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Original Research Paper

Effects of flavonoids derived from *Taxus yunnanensis* on p-glycoprotein and cytochrome P450 3A4

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ABSTRACT

The intestinal uptake of paclitaxel is hampered by trans-membrane efflux transporters such as P-glycoprotein (P-gp), and paclitaxel is mainly metabolized by cytochrome P450 3A4 (CYP3A4) presented in the liver. Our previous results demonstrated that flavonoids extracted from *Taxus yunnanensis* could improve the oral absorption of paclitaxel. The current study was purposed to investigate the effects of the flavonoid extracts on P-gp and CYP3A4 in vitro. The expression and activity of P-gp were detected by western blotting and intracellular rhodamine 123 accumulation assay in Caco-2 cells treated with the flavonoids extract. The expression of CYP3A4 was investigated by western blotting in mouse primary hepatocytes and the activity of CYP3A4 was detected by LC-MS/MS method using rat liver microsomes. Our results showed that the flavonoid extracts from *T. yunnanensis* could inhibit P-gp activity and concurrently decrease the expression and activity of CYP3A4. In conclusion, activity of P-gp and CYP3A4 could be inhibited by flavonoids extracted from *T. yunnanensis* which might be potential candidates for development of oral formulation of paclitaxel.

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1. Introduction

Paclitaxel was derived from the bark of the pacific yew tree (*Taxus brevifolia*) and worldwide used as one of the most important anticancer drugs for the treatment of various tumors such as breast, ovarian and lung cancer [1,2]. Until recently, paclitaxel is clinically formulated in a mixture of Cremophor EL and dehydrated ethanol for the intravenous infusion because its intestinal uptake is avidly hampered by transmembrane efflux transporter such as P-glycoprotein [3,4]. Since Cremophor EL itself could be toxic and the oral administration has many advantages over intravenous administration, oral paclitaxel formulation is still needed as a critical option for treating cancer [5]. Oral paclitaxel formulas have been tried for decades, including concomitant use of P-glycoprotein inhibitors, such as verapamil, tamoxifen,

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cyclosporine A [6–8]. In addition, paclitaxel is mainly metabolized by the liver, several studies have demonstrated that co-administration of CYP3A4 inhibitors could enhance the oral bioavailability of paclitaxel [9,10]. However, no oral formula of paclitaxel has been approved by far. The use of those drugs is limited because the required concentrations to modulate P-gp and CYP3A could result in cardiac toxicity and other adverse effects [11]. Therefore, compounds that can inhibit P-gp and CYP3A4 activity with low toxicity are highly desired to increase the systemic exposure of paclitaxel.

Taxus yunnanensis Cheng et L.K. Fu, which belongs to the plant family Taxaceae is widely distributed in south China. The branches, leaves and seeds of this plant have been used in traditional Chinese medicine for the treatment of kidney problem and diabetic conditions. In recent years, *T. yunnanensis* has been exploited a lot for its high content of paclitaxel. Meanwhile, many other components except for paclitaxel found in *T. yunnanensis* were shown biological activities [12,13].

Our previous pharmacokinetic results showed that oral bioavailability of paclitaxel was elevated by 10 times while the whole extract derived from *T. yunnanensis* containing paclitaxel was orally administrated to rats. Interestingly, flavonoid extract derived from *T. yunnanensis* was further confirmed to improve the oral bioavailability of paclitaxel (Data not show). According to our previous studies, flavonoids derived from *T. yunnanensis* might exert synergistic effect on paxlitaxel by affecting its pharmacokinetic behavior. However, the underlying mechanism is unclear yet. Many natural products have been widely reported to be able to increase the systemic exposure of paclitaxel by inhibiting the activity of P-glycoprotein and/or CYP3A [14–16]. Therefore, in this study, we investigated the effect of flavonoids derived from *T. yunnanensis* on P-gp and CYP3A4 to understand whether the flavonoids extract increased the systemic exposure of paclitaxel by inhibiting P-gp and/or CYP3A4.

2. Materials and methods

2.1. Materials

Barks and leaves of *T. yunnanensis* Cheng et L.K. Fu was supported by Zhongda Nanyao Co Ltd. Male Sprague–Dawley rats (weighing about 270–300 g) and C57 mice (weighing about 20–25 g) were obtained from the Experimental Animal Center of Sun Yat-sen University. DMEM (Dulbecco's Modified Eagle's medium) was obtained from HyClone. HBSS, PBS were obtained from GIBCO. Penicillin and streptomycin were from North China pharmaceutical Group Corporation. NADPH was obtained from Roche Diagnostics Ltd (Beijing, China). Trizol reagent, the Williams' medium E, fetal bovine serum and L-glutamine were purchased from Invitrogen (Carlsbad, CA). BSA was from Sangon Biotech (Shanghai) Co. Ltd. Antibodies of rabbit anti-mouse cyp3a11 and horseradish peroxidase-labeled anti-goat IgG were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Methanol and Tert-Butyl methyl ether of high performance liquid chromatography (HPLC) grade were purchased from Tedia Inc. (Beijing, China). All other reagents were of analytical grade or HPLC grade.

2.2. Extraction, isolation and analysis of flavonoids

Dried and pulverized barks and leaves of *T. yunnanensis* Cheng et L.K. Fu were extracted in back streaming apparatus with petroleum ether (b.p. 61 °C) and 70% ethanol/water for 3 times. The ethanol/water extract was concentrated and dried in the evaporator. Flavonoids were chromatographed over Sephadex LH-20 column with ethanol and determined by HPLC-UV and UV. Chromatographic fingerprint analysis of flavonoids was performed on Shimadzu LC-20 UFLC system (Shimadzu, Tokyo, Japan) equipped with an LC-20AD pump, a SIL-20A autosampler, a CTO-20A oven and an SPD-M20A detector. The separation and analysis of flavonoids was performed on a C18 Column (250 mm × 2.1 mm, 5 μm, Gemini, Phenomenex, USA) kept at 25 °C. The mobile phases was consisted of methanol (A) and water (B) as following gradient programs at 1.0 ml/min: 0–30 min (30%–60% A), 30–40 min (60%–80% A), 40–60 min (80% A), 60–65 min (80–100% A), 65–75 min (100% B), 75–80 min (100%–30% A), 80–90 min (30%). The eluent was monitored at wavelength of 190–600 nm. The injection volume for all samples was 20 μl.

2.3. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in H-DMEM containing 10% FBS in a humidified atmosphere of 5% CO_2 at 37 °C [17].

2.4. Isolation and culture of primary hepatocytes

The primary hepatocytes were isolated from C57 mice, which were supplied by the Laboratory Animal Service Center at Sun Yat-sen University (Guangzhou, China). Mouse was injected with 5 μl/g 0.7% chloralic hydras, and hepatic portal vein was perfused at 5 ml/min speed with digestion buffer to get excised liver. Then liver was cut up in the wash buffer and got through 100 × mesh sheet. Hepatocytes were obtained after spinning 600 r/min and seeded into 12-well plates. The cells were cultured with daily change of media (Williams' E medium supplemented with L-glutamine, and fetal bovine serum), after 48 h, the hepatocytes were treated with media containing the test chemicals [18].

2.5. Detection of expression of P-gp and CYP3A4 by western blotting analysis

P-gp expression was evaluated by western blot analysis in Caco-2 cells cultured for 48 h in presence or in absence of the flavonoid compounds. Caco-2 cells were digested in 0.25% trypsin and lysed for protein extraction. Protein concentrations of cells were determined using the lowry method [19] and bovine serum albumin as a standard. The protein sample (30 μg) was separated in 10% (w/v) SDS – polyacrylamide gel and blotted into a nitrocellulose membrane (Immobilon transfer membrane) at 250 mA for 90 min. The membranes were blocked for 2 h at room temperature in TBST containing 5% non-fat milk and then were incubated with P-gp antibody (1:500) or GAPDH (1:1000) overnight. Membranes were incubated with secondary antibody for 2 h. The protein bands were obtained from Gel image system. The expression of P-gp was

normalized by that of GADPH. Cyp3a11 (CYP3A4 in human) expression was evaluated using mouse primary liver cell and incubated with anti-cyp3a11 antibody.

2.6. *Effect of flavonoids on P-gp activity*

Caco-2 cells were incubated in 37 °C, 5% CO_2 flask for 1 week, and were further seeded into 96-well black plate with clear bottom at a density of 10,000 cells per well for 2 days. Then cells were treated with cyclosporine A (the positive control, 10 μM), vehicle (0.1% DMSO) and flavonoid components in three groups at concentrations of 0.5 μg/ml, 5 μg/ml and 50 μg/ml for 1 h or 48 h. Rhodamine 123 (10 μM) and MK571 (MRP inhibitor, 50 μM) was added. After 1 h, cells were kept on ice to stop P-gp activity and washed twice with ice-cold Hanks to remove extra cellular rhodamine 123. A total of 200 μl Hanks was added and fluorescence of intercellular rhodamine 123 was scanned by a CytoFlour fluorescence multiwall plate reader at an excitation wavelength of 485 nm and an emission wavelength of 535 nm [20].

2.7. *Preparation of rat liver microsomes*

Hepatic tissues were collected from healthy male Sprague–Dawley rats (230–310 g). Liver microsomes were prepared as described previously, resuspended in 0.125 M KCl/0.01 M potassium phosphate, stored at −80 °C, and used in one month [21].

2.8. *Effect of flavonoids on CYP3A4 activity*

The liver microsomes (0.25 mg/ml) were incubated in presence or in absence of the flavonoid compounds at different concentrations for 30 min, midazolam (MDZ) was added and after 15 min tert-butyl methyl ether/dichloromethane (75:25, v/v) was added to stop metabolism of MDZ. CYP3A4 activity was evaluated by amount of the metabolite of MDZ: 1-OH-midazolam (1-OH-MDZ), which was detected by liquid chromatography/tandem mass spectrometry [22].

2.9. *Transient transfection and reporter gene assay*

LS174T cells were seeded into 96-well plate and cultivated for 24 h. Transfection was performed in Phenol red-free RPMI 1640 medium supplemented with charcoal/dextran-treated FBS. The cells were transfected with 100 ng PSG5-PXR, 100 ng PGL3-CYP3A4-XREM-reporter, 5 ng PLR-TK as an internal control. 50 ng PSG5 was transfected instead of PSG5-PXR as vector control. After 6 h, cells were exposed to RIF at 10 μm, which is a known PXR transactivator, KTZ (25 μm, the positive control), and flavonoids at different concentrations (0.8, 4, 20, 100 μg/ml) for 24 h, the cells were harvested and analyzed for luciferase expression with a dual-luciferase reporter assay kit (Promega) [23].

3. Results and discussion

3.1. *Polychemistry analysis of extracts*

The flavonoid extracts were obtained through fractionation of ethanol extract of *T. yunnanensis* using macroporous absorbent resin chromatography and HPLC analysis. As shown in Fig. 1, chromatographic fingerprint analysis of flavonoids contained amentoflavone, bilobetin, 7-O-methylamentoflavone, ginkgetin, sciadopitysin.

3.2. *Effects of flavonoids derived from* T. yunnanensis *on P-gp*

Western bolt analysis was performed to observe the effect of flavonoid extracts on the expression of P-gp. As shown in Fig. 2, expression of P-gp was only inhibited slightly as concentration of flavonoids was up to 50 μg/ml. Furthermore, intracellular rhodamine 123 accumulation assay was conducted using Caco-2 cells to investigate the effect of flavonoids on the activity of P-gp. Inhibition of P-gp activity leads to decrease of the efflux of intracellular rhodamine 123 and increase of fluorescence associated with intracellular rhodamine 123. After

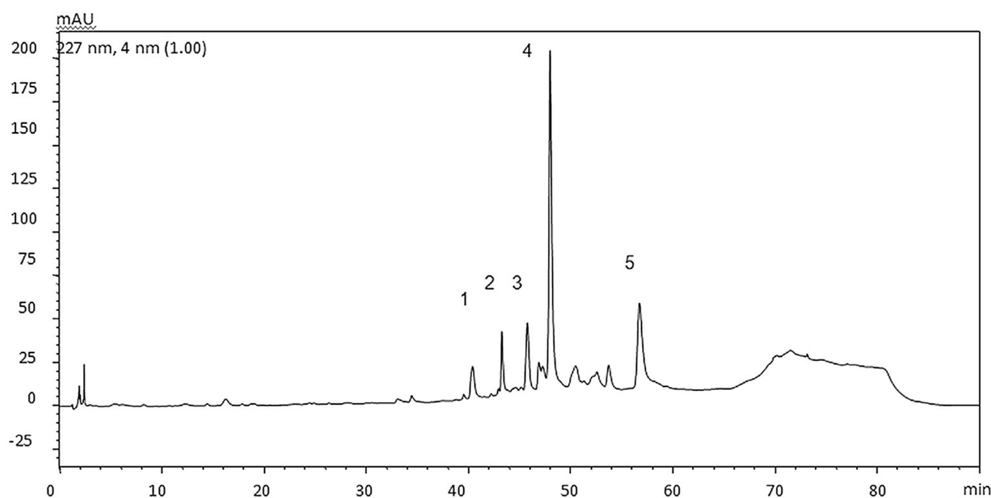

Fig. 1 – HDS extracts in 227 nm (characteristic wavelength for flavonoids): 1. amentoflavone, 2.bilobetin, 3. 7-O-methylamentoflavone, 4. ginkgetin, 5. sciadopitysin.

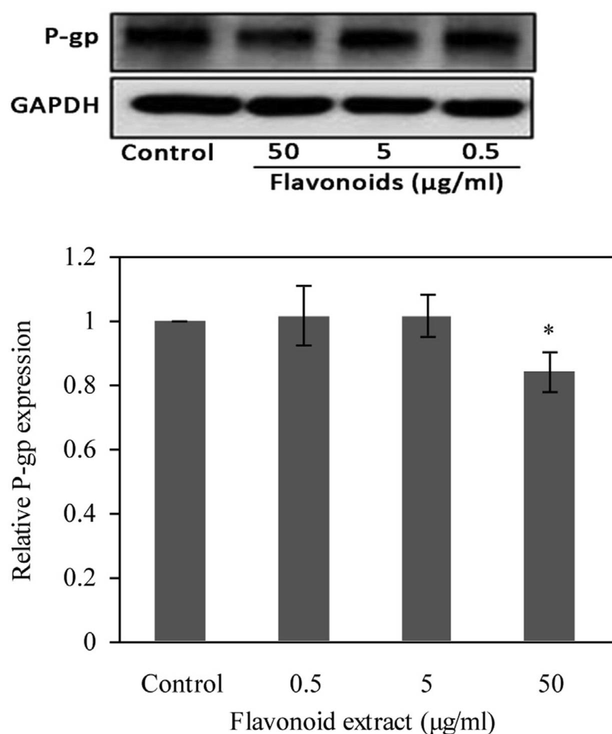

Fig. 2 – Effect of flavonoids derived from *Taxus yunnanensis* on P-gp protein expression.

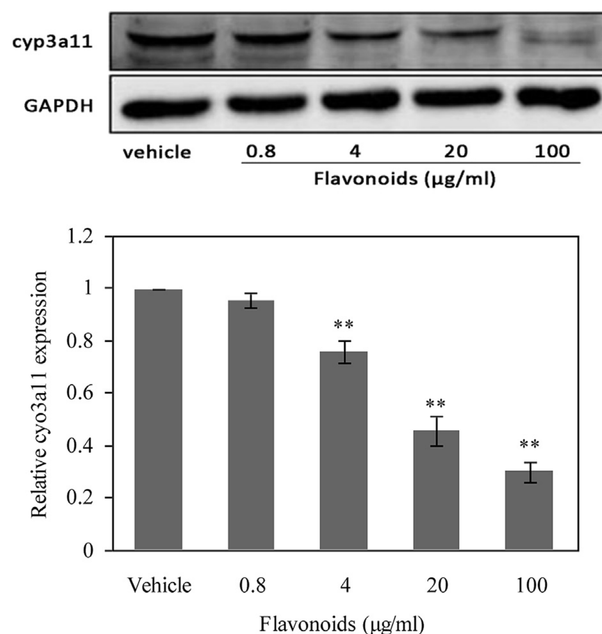

Fig. 4 – Analysis of the effect of flavonoids extract derived from *Taxus yunnanensis* on Cyp3a11 protein expression.

treatment with flavonoids for 1 h, fluorescence of intracellular rhodamine 123 increased to almost 1000 units comparing with 400 units in control group. To further study inhibitory effect of flavonoids on P-gp activity in long-term, Caco-2 cells were incubated with flavonoids for 48 h, the result showed that more rhodamine 123 was accumulated in cells and fluorescence of intracellular rhodamine 123 increased to more than 1500 units at concentration of 50 μg/ml (Fig. 3). Our results indicated that increase of intracellular rhodamine 123 by flavonoids was mainly through inhibiting P-gp activity but not protein expression. Interestingly, it should be noted that effects of the 5 flavonoids (see in Fig. 1) we identified from the extract on P-gp are still unclear, but this whole extract showed inhibitory effect on P-gp activity, which might be a kind of combined effect of the components in the extract.

3.3. Effects of flavonoids derived from *T. yunnanensis* on CYP3A4

Cytochrome P450 (CYPs) are the principal enzymes for oxidative metabolism of drugs and other xenobiotics. In human liver microsomes, CYP3A4 is the most abundant enzyme, and more than 50% of drugs (including paclitaxel) clinically used are oxidized by CYP3A4. Therefore, we further studied the effect of the flavonoid extract on the expression of CYP3A4 (cyp3a11 in mouse) using mouse primary hepatocytes. Fig. 4 showed that expression of cyp3a11 was markedly decreased in cells incubated with flavonoid extract for 48 h, and protein expression of cyp3a11 was reduced in a concentration dependent manner. To

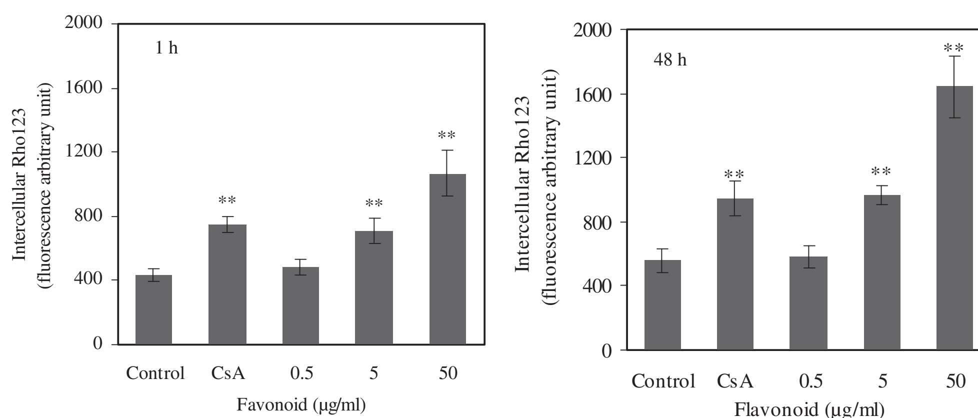

Fig. 3 – Effect of flavonoids derived from *Taxus yunnanensis* on P-gp activity by intracellular Rhodamine 123 accumulation assay.

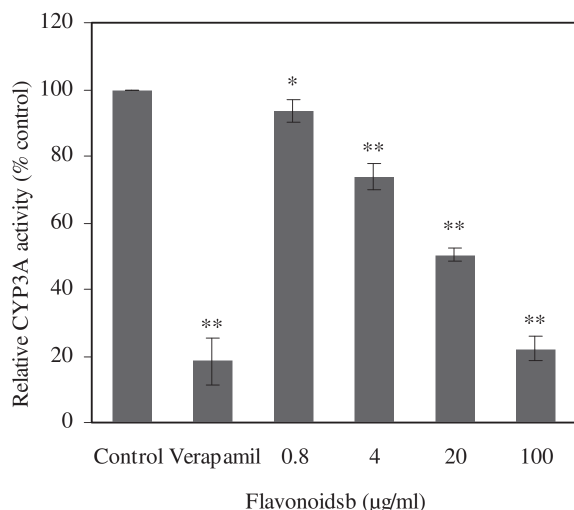

Fig. 5 – Analysis of the effect of flavonoids derived from *Taxus yunnanensis* on CYP3A activity.

further investigate the effect of flavonoid extract on the activity of CYP3A4, 1-OH-midazolam, the metabolite of typical CYP3A4 substrate of midazolam, was measured by our previously reported LC-MS/MS method after incubation with rat liver microsomes. After treatment with different concentrations of flavonoid extracts, the activity of CYP3A was dramatically inhibited in a concentration-dependent manner ($P < 0.01$) (Fig. 5).

It has been reported that flavonoid intake could enhance the bioavailability of some orally administered drugs [24,25]. In our previous studies, the bioavailability of paclitaxel was significantly enhanced by *T. yunnanensis* extract. As mentioned above, paclitaxel is a substrate of P-gp and is mainly metabolized by hepatic CYP3A, the pharmacokinetic interaction of paclitaxel may be attributed not only to the modulation of P-gp but also to CYP3A4. However, sometimes it is difficult to distinguish between the effect on P-gp or in CYP3A4 in vivo. Our in vitro study showed that flavonoids extract from *T. yunnanensis* significantly reduced expression of CYP3A and concurrently inhibited activity of CYP3A4. In addition, amentoflavone isolated from *Ginkgo biloba* has been reported to show a strong inhibitory effect on CYP3A4 [26], we supposed that amentoflavone might be the main inhibitory factor on CYP3A4 in the current flavonoid extract.

3.4. Inhibitory effects of flavonoids derived from T. yunnanensis on CYP3A4 expression through PXR-CYP3A4 pathway

Pregnane X receptor (PXR) is an important determinant of xenobiotics-induced CYP3A4 expression. To determine whether inhibition of CYP3A4 protein expression was regulated by PXR pathway, an in vitro PXR activation assay using a luciferase receptor was performed. Flavonoids were tested in the human PXR reporter gene system at the concentrations ranging from 0 to 100 μg/ml. As shown in Fig. 6, compared to the control group, RIF significantly induced CYP3A4 luciferase activity to 3.34-fold in transiently transfected LS174T cells ($P < 0.01$), and the induction by RIF markedly decreased while the transfected cells were co-treated with flavonoids and RIF. The result indicated that flavonoids inhibited gene expression of CYP3A4 through modulation of PXR pathway.

4. Conclusion

In the present study, flavonoid extract was derived from *T. yunnanensis* and was investigated to show inhibitory effects on both P-gp and CYP3A4, and gene expression of CYP3A4 was modulated through PXR pathway. Since paclitaxel is the substrate of P-gp and CYP3A4 and it also can be obtained from *T. yunnanensis*, our results suggested that flavonoid extract derived from *T. yunnanensis* might provide a pharmacokinetic benefit for development of oral paclitaxel formulation.

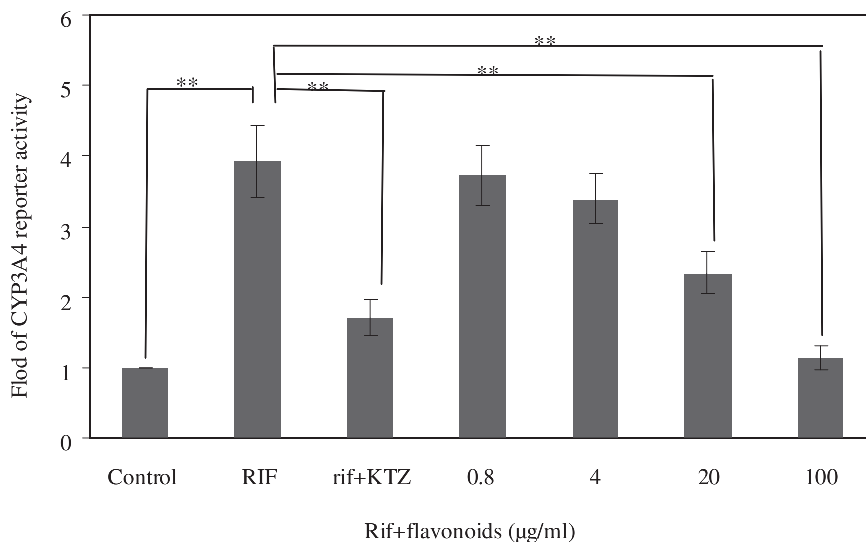

Fig. 6 – Effect of flavonoids extract on PXR activity.

Acknowledgments

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