatment of  
279 the cells with 0.8 mM indomethacin results in loss of attachment and rounding of their cell shape  
280 (Fig. 5A), a typical morphological change in apoptotic cells [25, 26]. When the cells were treated  
281 with 0.8 mM indomethacin plus 10 μM LPA, the indomethacin-induced morphological change  
282 was not observed. In fact, LPA caused cell spreading with an extended edge, indicating the  
283 disappearance of apoptotic symptoms (Fig. 5A). The protective action of LPA on indomethacin-  
284 induced cellular damage was quantified by directly counting PI-positive cells (dead cells). We  
285 found that LPA reduced the number of PI-positive cells in a dose-dependent fashion (Fig. 5B).  
286 LPA from peony root also significantly reduced cell death at 10 μM (Fig. 5B). The protective  
287 action of LPA against indomethacin-induced cellular damage was also confirmed by LDH  
288 leakage (Fig. 5C). To determine the involvement of G-protein-coupled receptors in these

289 observations, the effect of pretreatment of PTX, a specific inhibitor of Gi-protein, was examined.  
290 Flow cytometric analysis was applied for this experiment to quantify the population of dead cells  
291 in the total cells. Results showed that the effect of LPA against indomethacin-induced cell death  
292 was completely abolished by pre-treatment of PTX, indicating the involvement of Gi-coupled  
293 receptors in the LPA action (Fig. 5D). This is also confirmed by the morphological change of the  
294 cells (data not shown). We found that LPC did not show a protective effect on indomethacin-  
295 induced cell death (Fig. 5D).

### 296 **3.5 LPA stimulated proliferation of MKN74 cells**

297 Treatment of MKN74 cells with LPA induced proliferation in a dose-dependent manner (Fig.  
298 6A). LPA-induced cell proliferation was also confirmed by measuring incorporation of the  
299 thymidine analog BrdU into the cells (Fig. 6B). Peony root LPA also stimulated the proliferation  
300 of MKN74 cells (Fig. 6A, B). LPA-induced cell proliferation was partially and significantly  
301 inhibited by PTX (Fig. 6C). Again, LPC had no proliferative effect.

### 302 **3.6 LPA concentration in a mouse stomach fluid**

303 In order to better understand the role of LPA in the physiology of the stomach, it is necessary  
304 to know the concentration and molecular species of LPA in stomach fluid. The volume of  
305 stomach fluid was assumed to be 0.08 ml. This is based on the fact that the area of the mucus  
306 layer is 400 mm<sup>2</sup> and the thickness of mucus gel layer is 0.2 mm [27]. We found that the total  
307 LPA concentration in the stomach fluid was 2.4 μM (Fig. 7). The major species of LPA in the  
308 stomach fluid were found to be 16:0, 18:0, 18:1, and 18:2 LPAs (Fig. 7). The concentration of  
309 these LPA species was found to be 0.3–0.7 μM. Other LPA species, such as 16:1, 18:3, 18:4,

310 20:0, 20:1, 20:2, 20:4, 22:0, 22:4, and 22:6, were present in low (0.01–0.1  $\mu$ M) level  
311 (Supplementary Fig. 2).

#### 312 **4. Discussion**

313 Research in medicinal herbs has identified many active components that exert anti-ulcer effects  
314 with diverse mechanisms of action. These include compounds belonging to flavonoids, alkaloids,  
315 tannins, and saponins [28, 29]. **Dietary phospholipids, such as soy-derived PC, are also shown as**  
316 **reducing agents for NSAIDs-induced gastric ulcer [30].** In this study, we found that LPA in  
317 medicinal herbs is a potential component for prevention of gastric mucosal injury. This notion is  
318 based on several observations. Firstly, synthetic LPA and herbal LPA showed ameliorative  
319 activity against cytotoxic effect of indomethacin. Secondly, LPAs and LPA-rich lipid enhanced  
320 PGE<sub>2</sub> production, an important cytoprotective factor in GI mucosa. Thirdly, peony root lipid, an  
321 LPA-rich herbal lipid identified here, significantly ameliorated indomethacin-induced gastric  
322 lesions in mice. Lastly, there were many LPA-rich herbs in Chinese traditional medicines used  
323 for the treatment of GI disorders. We discuss on mechanisms of these effects in detail.

324 A well-known mechanism of NSAID-induced gastric mucosal lesions is the inhibition of  
325 COX-1 and COX-2 enzymes and a resulting decrease in gastroprotective PGE<sub>2</sub>. The importance  
326 of PGE<sub>2</sub> in the integrity of stomach mucosa is evident from the fact that common anti-ulcer drugs,  
327 such as rebamipide and geranylgeranylacetone, up-regulate COX-2, leading to the enhancement  
328 of PGE<sub>2</sub> production [31, 32]. Consistent with our previous study [16], LPA from medicinal herb  
329 was found to enhance PGE<sub>2</sub> production in human gastric cancer cell line, MKN74 cells. We also  
330 showed that peony root lipid, which has concentrated LPA, enhanced PGE<sub>2</sub> production with Gi-  
331 mediated manner. Surprisingly, the efficacy of PGE<sub>2</sub> production of peony root lipid is higher

332 than that expected from the LPA content in the lipid extract. At present, we do not know other  
333 components in peony root lipid that enhance PGE<sub>2</sub> production along with LPA. Considering that  
334 PGE<sub>2</sub>-enhancement was completely abolished by PTX, there may be components that increase  
335 LPA action in the lipid. Further study is needed for clarification of this point.

336 NSAIDs have been reported to induce apoptosis in gastric mucous cells [25, 26]. The  
337 mechanism of the cytotoxicity is uncoupling of mitochondrial oxidative phosphorylation and  
338 inhibition of the electron transport chain, leading to depletion of intracellular ATP, cellular Ca<sup>2+</sup>  
339 toxicity, and generation of reactive oxygen species [13]. It is also reported that NSAIDs  
340 chemically interact with cell membrane phospholipids, disrupt membrane permeability, and form  
341 membrane pores [13]. In this study, we found that pretreatment of MKN74 cells with LPA  
342 prevents indomethacin-induced cell shape change, LDH leakage and cell death. The protective  
343 effect of LPAs against indomethacin-induced cell death was completely abolished by PTX,  
344 indicating the involvement of Gi-coupled receptor.

345 It has been reported that LPA stimulates proliferation of diverse types of cells, including  
346 gastric cancer cells [33]. In this study, we found that LPA stimulated proliferation of MKN74  
347 cells. The proliferative effect of LPA was partially but significantly abolished by PTX. The  
348 partial inhibition of PTX in LPA-induced proliferation of MKN74 indicates the involvement of  
349 Gi as well as G12/13 and Gq/11/14 in the LPA response. Our result is consistent with previous  
350 reports showing that LPA stimulates proliferation of NIH3T3 in both PTX- sensitive and -  
351 insensitive manners [34].

352 We observed **membrane budding-like structure** in LPA-treated MKN74 cells (Supplementary  
353 Fig. 3). They form almost vesicles, and their size is considerably smaller than those of LPA-

354 induced membrane blebs reported by Valentine et al. [35]. At present, we do not know the  
355 biological significance of this phenomenon induced by LPA. Miyake et al. reported that MKN28  
356 cells secrete mucin when the plasma membrane of the cells is injured in the presence of calcium  
357 [36]. They also found microvilli on the plasma membrane of MKN28 cells. If the phenomenon  
358 observed here is one of the steps of mucin secretion from gastric cells, the physiological function  
359 of the LPA-induced vesicle secretion is to strengthen the mucus gel layer, a protective barrier of  
360 the stomach wall.

361 We found that LPA<sub>2</sub> and LPA<sub>5</sub> are predominant LPAR in MKN74 cells. This is good  
362 agreement with the expression profile of LPARs in human stomach tissue  
363 [<http://www.proteinatlas.org/>]. Here, we showed that LPA-induced PGE<sub>2</sub> production, anti-  
364 apoptosis, and proliferation in MKN74 cells were all Gi-mediated responses. LPA<sub>2</sub> has been  
365 known to couple with Gi in many cells [8, 37, 38]. On the other hand, LPA<sub>5</sub> seems to be coupled  
366 with Gq rather than Gi in many cells [39, 40]. Considering these facts, it is reasonable to assume  
367 that LPA<sub>2</sub> is involved in these observations and that LPA<sub>2</sub> expressed on the apical membrane of  
368 gastric mucous cells [16] plays important role in gastric mucosal integrity.

369 In this study, for the first time, we determined the LPA concentration in stomach fluid. We  
370 found that LPA concentration in stomach fluid was 2.4 μM. The LPA concentration in stomach  
371 fluid is three times higher than that in saliva (0.9 μM) [41] and similar or relatively higher level  
372 to that in human plasma or serum LPA (0.1–2.4 μM) [42-44]. The presence of LPA in stomach  
373 fluid is reasonable because LPA receptors are expressed in the apical side of gastric mucosal  
374 cells [14, 16]. We found that the level of LPA in the stomach fluid is in a range that induces a  
375 diverse response in gastric cells *in vitro* (Fig. 4, 5, 6). We also found that the abundant LPA  
376 species in the stomach fluid were 16:0, 18:0, 18:1, and 18:2 LPA. These LPA species are potent

377 agonists for LPA receptors [45] and showed potent gastro protective effects *in vivo* and *in vitro*  
378 (Fig. 3, 5 ). These results suggested that endogenous LPAs have a possibility to play vital roles in  
379 gastric epithelial cells, and that ingested LPAs contribute to mucosal integrity by augmentation  
380 of LPA in stomach fluid.

## 381 **5. Conclusion**

382 The present study revealed the abundant existence of LPA in medicinal herbs that are used for  
383 treatment of GI disorders. An LPA-rich herb, peony root had a significant gastro protective effect  
384 on NSAID-induced gastric ulcer. We also revealed that in addition of PGE<sub>2</sub> enhancement, LPA  
385 protects against NSAID-induced acute cell toxicity and stimulates the proliferation of gastric  
386 cells. LPA<sub>2</sub> in gastric mucosal cells are considered to be involved in these LPA actions.

## 387 **Conflicts of interest**

388 No conflicts of interest

## 389 **Author contributions**

390 S. A. is primary author of manuscript, conducted most of experiments and data analysis. S. W.,  
391 A. T., K. K. and T. T. designed this study. A. Y. contributed to analysis of medicinal herbs. K. F.,  
392 M. M. R., T. F., T. S. and T. I. contributed to cultured cell experiments. K. M. conducted mass  
393 spectrometric analysis of LPA in stomach fluid and receptor expression analysis. E. K. and K. T.  
394 conducted microscopic observation including electron microscope. All authors of the manuscript  
395 have approved this manuscript.

396

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407

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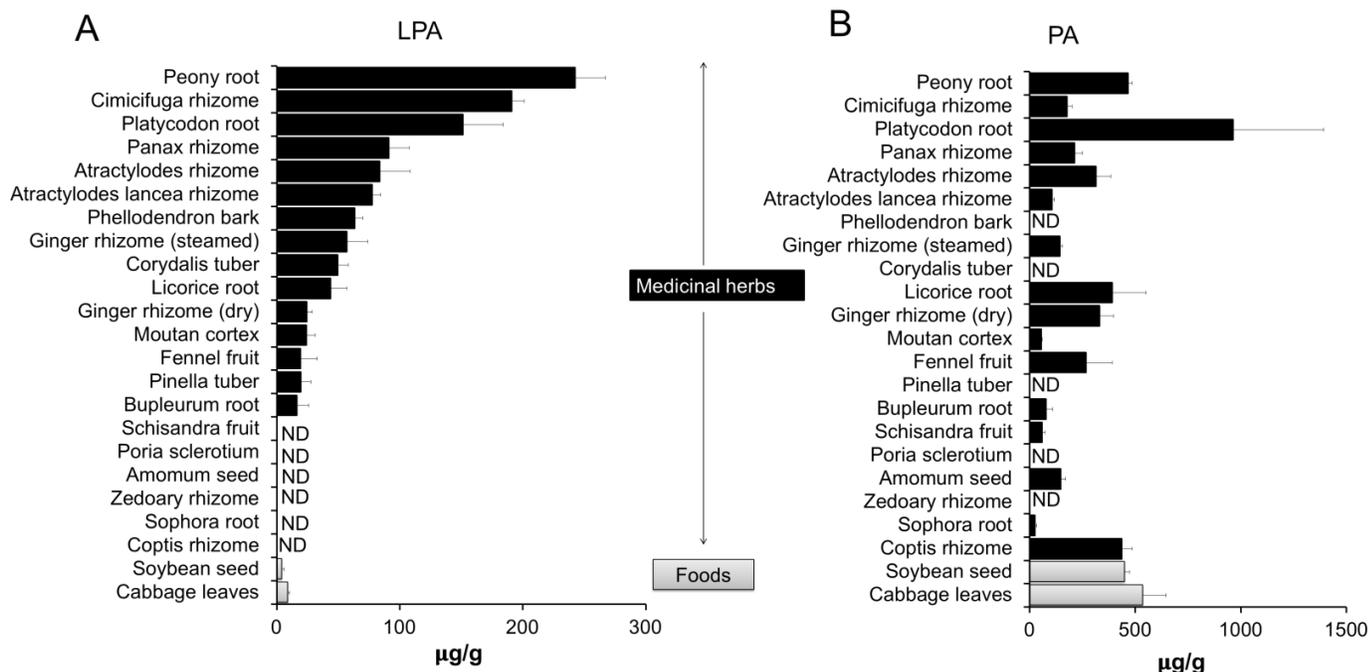
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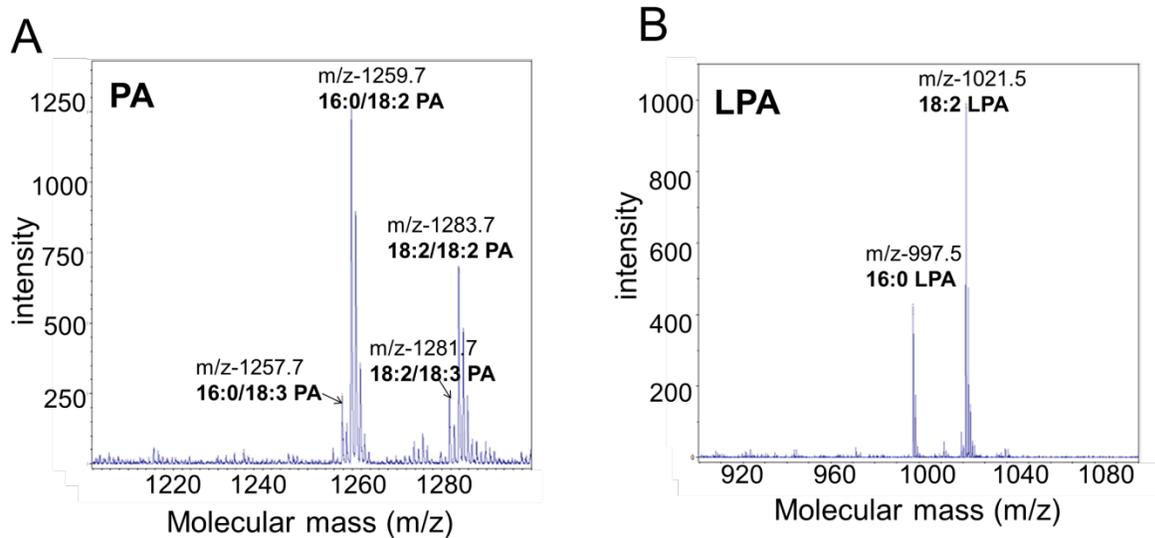
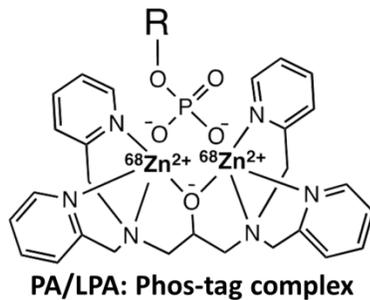


542

543 **Fig. 1. Abundance of LPA in medicinal herbs**

544 Amounts of (A) LPA and (B) PA isolated from herbs were determined by measurement of their  
 545 lipid phosphorus. Data represent means  $\pm$  SD of three independent experiments. Herbs and foods  
 546 except for cabbage leaves were dry weight. ND: Not detectable (less than 2  $\mu\text{g/g}$ ).

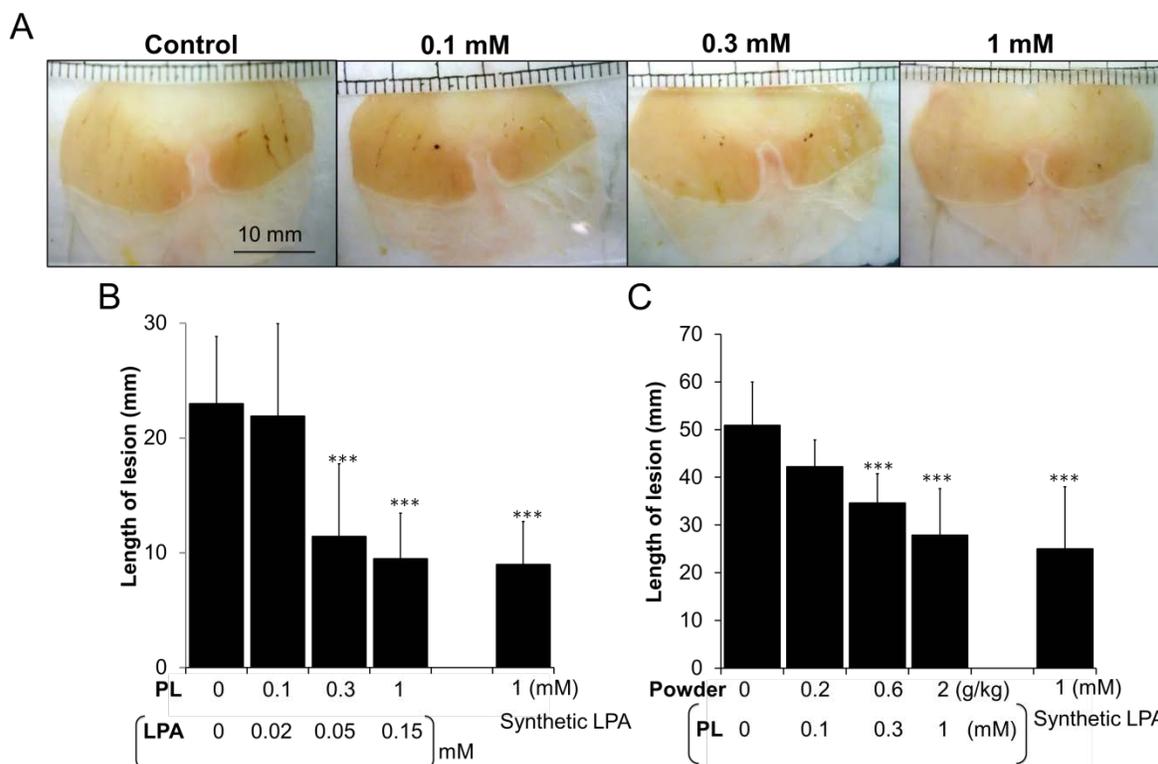
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549 **Fig. 2. MALDI TOF mass spectra of PA and LPA isolated from peony root**

550 (A) PA and (B) LPA from peony root were analyzed by MALDI-TOF MS as their Phos-tag  
 551 complexes. Chemical structure shown is a complex of a phosphate monoester compound with  
 552 Phos-tag.

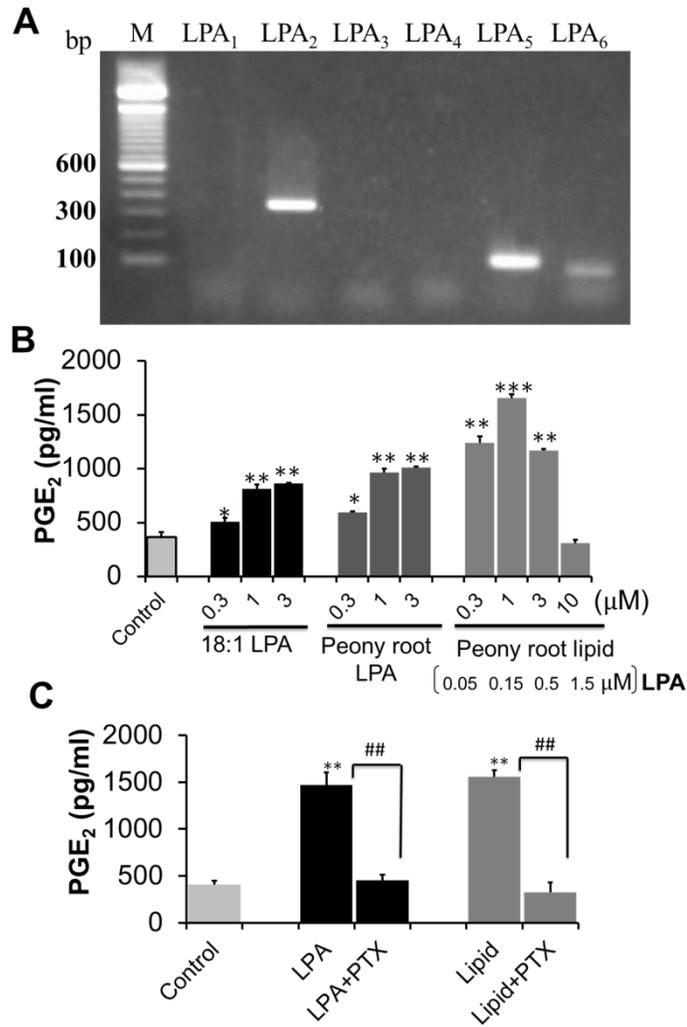


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554 **Fig. 3. Anti-ulcer effect of peony root lipid and powder**

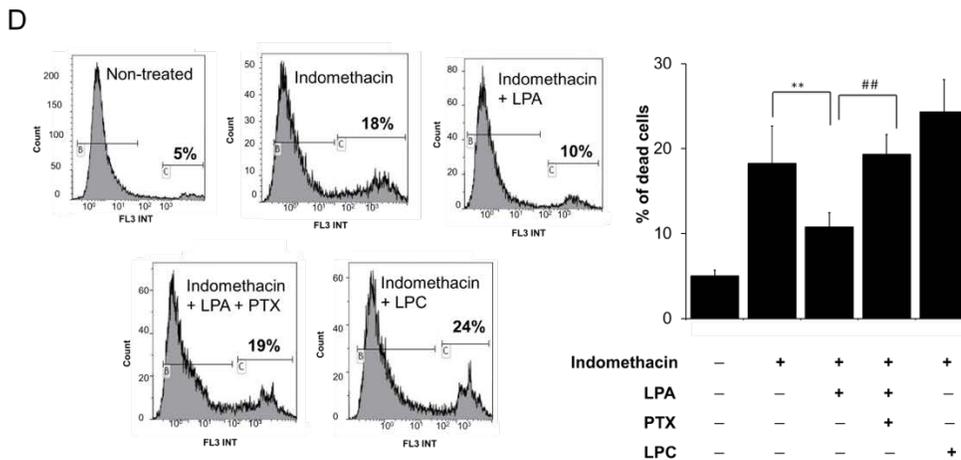
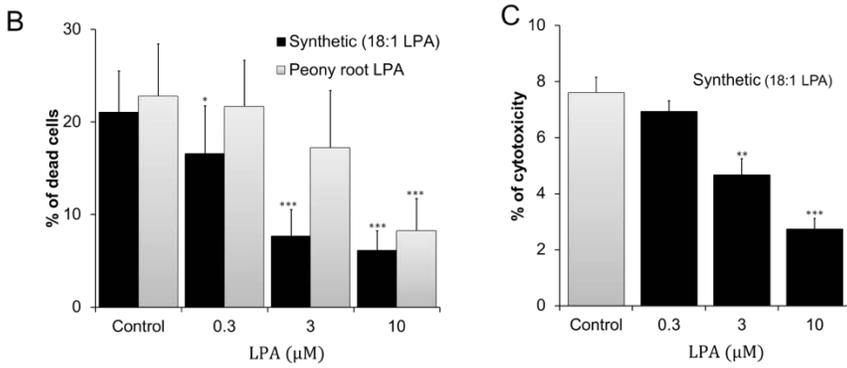
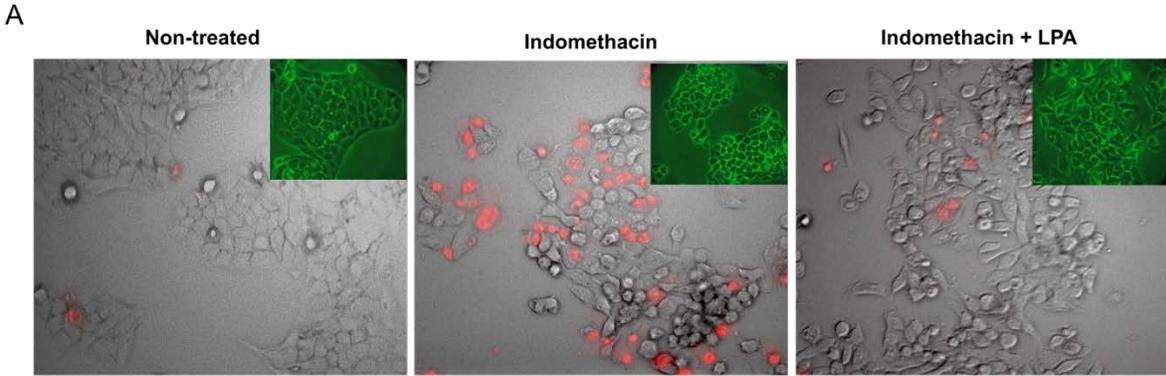
555 (A, B) Peony root lipid was suspended in 3% CMC. Aliquots of the suspension (0.2 ml) were  
 556 orally administered to mice. After 30 min, 0.2 ml of indomethacin (22.9 mg/kg) in 3% CMC was  
 557 intragastrically administered. The total lengths of lesions on the stomach wall were measured at  
 558 5 h after injection of indomethacin. (B) Indicated concentrations are based on the amounts of  
 559 total phospholipid. For example, “1 mM PL” indicates administration of peony lipid containing  
 560 0.2 μmol of phospholipid in 0.2 ml of the suspension. Values in the parenthesis indicate  
 561 concentrations of LPA in peony root lipid suspensions. This is deduced from Table1. The  
 562 numbers of mice of each group were 15 for control (0 mM) and 5–15 for others. \*\*\*P<0.005  
 563 versus control. (C) Mice were intragastrically administered 0.2 ml water or 0.2 ml peony root  
 564 powder suspended in water. Peony root powder in a dose of 2 g/kg body weight contains 1 mM  
 565 phospholipids. Mice were sacrificed 3 h after aspirin administration (300 mg/kg). The numbers  
 566 of mice of each group were 5–10. \*\*\*P<0.005 versus control. Synthetic LPA (16:0 LPA) at 1  
 567 mM (5.7 μmol/kg body weight) was used as positive control. Error bar represents SD.

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**Fig. 4. Enhancement of PGE<sub>2</sub> production by LPA and peony root lipid.**

(A) mRNA profile of LPARs (LPA<sub>1-6</sub>) of MKN74 cells. (B) MKN74 cells replated with arachidonic acid were preincubated with the indicated concentration of LPA or peony root lipid for 5 h before stimulation with 1 μM A23187 for 10 min. PGE<sub>2</sub> released into the culture media was measured using an ELISA kit. Indicated values in parenthesis are the concentration of LPA in the peony root lipid. Each value shown is mean ± SD. (C) MKN74 cells were incubated with 3 μM synthetic LPA (18:1 LPA) or 3 μM peony root lipid in the absence or presence of 100 ng/ml of PTX for 5 h before stimulation with 1 μM A23187 for 10 min. PGE<sub>2</sub> released into the culture media was measured using an ELISA kit. Each value shown is the mean ± SD. \*P<0.05, \*\*P<0.01, \*\*\*P<0.005 versus control and ## P<0.01.



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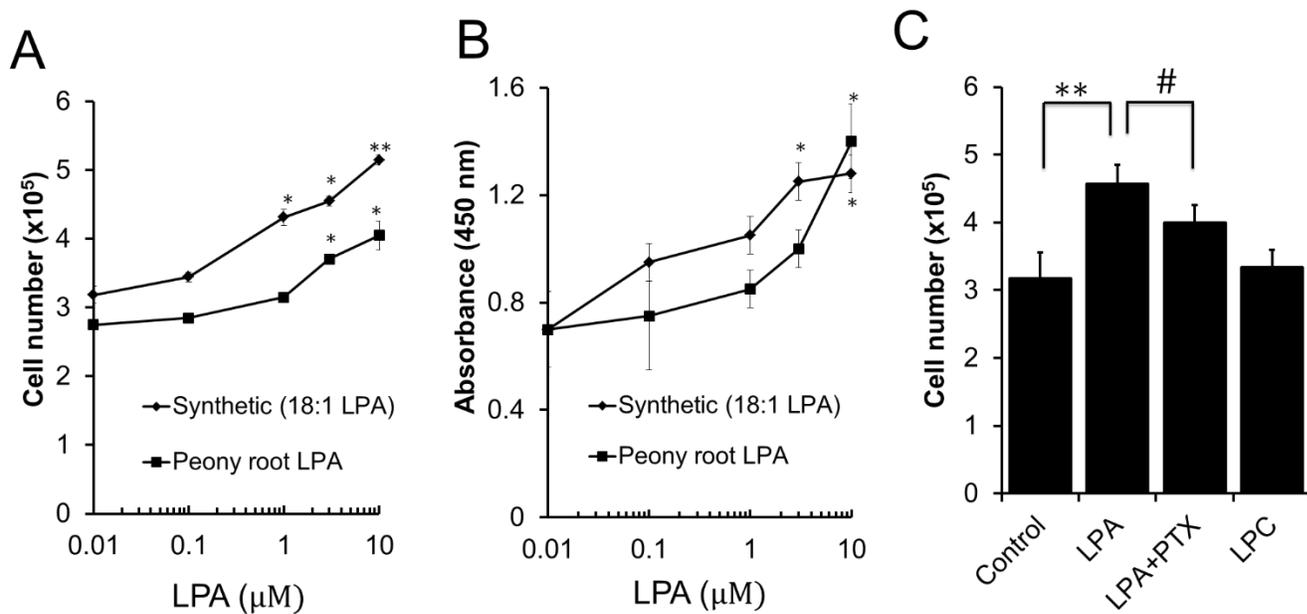
592 **Fig. 5. LPA protect indomethacin-induced cell injury in MKN74 cells.**

593 (A) Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin with or without 10  
 594 μM 18:1 LPA. After 30 h, dead cells are stained with propidium iodide (PI). The image shown is  
 595 phase-contrast microscopic photo merged with a fluorescent microscopic photo for indication of  
 596 PI-positive cells. (B, C) Serum-starved MKN74 cells were incubated with 0.8 mM indomethacin  
 597 in the absence or presence of increasing concentration of LPAs. (B) The percentage of dead cells

598 was calculated by counting PI-positive cells in several randomized subfields in each dish from  
599 three different experiments. (C) LDH leakage in the culture media was determined to know the  
600 extent of the cellular damage. (D) MKN74 cells were incubated with 0.8 mM indomethacin in  
601 the absence or presence of 10  $\mu$ M 18:1 LPA or 16:0 LPC with or without PTX (100 ng/ml). Flow  
602 cytometry was performed 30 h after indomethacin treatment. Values in the flow cytometry chart  
603 indicate the % of dead cells. Data represent means  $\pm$  SD of three independent experiments.  
604 \*P<0.05, \*\*P<0.01, \*\*\*P<0.005 versus control and ## P<0.01.

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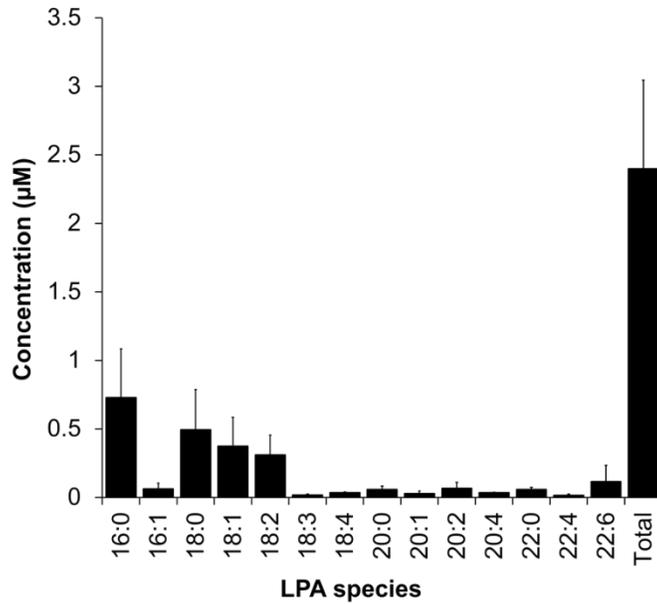


607

608 **Fig. 6. LPA stimulated proliferation of MKN74 cells**

609 (A, B) MKN74 cells were serum starved for 24 h, and then, treated with different concentrations  
610 of LPA. After 24 h, the extent of proliferation was determined by direct counting or BrdU  
611 incorporation. (C) The proliferation assay was conducted in the presence or absence of 100  
612 ng/ml of PTX or with 16:0 LPC (10 μM) instead of 18:1 LPA. Data represent means ± SD of  
613 three independent experiments. \*P<0.05, \*\*P<0.01 versus control and #P<0.05.

614



615

616 **Fig. 7. LPA concentration in a mouse stomach fluid**

617 Stomachs of fasted mice were isolated and cut along the greater curvature. The stomach mucosal  
 618 surface was washed with a small amount of PBS. Lipids were extracted from the stomach  
 619 washing solution and subjected to LC/MS/MS by using 17:0 LPA as an internal standard. Data  
 620 represent means  $\pm$  SD of three independent experiments.

621

622 **Table 1**

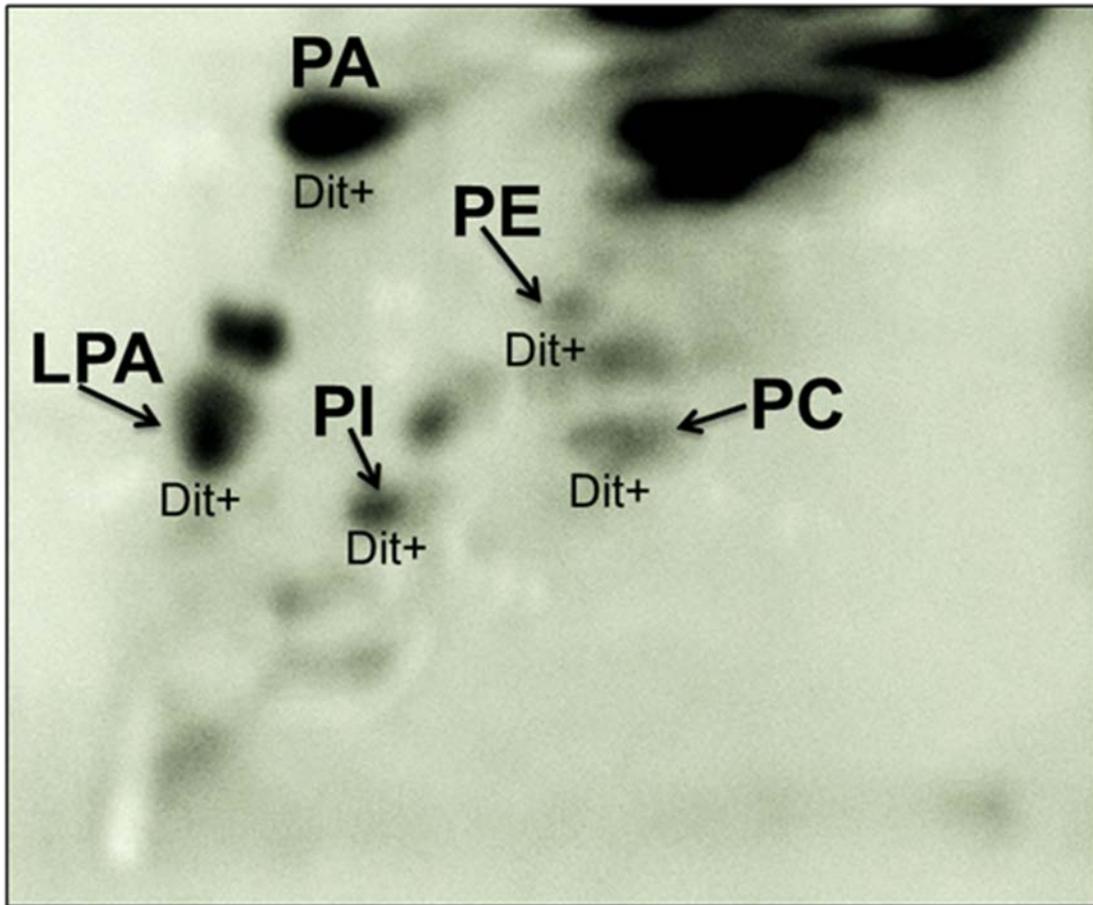
623 LPA is highly concentrated in the lipid fraction of peony root.

	Cabbage		Soybean		Peony root	
	$\mu\text{g/g}$	(%)	$\mu\text{g/g}$	(%)	$\mu\text{g/g}$	(%)
<b>Total</b>	$2300 \pm 160$	-	$13000 \pm 2700$	-	$2200 \pm 140$	-
<b>phospholipid</b>						
<b>PA</b>	$540 \pm 110$	(24)	$450 \pm 20$	(3.0)	$460 \pm 20$	(21)
<b>LPA</b>	$9 \pm 1$	(0.4)	$5 \pm 2$	(0.03)	$240 \pm 20$	(11)

624 Value in cabbage is wet weight. Values in soybean and peony root are dry weight. Values in

625 parentheses are percentage in total phospholipid.

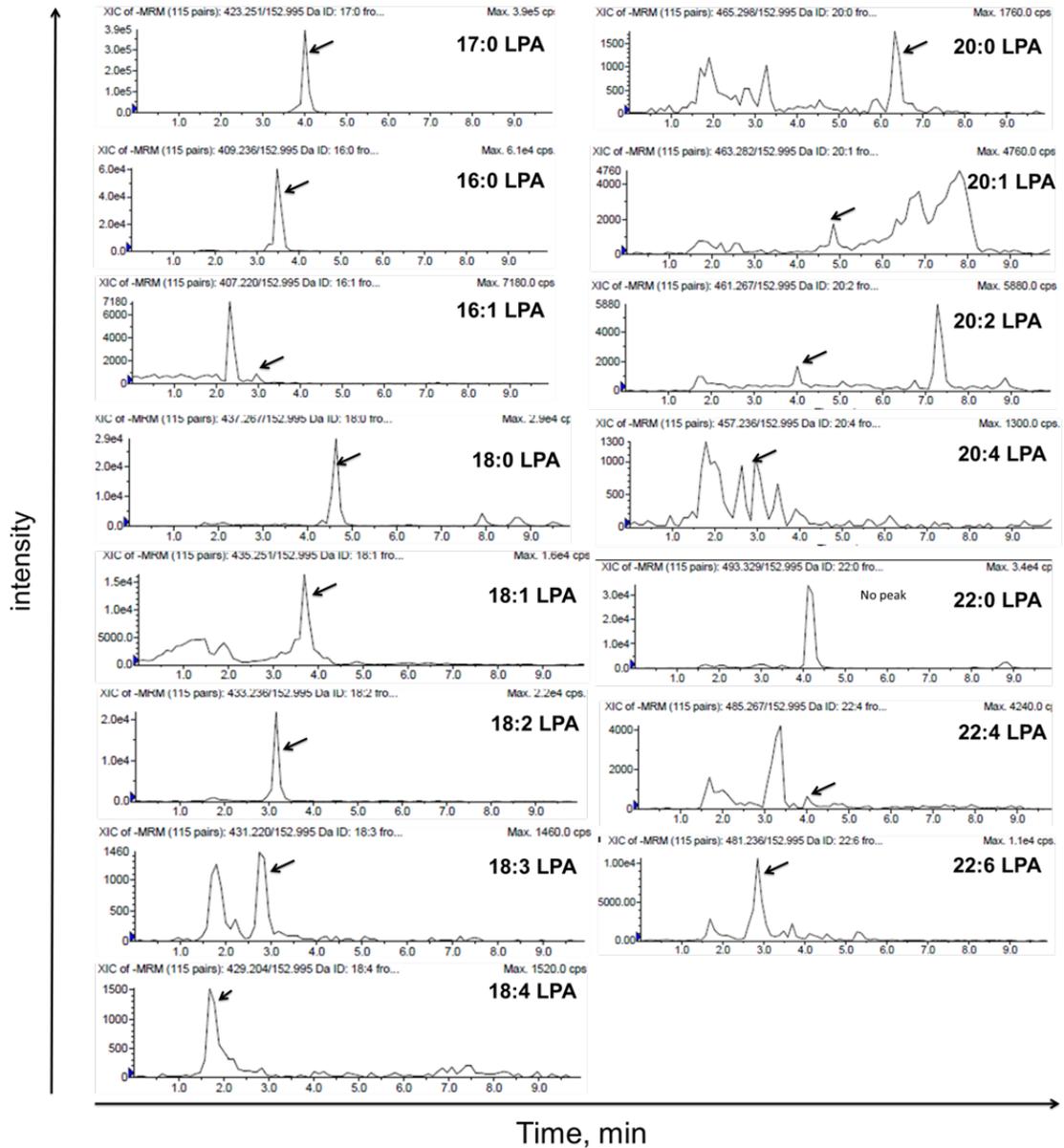
**Supplementary Fig. 1**



**Supplementary Fig. 1. Two-dimensional TLC of peony root lipid**

Peony root lipid was separated by two-dimensional TLC. The solvent systems for the first and second chromatography were chloroform/methanol/28% ammonia (60:35:8, v/v/v) and chloroform/acetone/methanol/acetic acid/water (50:20:10:13:5, v/v/v/v/v), respectively. PA, phosphatidic acid; LPA, lysophosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Dit, Dittmer-positive spot indicating phospholipid.

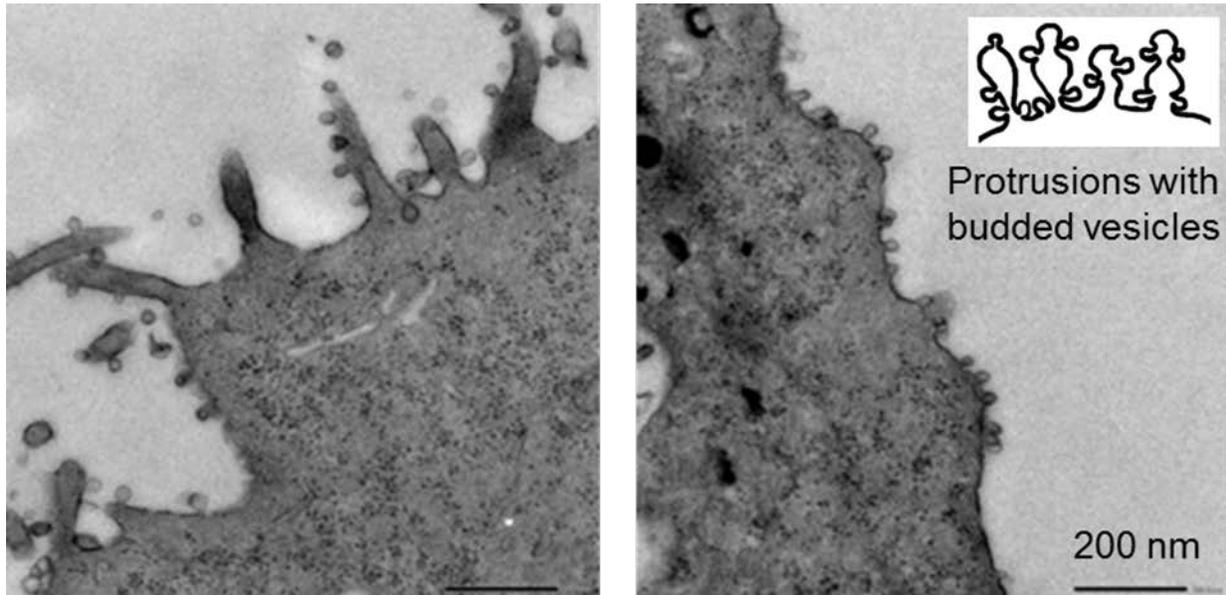
## Supplementary Fig. 2



### Supplementary Fig. 2. Determination of LPA molecular species in a mouse stomach fluid by LC/MS/MS.

A stomach of fasted mouse was isolated, and cut along the greater curvature. The stomach inner surface was washed with a small amount of PBS. Lipid was extracted from the washing solution, and subjected to LC/MS/MS for determination of LPA using 17:0 LPA as an internal standard.

**Supplementary Fig. 3**



**Supplementary Fig. 3. LPA-induced structural change in plasma membrane of MKN74 cells.**

MKN74 cells were treated with 10  $\mu$ M 16:0 LPA for 3 h and fixed with 3% glutaraldehyde. After treatment with 1% osmium, cells were stained with 2% uranyl acetate. Serial section of the cells in each 70–80 nm in thickness, were cut with an ultramicrotome and examined with an electron microscope. Large protrusions with budded vesicles were observed in plasma membrane of the cells.

### Supplementary Table 1.

Primer sequences and PCR conditions used in this study

Name	Sequence	Denaturation	Annealing	Extension
LPA <sub>1</sub>	Forward: 5'-GAGGAATCGGGACACCATGAT-3' Reverse: 5'-ACATCCAGCAATAACAAGACCAATC-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
LPA <sub>2</sub>	Forward: 5'-CATCATGCTTCCCGAGAACG-3' Reverse: 5'-GGGCTTACCAAGGATACGCAG-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
LPA <sub>3</sub>	Forward: 5'-GCTCCCATGAAGCTAATGAAGACA-3' Reverse: 5'-AGGCCGTCCAGCAGCAGA-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
LPA <sub>4</sub>	Forward: 5'-CAGTGCCTCCCTGTTTGTCTTC-3' Reverse: 5'-GAGAGGGCCAGGTTGGTGAT-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
LPA <sub>5</sub>	Forward: 5'-AGCAACACGGAGCACAGGTC-3' Reverse: 5'-CCAAAACAAGCAGAGGGAGGT-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec
LPA <sub>6</sub>	Forward: 5'-CCGCCGTTTTTGTTCAGTC-3' Reverse: 5'-GAGATATGTTTTCCATGTGGCTTC-3'	94°C/30 sec	67°C/30 sec	72°C/30 sec

**Supplementary Table 2.** Molecular species composition of PA in medicinal herbs

	*34:3	34:2	34:1	36:5	36:4	36:3	36:2
Peony root	9	53	—	10	28	—	—
Cimicifuga rhizome	—	75	—	—	25	—	—
Panax rhizome	—	60	16	—	24	—	—
Atractylodes rhizome	—	67	—	—	33	—	—
Atractylodes lancea rhizome	—	66	—	—	34	—	—
Phellodendron bark	—	—	—	—	—	—	—
Ginger rhizome (steamed)	—	—	—	—	100	—	—
Corydalis tuber	—	—	—	—	—	—	—
Licorice root	—	62	—	—	38	—	—
Ginger rhizome (dry)	16	75	—	—	9	—	—
Moutan cortex	—	55	—	—	45	—	—
Fennel fruit	—	32	16	—	16	22	14
Pinellia tuber	—	—	—	—	—	—	—
Bupleurum root	—	65	—	—	35	—	—
Schisandra fruit	—	25	—	—	45	28	—
Poria sclerotium	—	—	—	—	—	—	—
Amomum seed	15	40	45	—	—	—	—
Zedoary rhizome	—	—	—	—	—	—	—
Sophora root	—	53	—	—	47	—	—
Coptis rhizome	—	47	—	9	30	14	—

The possible assignable PA species are 16:0/18:3 (34:3), 16:0/18:2 (34:2), 16:0/18:1 (34:1), 18:2/18:3 (36:5), 18:1/18:3 or 18:2/18:2 (36:4), 18:1/18:2 (36:3), and 18:1/18:1 (36: 2). \*Total carbon number and number of double bonds in the fatty acid residues. The horizontal dashes lines indicate "not detectable". Our detection limit of PA in MALDI-TOF MS method is around 3 nmol/g herbs.

**Supplementary Table 3.** Molecular species composition of LPA in medicinal herbs

	16:0	18:1	18:2	18:3
Peony root	29	—	71	—
Cimicifuga rhizome	42	8	41	9
Platycodon root	12	—	83	4
Panax rhizome	42	—	58	—
Atractylodes rhizome	33	—	67	—
Atractylodes lancea rhizome	41	—	58	—
Phellodendron bark	36	—	56	8
Ginger rhizome (steamed)	23	41	36	—
Corydalis tuber	35	—	59	6
Licorice root	17	14	58	11
Ginger rhizome (dry)	44	—	46	—
Moutan cortex	21	14	57	8
Fennel fruit	41	27	32	—
Pinellia tuber	41	—	58	—
Bupleurum root	100	—	—	—
Schisandra fruit	—	—	—	—
Poria sclerotium	—	—	—	—
Amomum seed	—	—	—	—
Zedoary rhizome	—	—	—	—
Sophora root	—	—	—	—
Coptis rhizome	—	—	—	—

The fatty acyl moieties of LPA are designated in terms of the number of carbon atoms and double bonds. The horizontal dashes lines indicate "not detectable". Our detection limit of LPA in MALDI-TOF MS method is around 3 nmol/g herb.

## Highlights

- The concentration of LPA in mouse stomach fluid was determined to be 2.4  $\mu\text{M}$ .
- Peony root powder, a medicinal herb used for the treatment of gastrointestinal disorders, contained highly concentrated LPA.
- The lipid of peony root showed an ameliorative effect against indomethacin-induced gastric ulcer in mice.
- LPA and LPA-rich herbal lipid enhanced PGE<sub>2</sub> production and reduce cytotoxicity of NSAIDs in gastric cells.