Inhibition of Human Liver Cytochrome P450 by Star Fruit Juice

Jiang-Wei Zhang[1,2], Yong Liu[1], Jie Cheng[1,2], Wei Li[1,2], Hong Ma[1], Hong-Tao Liu[1,2], Jie Sun[3], Li-Ming Wang[3], Yu-Qi He[4], Yun Wang[4], Zheng-Tao Wang[4], Ling Yang[1,*]

[1]Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Zhongshan Road, Dalian, China [2]Graduate School of Chinese Academy of Sciences, Beijing, China [3]The Second Affiliated Hospital of Dalian Medical University, Dalian, China [4]University of Traditional Chinese Medicine, Shanghai, China

ABSTRACT- Purpose. To examine the inhibitory effects of star fruit (Averrhoa carambola) juice towards seven major cytochrome P450 (CYP) isoforms and NADPH-cytochrome P450 reductase (CPR). **Methods**. The inhibitory effects of star fruit juice (0.5 to 5%, v/v) against the activities of seven CYP isoforms including CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4 and CPR were examined in human liver microsomes. To identify time-dependent inhibition, star fruit juice (2.5%, v/v) was preincubated with microsomes and a NADPH-generating system for 0-15 min, and then the extent of inhibition towards seven CYP isoforms were examined. **Results.** Star fruit juice (5.0%, v/v) was found to inhibit all the activities of CYP isoforms tested by more than 70%. Based on the half inhibition values (%, v/v), the inhibitory effects towards different CYP isoforms were in the following order: CYP2A6 (0.9) > CYP1A2 (1.4) > CYP2D6 (1.6) > CYP2E1 (2.0) > CYP2C8 (2.2) > CYP2C9 (3.0) > CYP3A4 (3.2). Time-dependent inhibition was not observed towards any of the tested CYP isoforms. In addition, star fruit juice was found not to inhibit the activity of CPR. **Conclusions.** Star fruit juice inhibited the seven CYP isoforms tested, with the strongest inhibitory effect against CYP2A6 and the least towards CYP3A4.

INTRODUCTION

Fruit juice-drug interactions (FDIs) have been a concern since the discovery of the grapefruit juice-drug interaction and much attention has been paid to the inhibitory effects of all kinds of fruit juice towards cytochrome P450 (CYP) (1-5). Besides grapefruit juice, juice from other fruits such as banpeiyu (1), pomegranate (2), star fruit (3, 4), black berry, and wild grape (5) were also found to inhibit CYP3A *in vitro*.

Amongst the fruit juices mentioned, star fruit (Averrhoa carambola) juice was found to be the most potent inhibitor of CYP3A. Star fruit juice (5.0%, v/v) inhibited midazolam 1'-hydroxylation almost completely (3). *In vivo* evidence also suggested that star fruit juice inhibited the activity of CYP3A in rats (4). Compared to the control, the area under the concentration-time curve of carbamazepine (a typical CYP3A substrate) increased 1.3-fold following 2 mL star fruit juice administration (4). This indicated that FDI could occur in humans when star fruit juice is co-administered with drugs containing CYP3A substrates.

Star fruit juice was found to inhibit phenobarbital metabolism in rats (6), and phenobarbital was considered to be a substrate of CYP2C (7). This indicated that inhibition of CYPs by star fruit might not be limited to CYP3A. However, there was little information concerning star fruit juice's inhibitory effects of human CYP isoforms other than CYP3A4. Although CYP3A4 is the dominant CYP isoform, other CYP isoforms that are responsible for drug clearance account for a considerable amount of total CYP content in both liver and intestine (8, 9). Thus it is important to examine the inhibitory effect of star fruit juice towards major CYP isoforms besides CYP3A4. Moreover, NADPH-cytochrome P450 reductase (CPR), which facilitates efficient CYP activity, plays an important role in the process of electron transfer from NADPH to CYP (10). Therefore, it is also necessary to investigate whether star fruit juice inhibits such activity.

In this study, the FDIs potential of star fruit juice was investigated by examining its inhibitory effects towards CPR and seven major CYP isoforms, including CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 in human liver microsomes (HLMs).

Corresponding Author: Dr. Ling Yang, Laboratory of Pharmaceutical Resource Discovery, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian, China, e-mail: yling@dicp.ac.cn

MATERIALS AND METHODS

Chemicals.

D-glucose-6-phosphate, glucose-6-phosphate dehydrogenase, $NADP^+$, corticosterone, phenacetin, acetaminophen, 4'-hydroxydiclofenac, chlorzoxazone, paclitaxel, MTT-formazan, 7-hydroxycoumarin, 6-hydroxychlorzoxazone, MTT, and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO, USA). Testosterone was obtained from Acros Organics (Morris Plains, NJ, USA). Coumarin, diclofenac, and dextromethorphan were obtained from ICN Biomedicals, Inc. (Aurora, Ohio, USA) and 6α-hydroxypaclitaxel was purchased from BD Biosciences (Woburn, MA, USA). All other reagents were of HPLC grade or of the highest grade commercially available.

Human Liver Microsomes.

Human livers were obtained from three Chinese autopsy samples (male, ages 27, 29, and 42, respectively) from Dalian Medical University, with the approval of the university's ethics committee. The medication history of the donors was not known. Liver specimens were stored in liquid nitrogen until preparation of microsomes. Microsomes from three livers were prepared individually using differential ultracentrifugation as described previously (11). Protein concentrations of microsomes were determined by the Lowry method using bovine serum albumin as standards (12). Total CYP concentration was in the range of 0.16-0.35 nmol/mg protein determined according to Omura and Sato (13) with the use of a molar extinction coefficient of 91,000. Equal amounts of CYP from each of the liver sample were then pooled and the pooled HLMs were diluted to 10 mg/mL and stored at –80 °C.

Star Fruit Juice Samples.

Fresh star fruits from Taiwan or Thailand, were obtained from local supermarkets. The edible part of the fruit was placed in a juicer immediately after purchase and the extracted juice was filtered through a 0.45-μm membrane. Fresh juice samples were stored at –30 °C and thawed in ice-cold water before use. The inhibition studies were completed within one month of juice preparation.

Determination of Total Phenolic and Total Flavonoid Content in Star Fruit Juice.

The total phenolic content was determined according to the method of Shui et al (14) with slight modification. Gallic acid standard solution (2.0 mg/mL) was prepared by dissolving 0.100 g, accurately weighed, in 50 mL of distilled water. The solution was then diluted to working standard solutions in concentrations of 0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 mg/mL. A volume of 20 μL of star fruit juice or gallic acid standard was mixed with 0.9 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water). The mixture was allowed to stand at room temperature for 5 min, and then 0.6 mL of sodium bicarbonate (7.5%) was added. After standing for 10 min at 50 °C, the mixture was diluted to a total volume of 3 mL with distilled water. The absorbance at 765 nm was measured and results were expressed as mg/mL gallic acid equivalents.

Total flavonoid content was determined by a colorimetric method described previously (15). Quercetin standard solution (1.0 mg/mL) was prepared by dissolving 0.100 g, accurately weighed, in 100 mL of methanol. Fifty microliters of star fruit juice or quercetin standard solution (0, 50, 100, 150, 200, 250, 300 μL) was mixed with 150 μL of a 5% $NaNO_2$ solution. After 6 min, 150 μL of a 10% $AlCl_3 \cdot 6H_2O$ solution was added, and the mixture was allowed to stand for a further 5 min. After adding 0.5 mL of 1 M NaOH, the total volume was then brought up to 2.3 mL with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm. The results were expressed as mg/mL quercetin equivalents.

Assays of CYP Isoforms.

Assays of the CYP isoforms were based on the following probe reactions: phenacetin O-deethylation(CYP1A2), coumarin 7 - hydro xylation (CYP2A6), paclitaxel 6α-hydroxylation (CYP2C8), diclofenac 4'-hydro-xylation (CYP2C9), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A4) (16, 17). To characterize the pooled HLMs, marker reactions of seven CYP isoforms were examined, and apparent K_m values were estimated (Table 1). Assay methods including incubation and analytical conditions are listed in Tables 1 and 2, respectively. The incubation mixture (200 μL) included 100 mM potassium phosphate buffer (pH 7.4), NADPH-generating system (1.0 mM NADP+, 10 mM D-glucose-6-phosphate, 1.0 unit/mL of glucose-6-phosphate dehydrogenase, and 4.0 mM $MgCl_2$), HLMs, and individual probe substrate. All experiments were performed within the linear range of incubation time and protein concentration.

Dextromethorphan was dissolved in water, and other substrates were dissolved in methanol with a final concentration of 0.5% (v/v). After 3-min preincubation at 37 °C, the reaction was initiated by addition of the NADPH-generating system. After the defined incubation time, the reaction was then terminated by adding 100 μL acetonitrile (10% trichloroacetic acid for CYP2A6) together with internal standard. The reaction mixture was kept on ice until it was centrifuged at 20,000×g for 10 min at 4 °C. Aliquots of the supernatants were analyzed by HPLC (Table 2).

Inhibition of CYP Isoform Activities by Star Fruit Juice.
The inhibition study was conducted by adding star fruit juice (0.5% to 5.0%, v/v) to the reaction mixture before the addition of the NADPH-generating system. The incubation was carried out as described above. The inhibitory effect on the CYP activity was expressed as the percentage of the control activity in the absence of star fruit juice.

To examine whether star fruit juice inhibited the activity of CYPs in a time-dependent manner, star fruit juice (2.5%, v/v) was preincubated with HLMs, the NADPH-generating system, and buffer for 0, 5, 10, or 15 min at 37 °C. The reaction was initiated by addition of substrates.

Assay of CPR.
The activity of CPR was based on its ability to reduce3-(4,5-dimethylthiazol-2-yl)-2, 5 - diphen - yltetrazoli um bromide (MTT) to MTT-formazan in the presence of the NADPH-generating system (18). The assay of CPR was performed according to the method of Yim et al. (18) with slight modification. The reaction mixtures, with total volumes of 0.5 mL, consisted of 100 mM potassium phosphate buffer (pH 7.4), the NADPH-generating system (0.5 mM $NADP^+$, 5.0 mM D-glucose-6-phosphate, 0.5 unit/mL of glucose-6-phosphate dehydrogenase, and 4.0 mM $MgCl_2$), 0.5 mg/mL HLMs and 200 μM MTT. The reference cuvette contained reaction mixture without MTT. The amount of MTT-formazan was determined from a calibration curve (4.77, 9.54, 18.08, 28.62, 38.16 nmol). After 5-min preincubation at 37 °C, the reaction was initiated by the addition of MTT. Absorbance at 610 nm (A_{610}) was monitored continuously for 30 seconds (18) in a Jasco V-530 spectrophotometer (Tokyo, Japan) using a double beam model. The slopes of A_{610}–time plots in the first 30 seconds were used to calculate the formation rates of MTT-formazan.

Inhibition of CPR Activity by Star Fruit Juice.
The inhibition study was conducted by adding star fruit juice (1.0% to 5.0%, v/v) to the incubation mixture of CPR before the addition of MTT. The assay was conducted as described above. The inhibitory effects on the CPR activity were expressed as the percentage of the control activity in the absence of star fruit juice. All incubations were carried out in duplicate unless otherwise stated and the average of duplicate determinations was presented in the results, with standard deviations generally below 10 %.

Table 1. Isoforms tested, marker reactions, K_m, and incubation conditions used in the inhibition study

CYPs	Maker reactions	Estimated K_m (μM)	Substrate concentration (μM)	Protein concentration (mg/ml)	Incubation time (min)
1A2	Phenacetin O-deethylation	44	40	0.2	30
2A6	Coumarin 7-hydroxylation	1.0	1.0	0.1	10
2C8	Paclitaxel 6α-hydroxylation	13	10	0.5	30
2C9	Diclofenac 4'-hydroxylation	14	10	0.3	10
2D6	Dextromethorphan O-demethylation	4.8	25	0.25	20
2E1	Chlorzoxazone 6-hydroxylation	130	120	0.4	30
3A4	Testosterone 6β-hydroxylation	49	50	0.5	10

Table 2. HPLC conditions for the relevant P450 isoforms.

CYPs	Internal standard (Concentration, μM)	Mobile phase and gradient	Detection
1A2	7-Hydroxycoumarin (30 μM)	Methanol: Phosphate buffer (pH=3.0, 50 mM)= 34:66	UV 245 nm
2A6	-	Acetonitrile: Acetic acid (0.1%, v/v) =35:65	Fluo Ex/Em: 340nm/456nm
2C8	-	Methanol: Water=65:35	UV 230 nm
2C9	Coumarin (60 μM)	Acetonitrile (A): Phosphate buffer (pH=7.4, 100mM, B)=32:68, 0-9min, 68%B-32%B	UV 280 nm
2D6	-	Acetonitrile: Phosphate buffer (pH=3.0,50 mM)=25:75	Fluo Ex/Em: 235 nm /310 nm
2E1	Phenacetin (300 μM)	Acetonitrile: Acetic acid (0.5%, v/v) =22:78, 1-10min, 78%B-40%B	UV 287 nm
3A4	Corticosterone (20 μM)	Methanol: Water=52:48, 0-15min, 48%B-30%B; 15-22min, 30%B-20%B	UV 254 nm

RESULTS.

To control the quality of juice samples, total phenolic and total flavonoid content were measured. As shown in Table 3, the total phenolic content was in the range of 0.60 ± 0.02 to 1.01 ± 0.01 μg gallic acid mL^{-1} fruit juice, and the total flavonoid content in the range of 2.37 ± 0.05 to 5.03 ± 0.27 μg quercetin mL^{-1} fruit juice. The star fruits used in the inhibition studies were the samples bought on May 01, 2006 unless otherwise stated.

In the present study, the inhibitory effects of star fruit juice were investigated in HLMs. The star fruit juice's inhibitory effects towards seven CYP isoforms, including CYP1A2, CYP2A6, CYP2D6, CYP2C8, CYP2C9, CYP2E1, and CYP3A4, are shown in Figure 1. At 5.0% (v/v), star fruit juice showed different degrees of inhibition towards seven CYP isoforms. The values of half inhibition concentration (IC_{50}) were 1.4, 0.9, 2.2, 3.0, 1.6, 2.0, and 3.2% (v/v) for CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4, respectively. Based on the IC_{50} values, star fruit juice's inhibition effects towards different CYP isoforms were found to be in the following order: CYP2A6 > CYP1A2 > CYP2D6 > CYP2E1 > CYP2C8 > CYP2C9 > CYP3A4.

Star fruit juice inhibited CYP2A6 more potently than other isoforms tested. Subsequently further studies were performed to evaluate the inhibitory effects of star fruits obtained from different origin, purchase store, or purchase date. In spite of differences in origin, purchase store, or purchase time, juice made from the star fruits all inhibited CYP2A6 catalyzed coumarin 7-hydroxylation by more than 90% (Table 4).

Grapefruit juice is known to contain several mechanism-based inhibitors and grapefruit juice's inhibition has been characterized in a time-dependent nature (19). To examine whether star fruit juice inhibited the activity of CYPs in a similar way, the effect of preincubation time on the extent of inhibition was also studied. As shown in Figure 2, the extent of inhibition by star fruit juice (2.5%, v/v) towards CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 were not altered significantly during the of preincubation period (0-15 min), suggesting that star fruit juice did not inhibit the tested isoforms in a time-dependent manner.
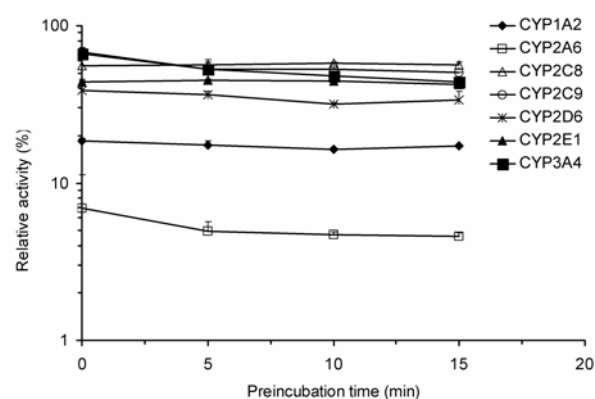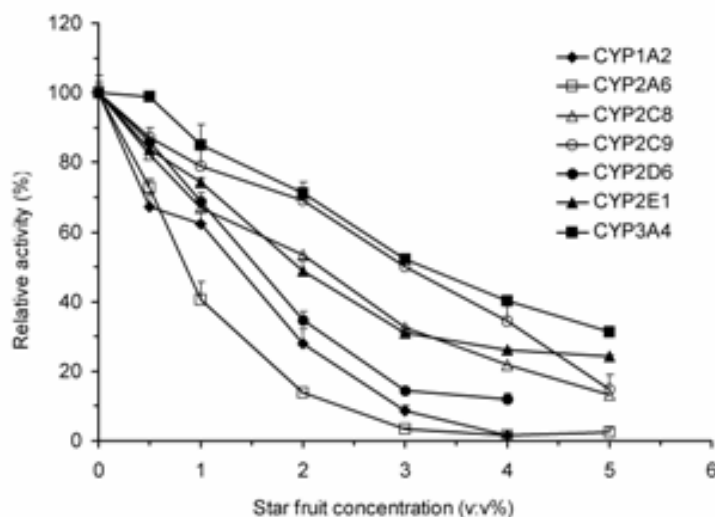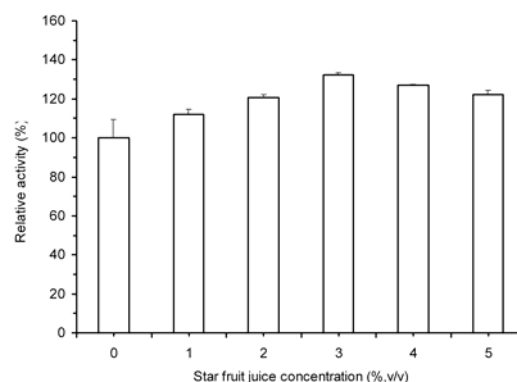
Because CPR played an important role in CYP-mediated reactions (10), it is possible that the non-selective inhibition of CYPs by star fruit juice could contribute to its inhibition of the CPR. To test this hypothesis, the inhibitory effect of star fruit juice towards CPR was evaluated. However, no inhibitory effect towards CPR was observed, as shown in Figure 3.

Table 3. Total phenolics and flavonoids in star fruit juice samples. Mean ± SD of quadruplicate determinations

Purchase date	Store	Origin	Total phenolics[a]	Total flavonoids[b]
May 01, 2006	New-mart1	Taiwan	0.60 ± 0.02	2.37 ± 0.05
May 09, 2006	Carrefour	Thailand	1.01 ± 0.01	5.03 ± 0.27
May 21, 2006	New-mart2	Taiwan	0.86 ± 0.02	4.33 ± 0.26
May 22, 2006	New-mart1	Taiwan	0.71 ± 0.02	2.85 ± 0.12

[a]mg gallic acid ml^{-1} fruit juice; [b]mg quercetin ml^{-1} fruit juice

Table 4. Reproducibility of coumarin 7-hydroxylation (CYP2A6) inhibition by star fruit juice (5.0%, v/v). Mean ± SD of triplicate incubations.

Purchase date	Store	Origin	Residual Activity (%)
May 01, 2006	New-mart1	Taiwan	5.6 ± 0.6
May 09, 2006	Carrefour	Thailand	6.7 ± 0.3
May 21, 2006	New-mart2	Taiwan	7.0 ± 0.2
May 22, 2006	New-mart1	Taiwan	7.5 ± 0.2

Figure 2. The effect of preincubation time (0, 5, 10, 15 min) on the inhibition potencies of CYP isoforms by star fruit juice (2.5%, v/v). Mean of duplicate incubations.


Figure 1. Inhibition of CYP isoforms by star fruit juice (0.5, 1.0, 2.0, 3.0, 4.0, 5.0%, v/v) in human liver microsomes. Mean of duplicate incubations.

Figure 3. Effects of star fruit juice (1.0, 2.0, 3.0, 4.0, 5.0%, v/v) on the activity of CPR in human liver microsomes. Mean of duplicate incubations.

DISCUSSION

Fruits, the main sources of antioxidant in our diet, are beneficial in preventing age-related diseases, cancers, obesity, and heart disease (20). Fruits are consumed everyday, thus FDIs might be daily occurrences. Star fruit, largely planted in Southeast Asia and Brazil, is a popular fruit around the world (14). It is recommended in folk medicine as a diuretic, expectorant, and cough suppressant (21-23). For Brazilians, it is common to drink 300 to 500 mL of star fruit juice in a single day (24, 25). Therefore, the probability of star fruit-drug interactions is high if star fruit juice inhibits CYPs.

Our findings that star fruit juice inhibits CYP2C9 are in agreement with the observed star fruit juice-phenobarbital interactions in rats (6). Phenobarbital-induced sleeping time was prolonged by star fruit juice in a dose-dependent manner (6). Phenobarbital metabolism was dependent on genetic polymorphism of CYP2C (7). Valproic acid, an inhibitor of CYP2C9 (26), inhibits phenobarbital metabolism in both human and rat (27, 28). All these findings suggest that CYP2C was involved in phenobarbital metabolism. It is reasonable that star fruit juice inhibits phenobarbital metabolism by inhibiting CYP2C.

CYP2A6 is recognized as being involved in the activation of promutagens in tobacco (29). CYP2A6 inhibitors, such as 8-methoxypsoralen could suppress tumorigenesis induced by nicotine-related chemicals (30, 31). In the present study, star fruit juice was found to be a potent inhibitor of CYP2A6. Therefore, one might speculate that star fruit juice could be used as a chemopreventive agent against nicotine-related cancer. However, this assumption is not supported by our study since the selective inhibition against CYP2A6 needs to be verified in vivo.

Previous studies have suggested that star fruit juice, similar to grapefruit juice, might contain some compounds that are CYP inhibitors (3, 4, 6). These compounds have not yet been identified. Some phenolics or flavonoids, such as gallic acid, catechin, (-)-epicatechin, proanthocyanidins, or their corresponding polymers were found in star fruit (20). It is possible that the inhibitory effect of star fruit juice is due to the presence of phenolics or flavonoids because certain phenolics (32) and flavonoids (33) are known to be CYP inhibitors. We therefore monitored the content of total phenolics and total flavonoids in the juice samples as comparators. However, further investigation is required to clarify the inhibitory

components against CYP in star fruit.

Several other CYP isoforms such as CYP2C19 and CYP2B6 were not included in the present study. It is not known whether these isoforms could be inhibited by star fruit juice. In conclusion, the following summarizes the findings from this study.

1) Star fruit juice inhibits not only CYP3A4, but also CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2D6, and CYP2E1,

2) The inhibitory effects of star fruit juice towards the CYP isoforms tested are not time-dependent and

3) Star fruit juice does not inhibit CPR. Since the inhibitory effect *in vitro* does not necessarily represent that which may occur *in vivo*, further research is necessary to investigate whether clinical relevant star fruit juice-drug interactions will occur *in vivo*.

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