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Original

Inhibitory Effect of *Polypodium Leucotomos* Extract on Cytochrome P450 3A-mediated Midazolam Metabolism

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Abstract: *Polypodium leucotomos* (PL) is a fern native to Latin America, and its extract is used as an oral sunscreen; however, its safety during use has not been adequately investigated. Therefore, the aim of this study was to evaluate food-drug interactions associated with PL extract mediated by cytochrome P450 3A (CYP3A) inhibition and induction. Inhibition of CYP3A-mediated midazolam (MDZ) 1'-hydroxylation activity by PL extract and its major phenolic components was evaluated *in vitro* using pooled human liver microsomes. In addition, MDZ pharmacokinetics were investigated in rats after a single dose, as well as after 1 week treatment with PL extract (30 mg/kg) in order to evaluate the inhibitory and inducible effects of PL on CYP3A *in vivo*, respectively. Serum MDZ concentrations were analyzed and pharmacokinetic parameters were compared between PL- and water (control)-treated groups. *In vitro*, PL extract decreased MDZ 1'-hydroxylation activity in a concentration-dependent manner. However, the major phenolic compounds in PL extracts, namely caffeic, chlorogenic, *p*-coumaric, ferulic, and vanillic acids, did not exhibit any marked inhibitory effects on MDZ 1'-hydroxylation activity. *In vivo*, administration of a single dose of PL extract to rats significantly increased the area under the serum concentration-time curve from time 0 to infinity ($AUC_{0-\infty}$) and the maximum serum concentration (C_{max}) of MDZ (by 57% and 88%, respectively; $P < 0.05$). In contrast, there were no significant changes in MDZ pharmacokinetic parameters after 1 week of treatment with PL extract. These results suggest that PL extract can cause a food-drug interaction by inhibiting CYP3A.

Key words: food-drug interaction, cytochrome P450, *Polypodium leucotomos*, CYP3A

Introduction

Sunscreens are widely used to protect against exposure to ultraviolet (UV) radiation, which causes sunburn, photoaging, and skin cancer. However, sunscreen needs to be reapplied frequently, and is difficult to use to achieve a demonstrably adequate effect. Therefore, oral

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supplements have recently attracted attention as an alternative and easier method of sunburn prevention than topical application¹.

Polypodium leucotomos (PL) is a fern native to Latin America, and its extract is expected to have photoprotective benefits through its antioxidative, chemoprotective, immunomodulatory, and anti-inflammatory effects². The major active ingredients of PL are phenolic compounds, such as caffeic, chlorogenic, and ferulic acids, which are effective for the treatment of vitiligo, chloasma, and polymorphous light eruption, as well as in the prevention of skin cancer²⁻⁸. In Japan, PL extract is easily purchased as an oral supplement on Internet shopping sites and in drugstores, so its use has become widespread. Therefore, it is possible that PL extract and other medicines may be taken simultaneously, but the safety of the combined use of PL with other medications has not been studied.

It is well known that drug-drug interactions may occur, and these need to be considered when coadministering multiple drugs. These drug-drug interactions may attenuate the effects of coadministered medicines or cause unexpected adverse effects, and so attention should be paid to avoid such interactions. Induction and inhibition of cytochrome P450 (CYP), a typical drug-metabolizing enzyme, are considered major causes of drug-drug interactions. Cytochrome P450 3A (CYP3A), a CYP isoform, is expressed not only in the liver, but also in the small intestine, and it is involved in the metabolism of many drugs, including erythromycin, cyclosporine, and nifedipine. Therefore, CYP3A is important when considering drug-drug interactions⁹⁻¹¹.

In addition to drug-drug interactions, several food-drug interactions have been identified. For example, grapefruit juice (GFJ) has been reported to inhibit CYP3A in the small intestine, thereby increasing blood concentrations of coadministered nifedipine, with a 1.5-fold increase in the area under the serum concentration-time curve (AUC)¹². In contrast, St. John's Wort (SJW) is known to induce hepatic and small intestinal CYP3A, markedly lowering blood concentrations of simvastatin and decreasing its AUC by approximately half¹³. Therefore, the effectiveness and safety of medicines may be altered in combination with foods. However, information regarding such potential interactions is currently limited. Because supplements are classified as "food" in Japan, it is not a legal requirement to conduct research on potential interactions between supplements and drugs. However, to prevent clinical adverse events, it is important to clarify potential food-drug interactions associated with CYP3A, which is involved in the metabolism of many drugs.

Although recently PL extract has become widely used by people of all generations as a simple and alternative method to the administration of sunscreen, its safety in combination with medicines has not been investigated. Against this background, the present study was conducted to clarify food-drug interactions mediated by inhibition and induction of CYP3A by PL extract.

Material and methods

Chemicals

Heliocare, which contains 240 mg PL extract in each capsule (480 mg), was purchased from Ferndale Healthcare (Ferndale, MI, USA). Caffeic, chlorogenic, and vanillic acids were purchased

from Tokyo Chemical Industry (Tokyo, Japan) and *p*-coumaric and ferulic acids were purchased from Sigma-Aldrich (St. Louis, MO, USA). Midazolam (MDZ) maleate was a kind gift from Nippon Roche (Tokyo, Japan). MDZ injection (Dormicum injection 10 mg / 2 ml) was purchased from Astellas Pharma (Tokyo, Japan), 1'-hydroxymidazolam was purchased from Daiichi Pure Chemicals (Tokyo, Japan), and diazepam and nitrazepam were purchased from Wako Pure Chemical Industries (Osaka, Japan). Pooled human liver microsomes were purchased from the Non Profit Organization Human and Animal Bridging Research Organization (Chiba, Japan). All other chemicals used were of analytical grade.

Assay of CYP3A activity

An *in vitro* study was undertaken to investigate the inhibitory effect of PL extract and its major phenolic components, namely caffeic, chlorogenic, *p*-coumaric, ferulic, and vanillic acids, on CYP3A-mediated MDZ 1'-hydroxylation (MDZ 1'-OH) activity using pooled human liver microsomes.

Heliocare was suspended in distilled water to a concentration of 240 mg / ml PL extract, vortexed for 5 min, centrifuged at $1,600 \times g$ for 10 min at room temperature, and the resultant supernatant was collected and considered as a 100% extract solution. Then, the solution was diluted with distilled water to final concentrations ranging from 2.5% to 10% and used for *in vitro* experiments; the five phenolic compounds were used at final concentrations ranging from 5 to 500 $\mu\text{g} / \text{ml}$.

The reaction mixtures consisted of pooled human liver microsomes (100 μg), 10 μM MDZ, and the nicotinamide adenine dinucleotide phosphate (NADPH)-generating system in 80 mM phosphate buffer (pH 7.4). Mixtures were incubated at 37°C for 5 min in the presence of either PL extract or any of the five phenolic compounds at the different concentrations tested. Reactions were quenched by the addition of a mixture of cold methanol: acetonitrile (35:21), and then nitrazepam (200 ng) was added as an internal standard. Then, 1'-hydroxymidazolam was analyzed using HPLC according to the methods of Wrighton and Ring¹⁴). Control activity was determined under the same conditions with reaction mixtures containing water instead of PL extract or phenolic compounds. Results are expressed as a percentage of control activity. Inhibition constant (K_i) values and inhibition modes were determined for PL extract.

In addition, a preincubation study was performed to determine whether metabolic intermediates produced from the PL extract components inactivate CYP3A. In these studies, PL extract (1.25%) was preincubated with pooled human liver microsomes at 37°C for 10, 20, or 30 min in the presence or absence of NADPH. After the preincubation step, 10 μM MDZ was added and MDZ 1'-OH activity was assayed as described above.

Animals

Male Sprague-Dawley rats weighing 200–230 g were obtained from Japan Laboratory Animals (Tokyo, Japan). Rats were housed in a temperature-controlled room under a 12-h light-dark cycle with free access to standard laboratory chow and water. Rats were starved overnight

before the day of the pharmacokinetic experiments. The study procedures were reviewed and approved by the Showa University Ethics Committee for Animal Care and Use and the experiments were conducted in accordance with guidelines established by the Institutional Animal Care and Use Committee of Showa University (Permit no.07002) and the Guide for the Care and Use of Laboratory Animals (8th edition) of the National Institutes of Health (NIH) <https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf>, accessed 2017-6-14).

Treatment and sampling

In the single treatment study, PL extract (30 mg/kg; corresponding to 60 mg/kg Heliocare) or water (control) was administered orally to rats ($n=3-4$) 30 min before oral administration of MDZ (15 mg/kg). In the 1-week treatment study, rats ($n=6$) were treated with PL extract (30 mg/kg) or water orally once a day for 1 week, and MDZ was administered 24 h after the last administration of extract or water. Blood samples (200 μ l) were collected from the jugular vein before and at 15, 30, 45, 60, 90, 120, 180, and 240 min after MDZ administration. Samples were centrifuged at $7,500 \times g$ for 10 min at 4°C , and each serum sample was stored at -80°C until analysis.

After blood sampling of rats treated for 1 week with PL extract, the rats were killed and their livers and small intestines excised. Liver and intestinal microsomes were prepared using a conventional fractional centrifugation method¹⁵⁾ and previously described methods with minor modifications^{16, 17)}, respectively. All microsomal fractions were frozen with liquid nitrogen and stored at -80°C until use. Protein concentrations were measured using the method of Lowry *et al*¹⁸⁾.

MDZ pharmacokinetic analysis

MDZ serum concentrations were determined using previously described HPLC methods¹⁹⁻²¹⁾ with slight modifications. The pharmacokinetic parameters of MDZ were assessed using non-compartmental analysis with MOMENT (Excel; Microsoft, Bellevue, WA, USA) based on the moment analytic method^{22, 23)}. The AUC from time 0 to infinity ($\text{AUC}_{0-\infty}$) was calculated according to the trapezoidal rule. The maximum serum concentration (C_{max}) was obtained from the actual data. The elimination half-life ($t_{1/2}$) was calculated by dividing $\ln 2$ by λ , which is the terminal elimination rate constant calculated using a linear regression analysis of at least three points from the terminal portion of the concentration-time plot.

Ex vivo study

Liver and intestinal microsomes, prepared 1 day after the last administration of PL extract in the 1-week treatment study, were used in the *ex vivo* study. Because MDZ is known to be predominantly metabolized to 4-hydroxymidazolam and, to a lesser extent, 1'-hydroxymidazolam by hepatic and intestinal CYP3A in rats, we analyzed MDZ 4-hydroxylation (MDZ 4-OH) activity as described previously^{24, 25)}.

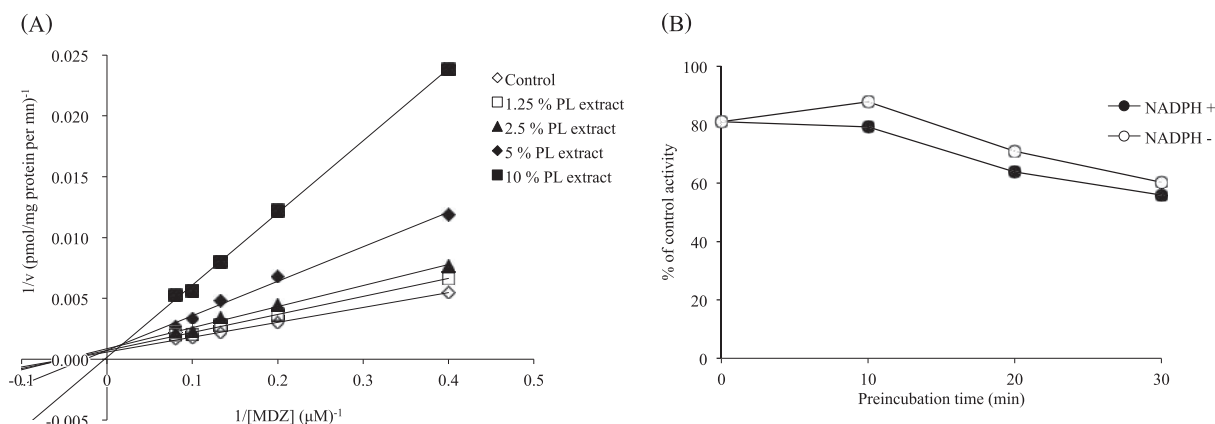


Fig. 1. Inhibitory effect of *Polypodium leucotomos* (PL) extract on cytochrome P450 3A (CYP3A) activity *in vitro* (A) The direct inhibitory effect of PL extract was investigated using a Lineweaver-Burk plot.

(B) Effect of preincubation with PL extract (1.25%) on midazolam 1'-hydroxylation (MDZ 1'-OH) activity in the presence (closed circles) or absence (open circles) of NADPH. Data show mean values of experiments performed in duplicate or triplicate.

Statistical analyses

In vitro data are expressed as mean values of experiments performed in duplicate. Data from *in vivo* and *ex vivo* experiments are presented as the mean \pm SD. The significance of differences between the groups was analyzed using the Mann-Whitney *U*-test and two-sided $P < 0.05$ was considered significant.

Results

CYP3A activity *in vitro*

PL extract inhibited CYP3A-mediated MDZ 1'-OH activity in pooled human liver microsomes in a concentration-dependent manner. Lineweaver-Burk plots showed that the inhibition mode was competitive with a K_i of 3.33% (Figure 1A). In contrast, the five major phenolic compounds contained in PL extract, namely caffeic, chlorogenic, *p*-coumaric, ferulic, and vanillic acids, did not exhibit any marked inhibitory effects on CYP3A activity (Figure 2).

To determine whether PL possibly forms metabolic intermediates that inhibit CYP3A activity during incubation, MDZ 1'-OH activity was assayed by including a 10- to 30-min preincubation step. The results of these experiments showed that including the preincubation step did not increase the inhibition of MDZ 1'-OH activity by PL extract (Figure 1B).

Pharmacokinetics of MDZ

Serum MDZ concentration-time profiles and pharmacokinetics 30 min after a single treatment with PL extract or water (control) are shown in Figure 3 and Table 1. The mean $AUC_{0-\infty}$ of MDZ increased from 480 ± 102 to 755 ± 61 ng·hr/ml per h, and the C_{max} increased from 462 ± 125 to 871 ± 164 ng/ml ($P < 0.05$ vs control), without any change in $t_{1/2}$. In contrast, in the 1-week treatment study, there were no significant differences in serum concentrations, $AUC_{0-\infty}$, C_{max} , or $t_{1/2}$.

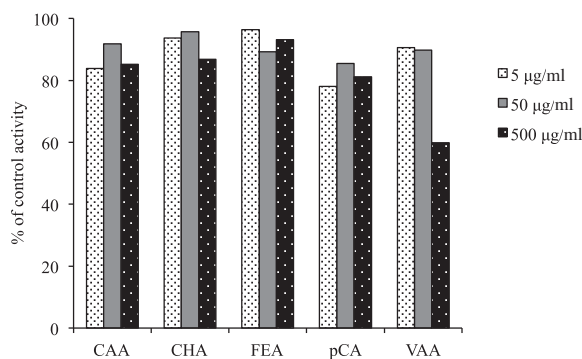


Fig. 2. Inhibitory effect of the major components of *Polypodium leucotomos* (PL) extract on midazolam 1'-hydroxylation (MDZ 1'-OH) activity *in vitro*. Components were added to the incubation mixture at final concentrations of 5–500 µg/ml. Data are mean values of experiments performed in duplicate. CAA, caffeic acid; CHA, chlorogenic acid; FEA, ferulic acid; pCA, *p*-coumaric acid; VAA, vanillic acid.

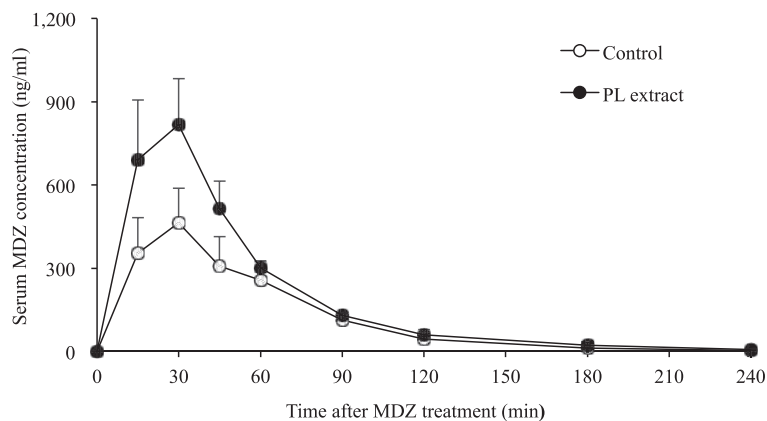


Fig. 3. Serum concentration-time profile of midazolam (MDZ) after treatment of rats with a single dose of *Polypodium leucotomos* (PL) extract. Rats were administered PL extract (30 mg/kg) or water (control) orally 30 min before oral administration of MDZ (15 mg/kg). Data show the mean \pm SD of three to four rats.

Table 1. Pharmacokinetic parameters of midazolam (MDZ) after a single treatment with *Polypodium leucotomos* (PL) extract in rats

	AUC _{0-∞} (ng·hr/ml)	C _{max} (ng/ml)	t _{1/2} (min)
Control	480 \pm 102	462 \pm 125	30 \pm 4
PL extract	755 \pm 61*	871 \pm 164*	40 \pm 15

Data are the mean \pm SD of three to four rats.

* $P < 0.05$ compared with control.

AUC_{0-∞}, area under the serum concentration-time curve from time 0 to infinity;

C_{max}, maximum serum concentration; t_{1/2}, half-life.

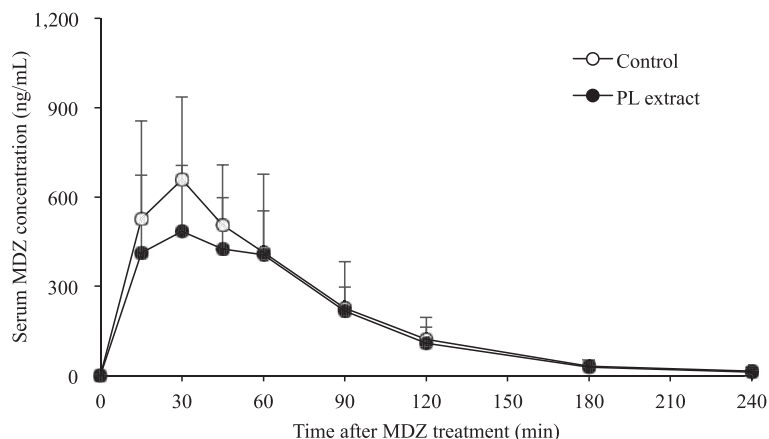


Fig. 4. Serum concentration-time profiles of midazolam (MDZ) after 1 week of treatment of rats with *Polypodium leucotomos* (PL) extract

Rats were pretreated with PL extract (30 mg/kg) or water (control) once a day for 1 week, and MDZ (15 mg/kg) was then administered orally 24 h after administration of the last dose of PL extract. Data are the mean \pm SD of six rats.

Table 2. Pharmacokinetic parameters of midazolam (MDZ) after 1 week treatment of rats with *Polypodium leucotomos* (PL) extract

	AUC _{0-∞} (ng·hr/ml)	C _{max} (ng/ml)	t _{1/2} (min)
Control	831 \pm 274	712 \pm 309	37 \pm 11
PL extract	603 \pm 146	542 \pm 215	37 \pm 6

Data are the mean \pm SD of six rats.

AUC_{0-∞}, area under the serum concentration-time curve from time 0 to infinity;

C_{max}, maximum serum concentration; t_{1/2}, half-life.

of MDZ between the PL- and water-treated groups (Figure 4; Table 2).

CYP activity ex vivo

After 1 week of treatment with PL, there were no significant differences in CYP3A-mediated MDZ 4-OH activity between the PL- and water-treated groups in either liver microsomes (922.82 \pm 247.61 vs 1,090.93 \pm 136.32 pmol/mg protein per min; Figure 5A) or intestinal microsomes (22.78 \pm 11.35 vs. 28.46 \pm 22.08 pmol/mg protein per min; Figure 5B).

Discussion

In the present study we investigated the inhibitory and inducing effects of PL extract on CYP3A to clarify potential food-drug interactions mediated by this enzyme. The results revealed that PL extract inhibited CYP3A-mediated MDZ metabolism both *in vitro* and *in vivo*.

First, our investigation of the inhibitory effect of PL extract on MDZ 1'-OH activity in human liver microsomes *in vitro* showed competitive inhibition of CYP3A activity. The extract primarily

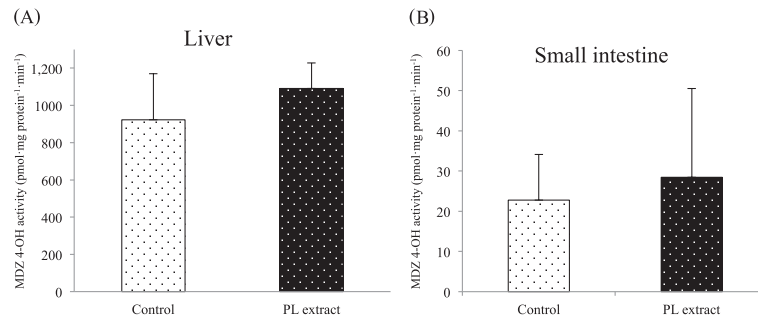


Fig. 5. Effects of 1 week treatment of rats with *Polypodium leucotomos* (PL) extract on cytochrome P450 3A (CYP3A) activity. Rats were pretreated with PL extract (30 mg/kg) or water (control) once a day for 1 week, and liver and small intestine microsomes were prepared 1 day after the last treatment. Midazolam 4-hydroxylation (MDZ 4-OH) activity was analyzed in (A) liver and (B) small intestine microsomes. Data are the mean \pm SD of six rats.

contains phenolic compounds, such as caffeic, chlorogenic, ferulic, hydroxycinnamic, *p*-coumaric, and vanillic acids, which have been reported to be the active ingredients mediating the sunscreen effect of PL extract²⁾. To investigate whether these compounds are involved in PL-induced inhibition of CYP3A, we examined the CYP3A-inhibiting activity of each of the compounds separately. In contrast with PL extract, none of the phenolic compounds significantly inhibited CYP3A activity. The concentration range of the compounds used in this study (5–500 $\mu\text{g/ml}$) is considered sufficiently high based on estimated amounts transported in the blood after treatment with PL extract²⁶⁾. Therefore, the lack of any significant inhibition of CYP3A by these five compounds suggests that other components of PL extract likely contributed to its inhibition of CYP3A.

The Heliocare used in this study contains 240 mg PL extract and it is recommended that 1–2 capsules be consumed per day. Although the types of ingredients and their amounts entering the blood after administration of PL extract are not known, we could assume that there are relatively high concentrations in the small intestine, indicating that possible drug interactions via intestinal CYP3A inhibition should be considered. In the present *in vitro* study, because the PL extract was suspended in water, as described in the Materials and Methods, and the supernatant was considered to be a 100% PL extract, we consider that there are likely to be fewer ingredients, in lower amounts, than that actually ingested after taking a Heliocare capsule. Therefore, it is fully conceivable that the inhibition of CYP3A observed *in vitro* was the result of treatment with the PL extract.

In this study, we also performed *in vivo* experiments using rats to clarify whether this CYP3A-mediated food-drug interaction occurs *in vivo*. Following treatment with a single dose of PL extract, there were significant increases in both the $\text{ACU}_{0-\infty}$ and C_{max} of MDZ compared with the control group. However, it should be noted that the 60 mg/kg dose of Heliocare used in our *in vivo* study (30 mg/kg as PL extract) was 7.5-fold higher than the usual dose in humans. However, efficacious drug doses are generally higher in rats than in humans. Therefore, the

dose of PL extract used in the present study was based on a previous report investigating the antioxidant effect of PL extract against UVB/UVA in rats²⁷). The results of the present study suggest that PL extract also increases blood MDZ concentrations *in vivo* by inhibiting CYP3A activity. Recently, PL extract has attracted attention as a sunscreen supplement and there are concerns about its coadministration with medicines. Because CYP3A is involved in the metabolism of many drugs, precautions are needed to avoid the development of unexpected adverse effects, especially with medicines that have a narrow therapeutic range.

In addition, mechanism-based inhibition (MBI) is a known mechanism of CYP inhibition. MBI is important clinically because the metabolic intermediates metabolized by CYP are irreversibly bound, resulting in potent and sustained inhibition. A typical example of MBI occurs with GFJ, known to inactivate CYP3A irreversibly with an effect that lasts at least for 3 days^{12, 28}). Therefore, we conducted an *in vitro* study including a preincubation step to investigate the possible occurrence of MBI with PL extract. However, no further inhibition of MDZ 1'-OH was observed when PL extract was preincubated with human liver microsomes and NADPH before the addition of MDZ. Therefore, we concluded that the mechanism by which PL extract inhibits CYP3A activity is direct rather than involving MBI.

PL extract may be ingested as a single treatment, but it is used for long periods for sun protection, especially in the summer season. Therefore, it is important to investigate food-drug interactions via induction of CYP3A in addition to the inhibitory effects of PL extract. For example, blood concentrations of simvastatin have been reported to be markedly decreased as a result of the induction of CYP3A by SJW¹³). In such situations, cotreatment with this supplement should be avoided because it may decrease the efficacy of medicines metabolized by this enzyme. Our examination of the potential induction of CYP3A by a 1-week of treatment of rats with PL extract showed no significant differences in the pharmacokinetic parameters of MDZ administered 24 h after the final PL administration compared with the control group. In addition, there were no changes in either liver or small intestine CYP3A activity after 1 week treatment with PL extract, suggesting that PL extract did not induce CYP3A. Meanwhile, in the 1-week treatment study, because MDZ was administered 24 h after the last dose of PL extract, it seems that the components had already been eliminated and no inhibition of CYP3A was observed. Further studies are needed to clarify the inhibitory effects of multiple doses of PL extract on CYP3A-mediated drug metabolism.

Recently, the self-medication concept has been expanding in Japan, and the use of herbal supplements has increased among the population. However, knowledge and information regarding the safety of the combined use of herbal supplements and conventional medicines is considered insufficient. Thus, the investigation of possible food-drug interactions is important for the safe and effective use of herbal supplements, which is the motivation for the present study. It is important to clarify whether the results of the present *in vitro* and *in vivo* rat experiments also occur in humans. In addition, experiments are needed to provide similar information on other CYP isoforms, such as CYP2C9 and CYP2D6, which metabolize many important medicines used clinically.

In conclusion, PL extract, which has recently drawn attention as a sunscreen supplement, inhibited CYP3A activity, indicating the possibility of increased blood concentrations of coadministered medicines that are metabolized by this enzyme. In contrast, PL extract was shown to be less likely to cause drug interactions as a result of CYP3A induction.

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Conflicts of Interest

The authors have no conflicts of interest to declare.

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