Research Article **Open-Access**

BCHD
Bioactive Compounds in Health and Disease

Fatty acids in *Plantago asiatica* seeds are responsible for the production of the pro-inflammatory mediator nitric oxide

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Submission Date: March 20th, 2024; **Acceptance Date:** May 9th, 2024; **Publication Date:** May 10th, 2024

Please cite this article as: Sholmire A., Ishii T., Leischner L., Garland B., Yamauchi Y., Shirako S., Nishidono Y., Ikeya Y., Corey L., Nishizawa M. Fatty acids in Plantago asiatica seeds are responsible for the production of the pro-inflammatory mediator nitric oxide. *Bioactive Compounds in Health and Disease* 2024; 7(5): 233-244.
DOI: https://doi.org/10.31989/bchd.v7i5.1337

ABSTRACT

Background: *Plantago asiatica* L. (Plantaginaceae) commonly grows in East Asia, with its seeds (*Shazenshi*), having been used as diuretic and anti-inflammatory drugs in traditional Japanese medicine. It is not known which constituents of *P. asiatica* seeds elicit the anti-inflammatory effects, such as reduced expression of the inducible nitric oxide synthase (iNOS) in interleukin (IL)-1β-treated hepatocytes which leads to a reduction in the pro-inflammatory mediator nitric oxide (NO).

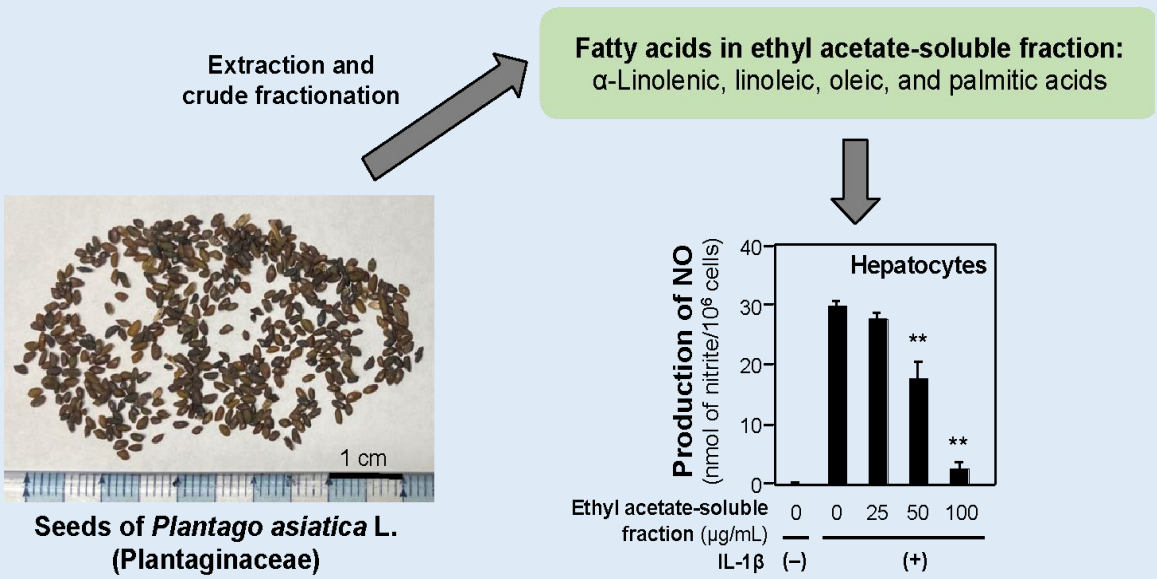
Objective: To identify the anti-inflammatory constituents of *P. asiatica* seeds, the anti-inflammatory activity of purified constituents was determined by assaying NO production in IL-1β-treated hepatocytes.

Methods: *P. asiatica* seeds were extracted with 50% methanol and successively fractionated into three crude fractions with ethyl acetate (EtOAc) and *n*-butanol. The compounds were methylated and analyzed by gas chromatography–mass spectrometry (GC–MS). Primary cultured rat hepatocytes were prepared by collagenase perfusion, and *P. asiatica* seed extract (PASE), a fraction, or a compound was added to the culture medium with IL-1β and incubated at 37 °C. Potency of each fraction was determined by the Griess method for measuring the levels of nitrite in the medium.

Results: PASE suppressed IL-1β-induced NO production without showing cytotoxicity, and an EtOAc-soluble fraction of PASE significantly inhibited NO production. GC–MS analysis detected 26 distinct fatty acids as their methyl esters in this fraction. Among them, three unsaturated fatty acids (linoleic, oleic, and α-linolenic acids) and palmitic acid were abundant. These unsaturated fatty acids are known to reduce NO levels. In contrast, acteoside and aucubin, which are thought to be present in the *n*-butanol-soluble fraction, showed only a low level of NO production suppression.

Conclusion: The EtOAc-soluble fraction of PASE included many fatty acids, which may suppress the production of NO. The results imply that the anti-inflammatory activity of *P. asiatica* seeds may be produced by three unsaturated fatty acids. Because the fatty acids are abundant in the seeds of medicinal plants, they are likely to contribute to anti-inflammatory activity of the seeds.

Keywords: Plantain seed, polyunsaturated fatty acid, Kampo medicine, hepatocyte, nitric oxide.



INTRODUCTION

Plantago asiatica L. (Plantaginaceae), which is known as *Obako* in Japan, commonly grows on roadsides in East Asian countries, such as Japan and China. The entire plant, along with its small seeds, have been used as crude drugs in Japanese Kampo medicines, duly

outlined in *the Japanese Pharmacopoeia* [1]. *P. asiatica* seeds (*Shazenshi* in Japanese) are thought to possess diuretic and anti-inflammatory effects. They are included into Kampo formulae such as *Goshajinkigan*, prescribed for conditions like frequent urination, leg pain, and numbness. In Europe and India, there exists other plantain species, like *Plantago psyllium* L. and *Plantago ovata* Forssk. [2]. These plantain seeds (known as Spanish or French psyllium) have been used as mild laxatives in traditional European medicine.

When *P. asiatica* seeds are soaked in water, the grain shell absorbs water, swells, and produces a hydrocolloid solution, which consists of polysaccharides including plantasan (major constituent) and plantago-mucilage A [3–4]. Additionally, *P. asiatica* seeds contain a diverse range of fatty acids, phenylethanoid glycosides, *e.g.*, acteoside (also known as verbascoside and kusaginin), and iridoid glycosides, *e.g.*, geniposidic acid and aucubin [3,5–6]. Acteoside and geniposidic acid are included at an average content of 0.577% and 0.557% of the seed extracts (by 60% methanol), respectively [3]. These two compounds are speculated to be pharmacologically active.

It has been reported that the water extract of *P. asiatica* seeds suppressed the nitric oxide (NO) production in a lipopolysaccharide (LPS)-treated mouse macrophage line RAW264.7 [7]. Both water and methanol extracts of *P. asiatica* seeds reduced NO production in the LPS-treated macrophage line J774.1 cells, as well as mouse peritoneal exudate macrophages that were treated with LPS and interferon-γ [8]. Furthermore, oral administration of *P. asiatica* seeds to mice inhibited the expression of inducible nitric oxide synthase (iNOS, also known as NOS2) and cyclooxygenase-2 (COX2), which is the prostaglandin-endoperoxide synthase 2 that synthesizes the pro-inflammatory mediator prostaglandin [9]. However, it remains unknown which constituent(s) function to suppress NO production by *P. asiatica* seeds.

Each crude drug used in Kampo medicine exhibits several pharmacological activities, making it challenging at times to identify the principal constituent(s) responsible for each activity. Primary cultured rat hepatocytes are an effective model system for evaluating anti-inflammatory effects because they produce NO, pro-inflammatory cytokines, and chemokines in response to the presence of interleukin (IL)-1β [10]. With the use of hepatocytes, we previously identified principal anti-inflammatory constituents in crude drugs, such as chlorogenic acid in the buds and flowers of *Lonicera japonica* [11] and atractylodin in *Atractylodes chinensis* rhizome [12], as well as in functional foods, such as adenosine in a standardized extract of cultured *Lentinula edodes* mycelia (ECLM, AHCC) [13].

To find the bioactive constituents, we extracted *P. asiatica* seeds and fractionated their extract by monitoring the NO production in IL-1β-treated hepatocytes. We targeted a specific crude fraction that demonstrated suppression of NO production and conducted analysis to identify constituents responsible for the anti-inflammatory effect of *P. asiatica* seeds.

MATERIALS AND METHODS

Plant materials: Raw seeds of *P. asiatica* L. collected from Jiangxi Province, China, were obtained from Tochimoto Tenkaido Co. Ltd. (Osaka, Japan). Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co., Ltd.) authenticated the samples as *Shazenshi* according to the Japanese Pharmacopoeia [1]. Voucher specimens were deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University under the code numbers RIN-PA-33.

Extraction of P. asiatica seeds and crude fractionation: Raw *P. asiatica* seeds (400 g) were ground and extracted by 50% methanol, *i.e.*, methanol:water (1:1 v/v) mixture, under reflux. The solvent was evaporated

in vacuo to yield 50% methanol extract (16.7 g; 4.17% yield from the plant material). According to a previously described method [11,14], the extract was suspended in water and successively partitioned with ethyl acetate (EtOAc) and then *n*-butanol. Resultant layers were evaporated *in vacuo* to provide EtOAc-soluble, *n*-butanol-soluble, and water-soluble fractions.

Analysis of fatty acids: Fatty acids in the EtOAc-soluble fraction were methylated before gas chromatography–mass spectrometry (GC–MS) analysis. Fraction A (20.1 mg) was methylated using a Fatty Acid Methylation Kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol, and extracted with *n*-hexane. The upper layer, which contained methylated fatty acids, was subjected to GC–MS analysis using a Nexis GC-2030 equipped with a DB-FastFAME column and a GCMS-QP2020 NX (Shimadzu Corporation, Kyoto, Japan). A sample (1 µL) was injected in a 10:1 split with the temperature set to 250 °C. The oven temperature, initially maintained at 60 °C for 1 min, was increased to 200 °C at a rate of 10 °C/min and kept for 3 min, and then increased to 250 °C at a rate of 25 °C/min and kept for 1 min. The transfer line to the mass spectrometer was kept at 250 °C. Mass temperature was set at 230 °C and ionization was performed by electron ionization (70 eV, 60 µA). Simultaneous scan and selected ion monitoring (SIM) measurement were conducted, utilizing the area values of peaks obtained through SIM for quantification purposes. The scan was performed in the range of *m*/*z* 50–500, with a cycle time of 0.1 sec, and SIM was measured *m*/*z* specific for each fatty acid methyl ester (FAME), with a cycle time of 0.2 sec. Supelco 37 Component FAME Mix (Merck KGaA, Darmstadt, Germany) was used for calibration standards of FAMEs.

Animals: All animal care and experimental procedures were performed in accordance with the laws and guidelines of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus (No. BKC2017-052 and BKC2020-045). Male Wistar rats (5.5 weeks old, specific pathogen-free; Charles River Laboratories Japan Inc., Yokohama, Japan) were housed at 21–23 °C under a 12-h light-dark cycle. Rats were fed a CRF-1 diet (γ-ray-irradiated; Charles River Laboratories Japan) and acclimated to the housing for a week. Water was available *ad libitum.*

Rat hepatocytes: According to a previously established method, hepatocytes were isolated from male Wistar rats [15]. Following *Clostridium histolyticum* (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) collagenase perfusion, the dispersed cells were centrifuged several times and resuspended. Microscopic observation confirmed greater than 99% hepatocyte purity (data not shown). The cells were seeded onto 35-mm-diameter dishes (1.2×10^6 cells per dish) and incubated at 37 °C for 2 h. Fresh Williams' E (WE) medium was added, and the hepatocytes, were incubated at 37 °C overnight until the subsequent assays on the next day (Day 1).

NO production and lactate dehydrogenase (LDH) activity*:* A fraction or a compound was dissolved in either water (for water-soluble fraction) or dimethyl sulfoxide (DMSO; for the others), then added to WE medium on Day 1. Hepatocytes were subsequently incubated for 8 hours. The final concentrations of DMSO were adjusted to less than 1.0% (v/v) in the medium, which did not affect NO production [16]. Because nitrite is a stable metabolite of NO, the concentrations of nitrite in the medium were measured using the Griess method [17–18]. Nitrite concentrations measured in the presence and absence of 1 nM rat recombinant IL-1β [15] were set as 100% and 0%, respectively. Nitrite concentration was measured in triplicate (n = 3 dishes) for at least three different concentrations of an extract, a fraction, or a

compound [18]. LDH activity of the medium was measured to estimate cytotoxicity using an LDH Cytotoxicity Detection Kit (Takara Bio Inc., Kusatsu, Japan). The half-maximal inhibitory concentration (IC_{50}) value was determined to assess the potency of the inhibition of NO production unless cytotoxicity was not observed. The NO level of at least one concentration should be >50% while the NO level of at least one concentration should be <50% [18]. NO levels from at least three concentrations were used to calculate an IC_{50} value [18]. Acteoside and aucubin were obtained from Extrasynthese S.A. (Geney, France) and FUJIFILM Wako Pure Chemical Corporation, respectively, and dissolved in DMSO.

Statistical analysis: The data are representative of three independent experiments that yielded similar results. The values were measured in triplicate and are presented as the means ± standard deviation (SD). Differences were analyzed using Student's *t* test followed by the Bonferroni correction. The statistical significance of probability was set to 0.05 and 0.01.

RESULTS

Extraction of P. asiatica seeds and crude fractionation:

The shells of raw *P. asiatica* seeds were crushed before the extraction. When absolute methanol was used to extract the crushed seeds (80.0 g), the yield of the extract was very low, *i.e.*, 0.66% of the extract by weight of the seeds. When 50% methanol was used, it improved the yield to 2.85%. Therefore, *P. asiatica* seeds (400 g) were extracted with 50% methanol and successively partitioned based on hydrophobicity with EtOAc and *n*-butanol (Figure 1).

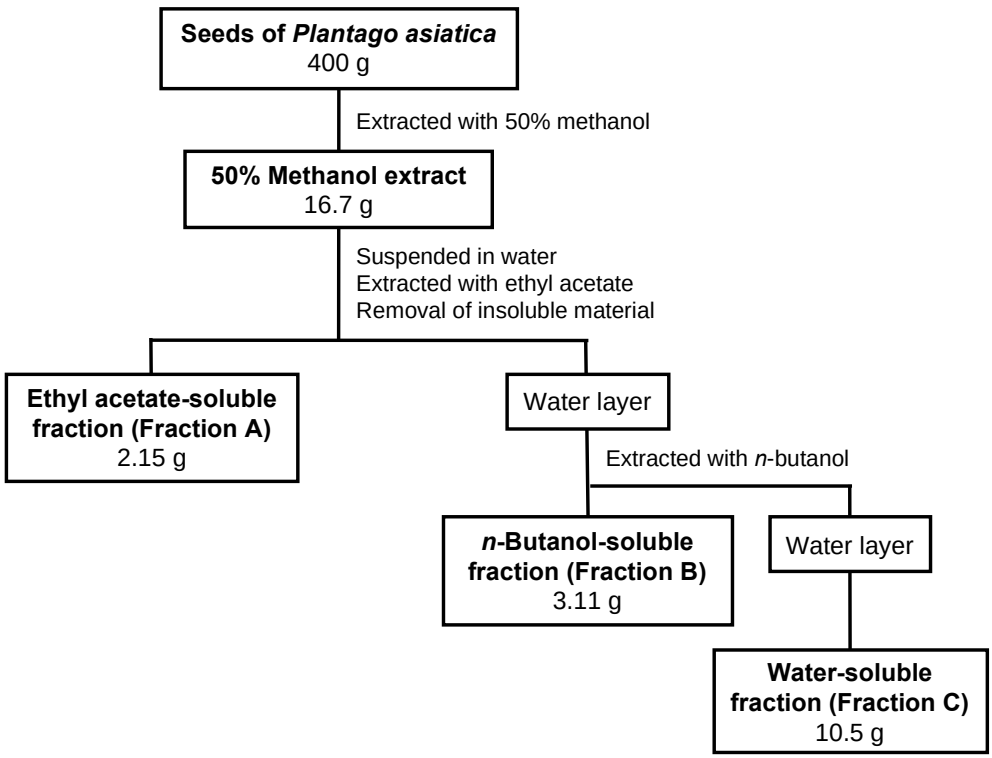


Figure 1. Extraction of *P. asiatica* seeds. A flowchart of the procedures for extraction and crude fractionation is shown. The extract was partitioned into Fraction A, which includes hydrophobic constituents, Fraction B, which includes amphipathic constituents, and Fraction C, which includes hydrophilic constituents.

The yield of each crude fraction of the extract is provided in Table 1. According to previous reports [11,14], polysaccharides of the seed shells seem to be included in the water-soluble fraction (Fraction C). These crude fractions were subjected to the NO assays using primary cultured rat hepatocytes.

Table 1. Fractionation of a *P. asiatica* seed extract and its effects on NO production.

Extract/crude fraction	Yield [%][a]	IC_{50} [μg/mL][b]
50% Methanol extract (PASE)	100	212 ± 25.8
EtOAc-soluble fraction (Fraction A)	13.6	49.1 ± 5.80
n-Butanol-soluble fraction (Fraction B)	19.7	144 ± 26.0
Water-soluble fraction (Fraction C)	66.7	NA

[a] Percentage calculated as the weight of each fraction divided by the sum of the three fractions. [b] The IC_{50} value of the production of nitrite (as NO) in IL-1β-treated hepatocytes (mean ± SD). To determine these values, at least three experiments were carried out. NA, not applicable.

Effect of P. asiatica seed extract (PASE) and crude fractions on NO production: The 50% methanol extract of *P. asiatica* seeds was added to the medium of hepatocytes, leading to significantly decreased NO production (Figure 2A). The LDH activity of the medium was less than 5% of the activity of the entire cell extract, suggesting that PASE was not cytotoxic to hepatocytes. We then prepared crude fractions from PASE.

When the EtOAc-soluble fraction (Fraction A) of PASE was added to the medium, it inhibited NO production in IL-1β-treated hepatocytes (Figure 2B). The *n*-butanol-soluble fraction (Fraction B) also reduced IL-1β-induced NO production, while Fraction C did not significantly reduce NO production. The LDH assay indicated that these three crude fractions were not cytotoxic at the concentrations applied (data not shown). The IC_{50} values for inhibition of NO production were calculated and summarized. As shown in Table 1, Fraction A showed the lowest IC_{50} value of NO production, which is 2.93-fold less than that of Fraction B. The IC_{50} value of Fraction C could not be calculated due to low activity.

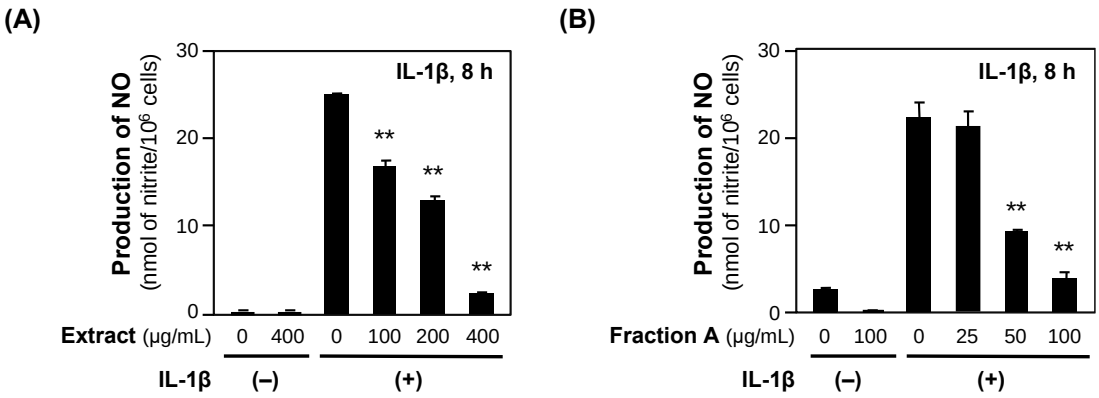


Figure 2. Effects of *P. asiatica* seed extract (PASE) and Fraction A on NO production in hepatocytes. (**A**) PASE reduces IL-1β-induced NO production. Hepatocytes were treated with 1 nM IL-1β in the absence or presence of PASE (50% methanol extract) for 8 h. The NO concentration was measured as nitrite in the medium. Results are presented as the mean ± SD (n = 3). ** P < 0.01 *versus* IL-1β alone. (**B**) Fraction A reduces IL-1β-induced NO production. Cells were treated with IL-1β in the absence or presence of Fraction A for 8 h. The NO concentration was presented as the mean ± SD (n = 3). ** P < 0.01 *versus* IL-1β alone.

Analysis of constituents in Fraction A: Fraction A underwent further fractionation through silica gel column chromatography and was eluted with an *n*-hexane: EtOAc mixture (100:0) to (0:100) to yield 10 subfractions. Thin-layer chromatography (TLC) of these subfractions gave broad spots (data not shown). It was speculated that many fatty acids are present in Fraction A. Therefore, the fatty acids present in Fraction A were methylated and analyzed using GC–MS.

Fatty acids present in Fraction A were methylated,

leading to the formation of methyl esters of fatty acids (*i.e.*, FAMEs) which were then subjected to GC–MS analysis. Compared to 37 FAME standards, 26 FAMEs were detected in this fraction (Figure 3), suggesting that fatty acids are abundant and diverse. In the scan chromatogram, the peaks corresponding to palmitic acid (C16:0), oleic acid [C18:1(9*c*)], linoleic acid [C18:2(9*c*,12*c*)], and α-linolenic acid [C18:3(9*c*,12*c*,15*c*)] were prominent. The unsaturated fatty acids present (oleic, linoleic, and α-linolenic acids) were *cis* fatty acids (Figure 4); *trans* fatty acids were not detected by this analysis

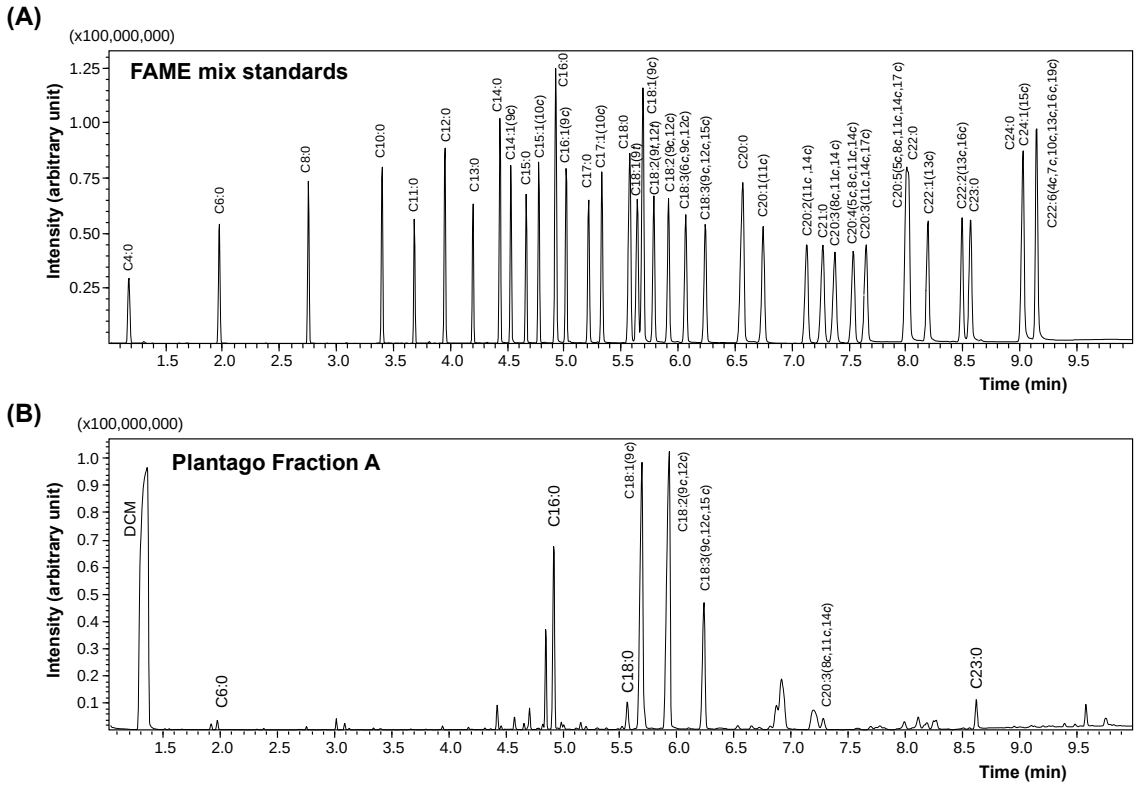


Figure 3. Detection of fatty acids in Fraction A of PASE. (**A**) GC–MS separation profiles of 37 FAMEs. Total ion chromatogram (TIC) of 37 FAME mix standards with scan measurement were shown. Separated peaks are indicated by the abbreviations of chemical structures. DCM: dichloromethane, *c*: *cis*, *t*: *trans*. (**B**) GC–MS scan chromatogram of Fraction A. TIC of FAMEs in Fraction A and its corresponding peaks are shown with the abbreviations.

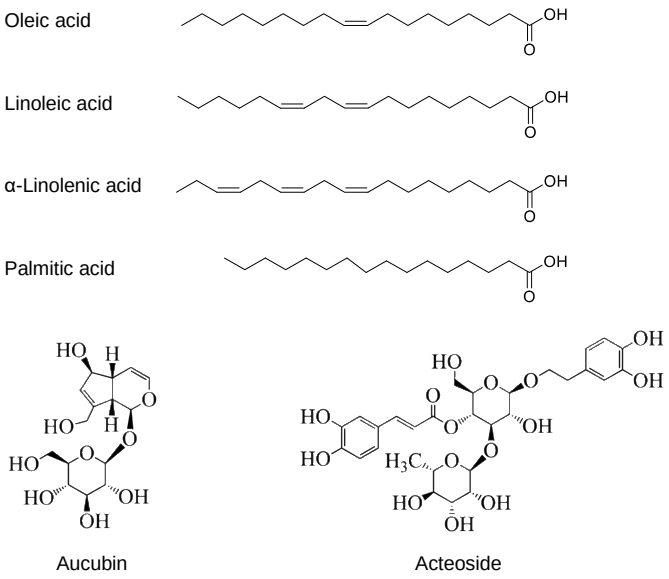


Figure 4. Chemical structures of constituents in *P. asiatica* seeds: unsaturated fatty acids (oleic, linoleic, and α-linolenic acids), a saturated fatty acid (palmitic acid), glycosides (aucubin and acteoside).

Next, the quantitative analysis by SIM was carried out for the high peaks corresponding to four fatty acids, *i.e.*, linoleic, oleic, α-linolenic, and palmitic acids. Because each peak in the TIC (Figure 4) has a specific *m*/*z* value, the content can be estimated by the quantitative analysis by SIM, even when the peaks are overlapping in TIC. The content estimated by this analysis was shown in Table 2. It is implied that these four fatty acids were abundant in Fraction A.

Table 2. The content of the top 4 fatty acids in Fraction A of PASE.

Fatty acid	Abbreviation [a]	Content in Fraction A [%] [b]
Linoleic acid	C18:2(9*c*,12*c*)	2.52
Oleic acid	C18:1(9*c*)	2.21
Palmitic acid	C16:0	1.34
α-Linolenic acid	C18:3(9*c*,12*c*,15*c*)	0.86

[a] The number of carbon atoms of a fatty acid and the number of double bonds after the colon. The positioning of the first double bond from the omega end in parenthesis. *c*: *cis* (= *Z*). [b] the content of each fatty acid was evaluated by GC–MS analysis (SIM mode) and is depicted as the percentage of the dry weight of Fraction A.

Constituents, other than fatty acids, were analyzed by the GC–MS analysis of Fraction A of PASE. Phenylpropanoids, such as isovanillic, phloretic, and ferulic acids were detected. However, the content of these phenylpropanoids was much less than that of fatty acids (data not shown).

Effects of acteoside and aucubin on NO production: The comparison of the IC_{50} values demonstrated that Fraction B had much less potency in the suppression of NO production than Fraction A (Table 1). Fraction B is thought to contain amphipathic glycosides, such as acteoside and aucubin (Figure 4). Because aucubin and acteoside are considered to possess anti-inflammatory activity [3,5–6,19], we examined the effects of these components of Fraction B on NO production in hepatocytes. When acteoside was added to the medium up to 600 μM (= 375 μg/mL) with IL-1β, it inhibited NO production to some extent without showing cytotoxicity to hepatocytes (Figure 5). However, after repeated NO assays, an IC_{50} value of acteoside could not be calculated according to the criteria described in the Materials and Methods. Although aucubin significantly suppressed NO induction at a concentration of 600 μM, the inhibiting activity was very low (Figure 5).

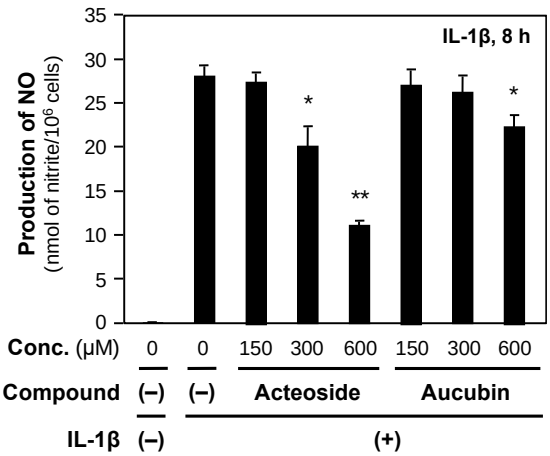


Figure 5. Effects of acteoside and aucubin on NO production in hepatocytes. Rat hepatocytes were treated with IL-1β in the absence or presence of acteoside or aucubin for 8 h. The NO levels were then measured as nitrite in the medium. The results presented as mean ± SD (n = 3). * $P < 0.05$ and ** $P < 0.01$ *versus* IL-1β alone.

DISCUSSION

The EtOAc-soluble fraction (Fraction A) is an active crude fraction extracted from *P. asiatica* seeds. We have clearly shown that PASE and Fraction A inhibited IL-1β-induced NO production in primary cultured rat hepatocytes. These results are in accordance with previous reports using LPS-treated macrophage lines. A water extract of *P. asiatica* seeds decreased NO production in RAW264.7 cells [7]. A methanol extract decreased both NO production and iNOS mRNA levels in J774.1 cells and did not have *in vitro* scavenging activity on NO radicals [8]. In general, the solvents for the preparation of extracts change the partition ratios of Fractions A, B, and C. When water is used, hydrophilic and amphipathic compounds are efficiently extracted, leading the increases in the ratios of these compounds in Fractions B and C. The content of glycosides, including acteoside and geniposidic acid, in a water extract may be higher than that in our 50% methanol extract. Therefore, it is difficult to elucidate which compounds in the extracts are responsible for the suppression of NO production.

Plant seeds and nuts are rich in diverse fatty acids. For example, the composition of oleic acid is more than 50% in hazelnut, almond, macadamia, and pistachio, while that of linoleic acid is 62% in walnut [20]. Special fatty acids are sometimes included in plants, such as punicic acid (also known as trichosanic acid) present in pomegranate seed oil, which is an isomer of conjugated α-linolenic acid and a ω-5 polyunsaturated fatty acid [21]. Fatty acids are analyzed by GC–MS or a rapid GC–FID/MS method that was recently reported [22]. According to our expectation that *P. asiatica* seeds contain many fatty acids, GC–MS analysis indicated that three unsaturated fatty acids (*i.e*., oleic, linoleic, and α-linolenic acids) and the saturated fatty acid, palmitic acid were rich in Fraction A of PASE (Figure 3, Table 2). Oleic acid decreased the expression of mRNAs encoding pro-inflammatory cytokines, such as iNOS, tumor necrosis factor α (TNF-α), IL-1β, and IL-6 [23]. Linoleic and α-linolenic and acids significantly decreased NO levels in the medium of RAW264.7 cells [24].

Which fatty acid is more effective in suppressing inflammatory responses? Ohata *et al.* compared the potency of inhibition of NO production in LPS-treated RAW264.7 cells; Dose-response curves indicated that α-linolenic acid (*i.e*., ω-3 polyunsaturated fatty acid) inhibited NO production more efficiently than linoleic acids, whereas oleic acid did not at the concentrations up to 100 μM [25]. However, the IC_{50} values of the fatty acids were not indicated. Ringbom *et al*. reported the inhibition of prostaglandin production by fatty acid [26]. An *in vitro* prostaglandin production assay using purified COX2 protein demonstrated that α-linolenic acid more efficiently inhibited the conversion of arachidonic acid to prostaglandin than other fatty acids; therefore, α-linolenic acid > linoleic acid >> oleic acid = palmitic acid [26]. We could not find any report that palmitic acid reduces NO production in the literature that we have searched to date. Other ω-3 polyunsaturated fatty acids, *i.e*., docosahexaenoic acid (DHA; C22:6 ω-3) and eicosapentaenoic acid (EPA; C20:5 ω-3), inhibited NO production at the similar efficiency with that of α-linolenic acid [26–27]. EPA and DHA are spontaneously peroxidized in the air, and peroxidized EPA and DHA inhibited NO production more efficiently than unoxidized ones in rat hepatocytes [28].

Acteoside and aucubin are glycosides in PASE, both of which are thought to be included in Fraction B. They had much less potency of the suppression of IL-1β-induced NO production (Figure 5), suggesting that they possess less anti-inflammatory activity than the unsaturated fatty acids. Ferulic acid, a phenylpropanoid, was detected at a low content in

Fraction A and had an IC_{50} value of 474 μM [11]. It seems likely that phenylpropanoids may have little contribution to the suppression of NO production. When *P. asiatica* seeds were extracted with ethyl ether, its extract was rich in essential oils, *e.g.*, eugenol, linalool, and bicyclogermacrene [29]. However, we could not detect terpenoids in Fraction A of PASE as 50% methanol extract by GC–MS analysis (data not shown). Collectively, the three unsaturated fatty acids are largely responsible for the suppression of IL-1β-induced NO production and may contribute to anti-inflammatory effects of *P. asiatica* seeds.

Anti-obesity and antidiabetic effects of *P. asiatica* seeds were previously reported [30–31]. When C57BL/6 mice were fed a high-fat diet for 16 weeks and then administered PASE as 60% ethanol extract, the levels of lipid accumulation and hyperglycemia were improved [30]. Acteoside and geniposidic acid were the main bioactive compounds in PASE and may be involved in a hypoglycemic effect of PASE on high-fat diet-induced mice [31]. Therefore, we performed a week-long experiment using leptin-deficient mice (genotype $Lep^{ob/ob}$, *ob*/*ob* mice), which is another model of obesity and type 2 diabetes mellitus [32]. Each crude fraction (Fraction A, B, or C) of PASE (50% methanol extract) was orally administered to *ob*/*ob* mice, and a standard diet alone was fed to mice as negative controls. There were no significant differences in the body weight, blood glucose levels, and serum triglyceride concentrations between the three test groups and the control group (LL, LC, and MN, unpublished data). It should be noted that the composition of extracts is affected by the solvents (*e.g.*, ethyl ether, methanol, ethanol, and water) and the content of each bioactive compound may be different. Comparison of these results is difficult due to the differences in the solvents of extraction. Selection of animal models (feeding of high fat diet or *ob*/*ob* mice) is the second factor that may affect the results. Therefore, more studies are required using animals to elucidate anti-obesity and antidiabetic effects of *P. asiatica* seeds.

CONCLUSION

The EtOAc-soluble fraction displayed high potency to inhibit NO production in hepatocytes. GC–MS analysis indicated that α-linolenic, linoleic, oleic, and palmitic acids were abundant in this fraction. The three unsaturated fatty acids are known to efficiently inhibit NO production in macrophages and may largely contribute to the anti-inflammatory activity of *P. asiatica* seeds. It is not clear whether *P. asiatica* seeds have anti-obesity and antidiabetic effects, which will be investigated in the future.

List of Abbreviations: NO: nitric oxide, LPS: lipopolysaccharide, iNOS: inducible nitric oxide synthase, IL: interleukin, EtOAc: ethyl acetate, GC–MS: gas chromatography–mass spectrometry, SIM: selected ion monitoring, PASE: *Plantago asiatica* seed extract, FAME: fatty acid methyl ester, LDH: lactate dehydrogenase, IC_{50}: half-maximal inhibitory concentration, SD: standard deviation.

Author contributions: Ashley Sholmire, Lauren Leischner, and Brendhan Garland performed the experiments and data collection in Japan as research intern. Toshinari Ishii and Yuko Yamauchi performed the experiments and data collection as graduate students. Saki Shirako carried out the experiments and analyzed the data. Yuto Nishidono reviewed and edited the manuscript. Yukinobu Ikeya, Laure Corey, and Mikio Nishizawa participated in the design of the study, supervised the study, and provided oversight in the drafting of the manuscript. All authors were involved in the performance of experiments and the preparation of the manuscript.

Competing Interests: No authors have financial interests or conflicts of interest.

Acknowledgments and Funding: We thank Ms Airi Fujii and Ms Mizuki Kajimura for technical assistance, Dr. Tetsuya Okuyama for invaluable advice, and Ms Noriko Kanazawa for secretarial assistance. This work was supported in part by research grants from the Henry R. Luce Foundation's Luce Initiative on Asian Studies and the Environment (LIASE) and the Asia-Japan Research Institute (AJI), Ritsumeikan University (Ibaraki, Osaka, Japan). The stay in Japan for Ashley Sholmire, Lauren Leischner, and Brendhan Garland was supported by the grant from LIASE.

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