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

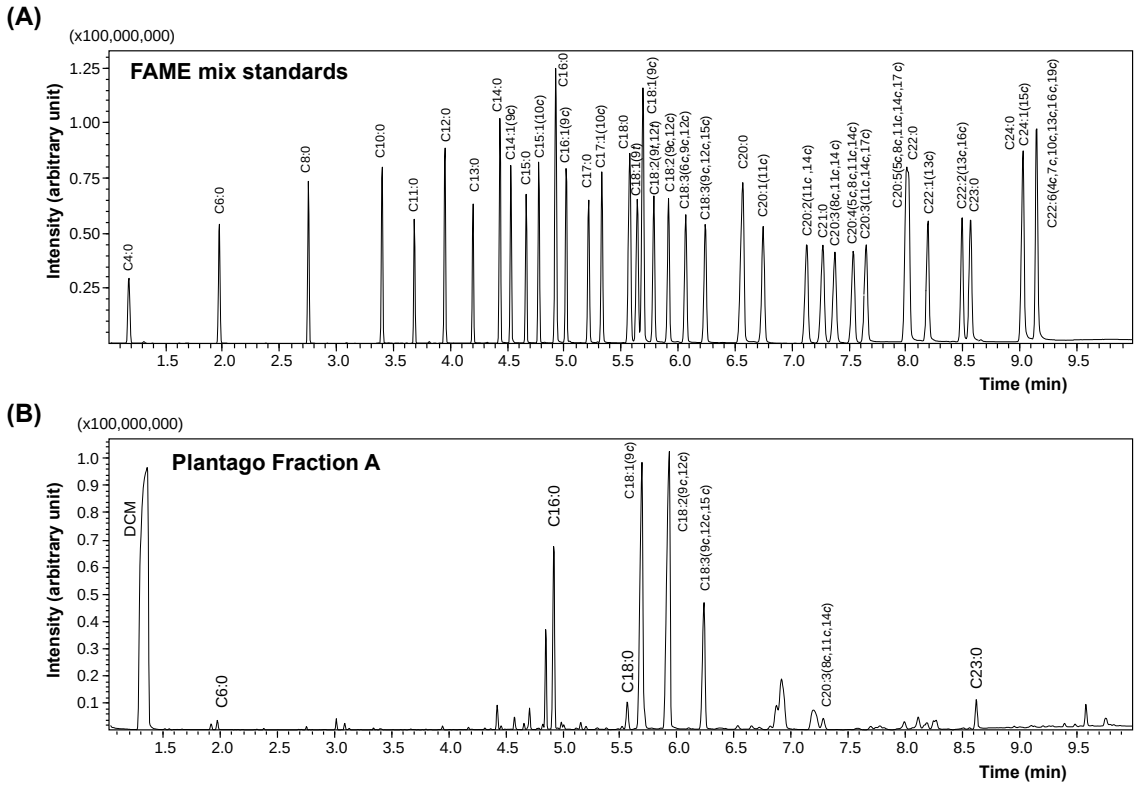

Figure 3. Detection of fatty acids in Fraction A of PASE. (**A**) GC–MS separation profiles of 37 FAMEs. Total ion chromatogram (TIC) of 37 FAME mix standards with scan measurement were shown. Separated peaks are indicated by the abbreviations of chemical structures. DCM: dichloromethane, *c*: *cis*,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

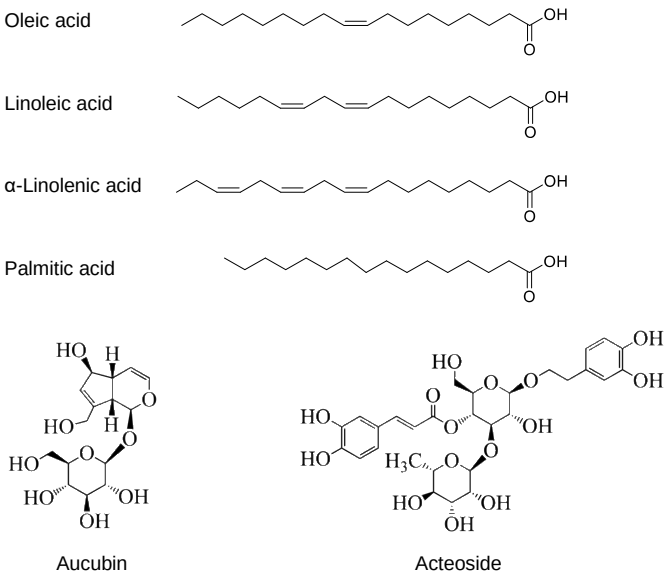

Figure 4. Chemical structures of constituents in *P. asiatica* seeds: unsaturated fatty acids (oleic, linoleic, and α-linolenic acids), a saturated fatty acid (palmitic acid), glycosides (aucubin and acteoside).

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).

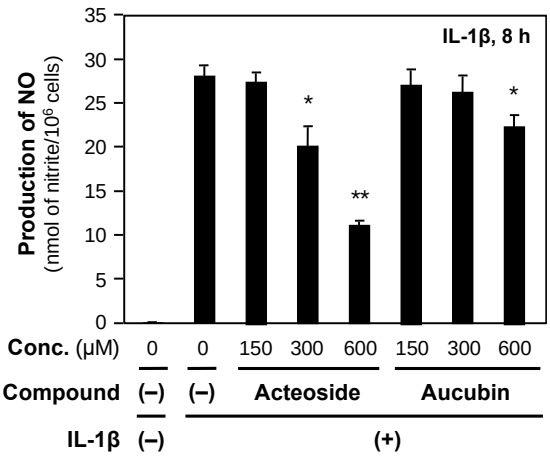

Figure 5. Effects of acteoside and aucubin on NO production in hepatocytes. Rat hepatocytes were treated with IL-1β in the absence or presence of acteoside or aucubin for 8 h. The NO levels were then measured as nitrite in the medium. The results presented as mean ± SD (n = 3). * $P < 0.05$ and ** $P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

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