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ORIGINAL PAPER

Structure–function relationships of inhibition of mosquito cytochrome P450 enzymes by flavonoids of *Andrographis paniculata*

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Abstract The cytochrome P450 monooxygenases are known to play a major role in pyrethroid resistance, by means of increased rate of insecticide detoxification as a result of their overexpression. Inhibition of detoxification enzymes may help disrupting insect detoxifying defense system. The *Anopheles minimus* CYP6AA3 and CYP6P7 have shown pyrethroid degradation activity and been implicated in pyrethroid resistance. In this study inhibition of the extracts and constituents of *Andrographis paniculata* Nees. leaves and roots was examined against benzyloxyresorufin *O*-debenzylation (BROD) of CYP6AA3 and CYP6P7. Four purified flavones (5,7,4′-trihydroxyflavone, 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone), one flavanone (5-hydroxy-7,8-dimethoxyflavanone) and a diterpenoid (14-deoxy-11,12-didehydroandrographolide) containing inhibitory effects toward both enzymes were isolated from *A. paniculata*. Structure–function relationships were observed for modes and kinetics of inhibition among flavones, while diterpenoid and flavanone were inferior to flavones. Docking of flavones onto enzyme homology models reinforced relationships on flavone structures and inhibition modes. Cell-based inhibition assays employing 3-(4,5-dimethylthiazol-2-y-l)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays revealed that these flavonoids efficiently increased susceptibility of CYP6AA3- and CYP6P7-expressing *Spodoptera frugiperda* (Sf9) cells to cypermethrin toxicity, due to inhibition effects on mosquito enzymes. Thus synergistic effects on cypermethrin toxicity of *A. paniculata* compounds as a result of enzyme inhibition could be useful for mosquito vector control and insecticide resistance management in the future.

Keywords *Anopheles minimus* · *Andrographis paniculata* · Cytochrome P450 · Flavonoids · Inhibition · Synergism

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Introduction

Control of insect vectors by chemical insecticides is an efficient strategy in preventing transmission of vector-borne diseases including malaria. However, long-term use of synthetic insecticides often leads to insecticide resistance and hinders control of associated diseases, especially resistance to the widely used pyrethroid insecticides has been found widespread among mosquito vectors (Hemingway and Ranson 2000; Nkya et al. 2013). Resistance toward insecticides can be due to a number of mechanisms which are reduced sensitivity of the target site, reduced penetration of insecticide, and

increased metabolisms of insecticides by detoxification enzymes such as cytochrome P450 monooxygenases (P450s), esterases and glutathione transferases (Hemingway and Ranson 2000; Li et al. 2007). Overexpression of P450s has frequently been shown associated with pyrethroid resistance in mosquitoes as a result of increased rate of pyrethroid metabolism (Feyereisen 1999; David et al. 2013). Thus inhibition of the P450 enzyme defense mechanism by insecticide synergist may increase the effectiveness of insecticides. For instance, piperonyl butoxide (PBO), which is an inhibitor of detoxification enzymes, has been shown to enhance the toxicity of pyrethroids against mosquitoes (Vijayan et al. 2007; Pennetier et al. 2013). An alternative strategy could be the use of naturally occurring phytochemicals that are generally regarded as low mammalian toxicity, minimal environmental persistence and have reduced effects on non-target organisms as synergists in order to reduce the use of synthetic insecticides. Examples have been shown for various agricultural insect species and disease vectors. In a study with the house fly *Musca domestica*, the dillapiole oil from *Anethum graveolens* and parsley seed oil from *Petroselinum crispum* exhibiting inhibition of oxidases in vitro has been reported potential synergist with pyrethrum extract (Joffe et al. 2011). In *Aedes aegypti* (Linn.) synergistic effects of insecticidal essential oils with PBO were observed together with inhibition of P450-mediated oxidation and reduction of glutathione *S*-transferase activity (Waliwitiya et al. 2012). Alternatively combinations of insecticides and botanical compounds with different modes of action against insects could be used. Leaf extracts from *Jatropha gossypifolia* and *Melia azedarach*, for example, have antifeedant activity and inhibition effects on P450, esterase and acetylcholine esterase enzymes and effectively synergize with cypermethrin in *Spodoptera frugiperda* (Bullangpoti et al. 2011).

We have isolated two P450s, CYP6AA3 and CYP6P7, from laboratory-selected pyrethroid-resistant *Anopheles minimus*, a major malaria mosquito vector in Thailand (Rodpradit et al. 2005; Rongnoparut et al. 2003). Enzymatic assays in vitro and homology modeling of enzymes support the role of CYP6AA3 and CYP6P7 in metabolisms of pyrethroids (Boonseupsakul et al. 2008; Duangkaew et al. 2011b; Lertkiatmongkol et al. 2011). Using cell-based 3-(4,5-dimethylthiazol-2-y-l)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assays, we observed that expression of CYP6AA3 or CYP6P7 in *Spodoptera frugiperda* (Sf9) cells efficiently cytoprotect cells against pyrethroid toxicity due to pyrethroid degradation by both enzymes (Duangkaew et al. 2011a). Addition of PBO abolished cytoprotection by both enzymes against pyrethroid toxicity (Duangkaew et al. 2011a). Cell-based assay expressing human P450s has also been successfully established to assess cytotoxicity of compounds and drugs (Lahoz et al. 2013). Using a combination of in vitro enzymatic assay and cell-based cytotoxicity assay, a search of mosquito P450 inhibitory agents in natural products that readily comprise insecticidal activity could be performed, especially when the search compounds are present in small amount. With this approach we recently obtained three naphthoquinones (rhinacanthin-A, -B, and -C) from *Rhinacanthus nasutus* that displayed potent inhibition effects and different modes of inhibition on CYP6AA3 and CYP6P7 (Pethuan et al. 2012). Inhibition of P450s in general can be in reversible and irreversible modes. In particular, a mechanism-based inactivation by covalent modification of the heme group of P450s or the active site of enzymes can result in irreversible loss of enzyme activity (Correia and Ortiz de Montellano 2005) and could effectively prevent insecticide detoxification. The mechanism-based inhibition requires a catalysis process mediated by the enzyme and time to generate active inhibitor molecule and is initially characterized by NADPH-, inhibitor concentration- and time-dependent inhibition pattern (Correia and Ortiz de Montellano 2005).

In this study, we preliminarily evaluated inhibition of plant extracts in Thailand against *An. minimus* benzyloxyresorufin-*O*-debenzylation (BROD) mediated by CYP6AA3 and CYP6P7. The enzymatic assay employed in vitro reconstitution of each P450 with cytochrome P450 reductase (CPR) redox partner enzyme, using NADPH as electron donor and benzyloxyresorufin as substrate (Duangkaew et al. 2011b). Plant extracts tested included *Calotropis procera* Ait. (Asclepiadaceae) leaves, *Citrus reticulata* (Rutaceae) seeds, *Curcuma longa* Linn. (Zingiberaceae) rhizomes, and aerial parts of *Andrographis paniculata* Nees. (Acantaceae), *Derris trifoliata* Lour. (Leguminosae), and *Stemona* sp. (Stemonaceae). These were previously shown possessing mosquitocidal activity (Omena et al. 2007; Elimam et al. 2009; Kaltenegger et al. 2003; Shaalan et al. 2005;). Among these, *A. paniculata* that effectively inhibited both enzymes was chosen for further investigation. Larvicidal and adulticidal activities of *A. paniculata* have been reported in *Culex quinquefasciatus* (Say) and *Ae. aegypti* mosquitoes (Govindarajan 2011; Govindarajan and Sivakumar 2012). Moreover extracts of *A. paniculata* leaves and roots displayed synergistic activity with deltamethrin toxicity in *An. stephensi* (Liston) (Chenniappan and Kadarkarai 2008). *A. paniculata* contains diterpenoids, flavonoids and polyphenols as the bioactive components (Chao and Lin 2010), but their effects on insect detoxification enzymes have not been investigated. The current study isolated, identified, and purified chemical constituents with inhibitory activity against both mosquito enzymes. Mode of inhibition and kinetic parameters of purified compounds on CYP6AA3 and CYP6P7 were determined, molecular docking of flavonoids on homology models of both P450s were performed, and synergistic effect on susceptibility to cypermethrin cytotoxicity of Sf9 cells expressing mosquito P450s was investigated.

Materials and methods

Chemicals

Benzyloxyresorufin, cypermethrin, nicotinamide adenosine diphosphate reduced form (NADPH), 1,2-didodecanoyl-*rac*-glycero-3-phosphocholine (DLPC), dimethyl sulfoxide (DMSO), curcumin, rotenone, 5,7-dihydroxy-8-methoxyflavone and andrographolide were purchased from Sigma-Aldrich (USA). 5,7-dihydroxyflavone (chrysin) was obtained from Acros Organics (USA). Analytical grade *n*-hexane, ethyl acetate (EtOAc), ethanol, methanol, diethyl ether, and high performance liquid chromatography (HPLC) grade acetonitrile were supplied by RCI Labscan (Bangkok, Thailand).

Preparation of crude plant extracts

Preliminary screening of selected plant extracts for inhibition effects on CYP6AA3 and CYP6P7 enzyme activities were performed with *C. longa* rhizomes, *C. procera* leaves, *C. reticulata* seeds and aerial parts of *A. paniculata*, *D. trifoliata*, and *Stemona* sp. These were purchased from traditional medicine market (Bangkok, Thailand). Crude ethanol extracts of these plants were prepared as previously described (Kuppusamy and Murugan 2010; Rao et al. 2004) with modifications. Briefly dried plant parts were homogenized and macerated in 95 % ethanol at room temperature. The ethanol extract of each plant was filtered through filter paper (Whatman no. 1) and the filtrate was re-extracted. Solvent was subsequently removed from each filtrate by rotary evaporator under reduced pressure.

Extraction and bioassay-guided isolation of bioactive compounds from *A. paniculata*

The *A. paniculata* leaves and roots were obtained from Amphur Muang, Surin Province, Thailand and identified by Dr.Thaya Jenjitikul, Department of Plant Science, Faculty of Science, Mahidol University, Bangkok, Thailand. A voucher sample (7802) was deposited at Suanluang Rama IX herbarium, Bangkok. The air-dried material (2 kg) was subjected to maceration with 95 % ethanol as described above to obtain 200 g ethanol extract, and successively partitioned with hexane and EtOAc, yielding hexane (30 g), EtOAc (100 g) and aqueous (70 g) extracts.

Stepwise gradient elution of the hexane extract was done in silica gel column chromatography (silica 60; 0.063–0.200 mm; Merck, Germany) using a hexane/EtOAc/methanol solvent system to yield five fractions (fr.1: hexane, 0.75 g; fr.2: hexane–EtOAc (17:3), 3 g; fr.3: hexane–EtOAc (7:3), 9 g; fr.4: hexane–EtOAc (1:1), 5.25 g; fr.5: EtOAc, 12 g). Each of the fractions (10 μg) containing at least 50 % inhibition effect against CYP6AA3 and CYP6P7-mediated BROD was subjected to thin layer chromatography (TLC) on precoated Kiesel gel 60 F_{254} sheets (Merck, Germany) using hexane–diethyl ether (4:6, vol/vol) as mobile phase to give eight subfractions. Each subfraction was subjected to HPLC (Symmetry RP-18 column; 3.9×150 mm; Waters, Ireland) with detection at 254 nm. A mobile phase (1.5 ml/min) consisting of ACN and water was set as follows: 0 min, 50 % v/v ACN/water; 0–10 min, a linear gradient from 50 % ACN/water to 60 % ACN/water; 10–15 min, 60 % ACN/water to 100 % ACN; 15–20 min, 100 % ACN; 20–25 min, linear gradient returning to 50 % ACN; 25–30 min, a final equilibration at 50 % ACN. Each HPLC peak associated with potent inhibition effect was collected. The purity of each compound was checked by TLC with hexane–diethyl ether (4:6, vol/vol) and nuclear magnetic resonance (NMR; Bruker AVANCE 400 MHz NMR Spectrometer, Bruker, Germany). Two compounds obtained from fr.3 were eluted at retention times 12.43 and 13.19 min under HPLC conditions described and identified as 5-hydroxy-7,8-dimethoxyflavanone and 5-hydroxy-7,8-dimethoxyflavone, respectively, based on spectrum, total mass and NMR spectra previously reported (Kuroyanagi et al. 1987). Three compounds in fr.4 eluted from HPLC at 3.63, 7.91 and 11.77 min were identified as 5,7,4′-trihydroxyflavone (known as apigenin), 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone, and 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, respectively.

The EtOAc fraction was subjected to separation by TLC using diethyl ether–EtOAc (2:8, vol/vol) as mobile phase to give eight fractions and only one fraction contained inhibition effect against CYP6AA3- and CYP6P7-mediated BROD. The fraction was subjected to HPLC and a mobile phase (1.5 ml/min) was ACN/water setting as follows: 0 min, 25 % ACN/water (v/v); 0–10 min, a linear gradient of 25 % to 100 % ACN; 10–15 min, 100 % ACN; 15–20 min a linear gradient from 100 % ACN to 25 % ACN; 20–25 min, a final equilibration at 25 % ACN. The purified compound eluted at 9.76 min on HPLC was identified as 14-deoxy-11,12-didehydroandrographolide, based on spectrum, total mass and NMR spectra previously reported (Siripong et al. 1992).

In vitro inhibition assays of CYP6AA3 and CYP6P7

The *A. paniculata* extracts and constituents were tested for inhibitory activity on CYP6AA3- and CYP6P7-mediated BROD using enzymatic reconstitution assay. CYP6AA3 and CYP6P7 were expressed using baculovirus-mediated insect cell expression system in Sf9 cells as previously described (Duangkaew et al. 2011b; Kaewpa et al. 2007). Membrane fractions containing either CYP6AA3 or CY6P7 were prepared and reconstituted with the purified *An. minimus* CPR redox partner in the ratio of 3:1 in 500 μl 50 mM Tris–HCl (pH 7.5) and the presence of DLPC using benzyloxyresorufin

as substrate (approximately K_m values of 2 and 0.5 μM for CYP6AA3 and CYP6P7, respectively). Each reaction was incubated at room temperature for 10 min prior to initiation of reaction via addition of NADPH (as electron donor) to a final concentration of 50 μM. Formation of the resorufin product was measured at λ_{ex}=530 and λ_{em}=590 nm using RF-5301 PC spectrofluorometer (Shimadzu, Kyoto, Japan). Rate of resorufin formation was calculated based on the resorufin standard curve and was expressed as pmol resorufin/min/pmol P450. Different concentrations of *A. paniculata* extracts and test compounds (10 μg each of fractions and sub-fractions, and 0–120 μM each of compounds) were each co-incubated with benzyloxyresorufin substrate in the reaction mixture and enzyme activities determined. Inhibitory effect was calculated as percent relative inhibition compared with the ethanol vehicle control reaction and quantified to obtain IC_{50} values, determined graphically by nonlinear regression analysis of logarithm of extract concentrations against the relative residual enzyme activity using GraphPad Prism 5 (GraphPad Co. Ltd., USA).

Time- and concentration-dependent inhibition of P450 activity was investigated to preliminarily determine whether inhibition by plant extracts and compounds was conformed to a mechanism-based inhibition. Pre-incubation of various concentrations of each extract or test compound was allowed to occur for 30 min in the presence or absence of NADPH before addition of substrate. The indication of mechanism-based inhibition is the IC_{50} shift to lower range in pre-incubation with inhibitor in the presence of NADPH compared with co-incubation assay (Fowler and Zhang 2008).

Determination of inhibition kinetics

Apparent K_i values and mode of inhibition were determined from enzymatic assays with various concentrations of benzyloxyresorufin substrate (0.5, 1, 2, 4, 8 μM for CYP6AA3 and 0.125, 0.25, 0.5, 1, 2 μM for CYP6P7) conducted with different concentrations of each purified compounds from *A. paniculata* (1–4 μM of 5,7,4′-trihydroxyflavone, 8–32 μM of 5-hydroxy-7,8-dimethoxyflavanone, 30–120 μM of 14-deoxy-11,12-didehydroandrographolide, and 4–16 μM of 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone) and two purchased compounds (1–4 μM of 5,7-dihydroxyflavone or chrysin and 8–32 μM of 5,7-dihydroxy-8-methoxyflavone). Mode of inhibition of these compounds was estimated graphically from a double reciprocal plot of velocity against substrate concentrations (Lineweaver–Burk plot). K_i values were calculated via second plots of the slopes from Lineweaver–Burk plots versus inhibitor concentrations. Following time- and NADPH-dependent inhibition characteristics of 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone, the kinetic constants for mechanism-based inhibition, K_I and k_{inact}, were determined following the inactivation assay as described using pre-incubation times at 0, 20, 40, and 60 min (Pethuan et al. 2012). The maximal inactivation rate constant (k_{inact}) and the inhibitor concentration required for half-maximal rate of inactivation (K_I) were calculated from double reciprocal plots of k_{obs} versus inhibitor concentrations by linear regression analysis using GraphPad Prism 5.

Inhibition of P450 activities in P450-expressing Sf9 cells

To determine the synergistic action of *A. paniculata* extracts and test compounds, cell-based inhibition assay was performed with CYP6AA3- or CYP6P7-expressing Sf9 cells as previously described (Pethuan et al. 2012). Before the assays, cytotoxicities of cypermethrin and test compounds toward the Sf9 parent cells were evaluated by MTT assays as described (Duangkaew et al. 2011a). The amount of compounds that caused approximately 20 % mortality in Sf9 parent cells were used in cell-based inhibition assays. In inhibition assays, CYP6AA3- and CYP6P7-expressing cells and Sf9 control cells were seeded at 2×10^5 cells per well in 24-well culture plates containing SF-900 serum-free medium (Invitrogen, USA) and supplemented with 10 μg/ml streptomycin and 6.04 μg/ml penicillin. Cells were allowed to attach for 3 h before addition of selected doses of each test compounds to each well and DMSO was vehicle control, followed by addition of various concentrations of cypermethrin (3–500 μM) before performing MTT assay. The formazan product formation was measured at 540 nm using Multiskan EX microtiter plate reader (Thermo Labsystems, Finland). Cell viability assessed by MTT assay was expressed as percentage of viable cells relative to cells treated with DMSO alone (assigned as 100 % viability). Inhibition of P450s was measured as cytotoxicity of cypermethrin to cells in the presence of test compound, after normalization with that of cells treated with test compound alone, compared to cells treated with cypermethrin alone.

Molecular docking analysis

The molecular docking program AutoDock Vina v.1.1.2 (Trott and Olson 2010) was used to dock flavones to the active sites of CYP6AA3 and CYP6P7 homology models previously built (Lertkiatmongkol et al. 2011). The 3D structure of compounds was constructed by Chem3D Ultra 8.0 (CambridgeSoft Corporation, USA). Docking was carried out with the grid size set to 60×60×60 xyz points with grid spacing of 0.375 Å and the grid centered at the heme iron of each P450. The poses with lowest energy of binding and those with closest interaction with heme iron of CYP6AA3 and

CYP6P7 were selected. The simulation results were illustrated by PyMOL Molecular Graphics System v.1.3 (Schrodinger, USA).

Statistical analysis

One-way analysis of variance (ANOVA) with Tukey's multiple comparison tests were used to analyze data on GraphPad Prism 5. Results with $P \leq 0.05$ were considered to be significantly different.

Results

To investigate the effect of plant ethanol extracts on CYP6AA3- and CYP6P7-mediated BROD, *in vitro* reconstitution reactions of either CYP6AA3 or CYP6P7 were conducted in the presence of benzyloxyresorufin substrate and each crude extract of various plants including *C. procera* leaves, *C. reticulata* seeds, *C. longa* rhizomes, and aerial parts of *A. paniculata*, *D. trifoliata,* and *Stemona* sp. Inhibition by *C. longa* rhizomes was strongest with the IC_{50} values of 1.83± 0.04 and 0.89±0.09 μg/ml against CYP6AA3 and CYP6P7, respectively, followed by *A. paniculata* and *D. trifoliata* extracts with IC_{50} values ranging about 8–10 and 14–19 μg/ml, respectively (electronic supplementary material, Table). A much less inhibitory effect was observed with *C. procera*, *Stemona* sp. and *C. reticulata*. There was no inhibitory activity against CPR-mediated cytochrome c reduction by crude extracts (data not shown), suggesting inhibition effect was not attributed to CPR redox partner enzyme.

Investigation of curcumin, a major compound of *C. longa* rhizomes (Green et al. 2008), revealed it caused high inhibitory effect against CYP6AA3 with IC_{50} values of 0.74 μM and CYP6P7 of 0.47 μM (electronic supplementary material, Table), suggesting curcumin was a major inhibitory compound in *C. longa* rhizomes. Similarly inhibition by *D. trifoliata* extract could be contributed by rotenone, a major compound (Hien et al. 2003), with IC_{50} values of 5.51 and 5.27 μM against CYP6AA3 and CYP6P7, respectively. In contrast, andrographolide, a major constituent of *A. paniculata*, was not a major contributor to inhibition since it displayed low inhibition efficiency against both enzymes (IC_{50} values of 300 and 400 μM against CYP6AA3 and CYP6P7, respectively). Thus, *A. paniculata* extract was chosen for further investigation and isolation of active inhibitory compounds.

Fractionation of the crude ethanol extract of *A. paniculata* leaves and roots demonstrated that hexane fraction inhibited CYP6AA3 and CYP6P7, under co-incubation, about 3.3- and 6.8-folds, respectively, better than EtOAc fraction (Table 1). This implied that more efficient compounds resided in hexane fraction. The aqueous fraction showed absence of inhibition up to 400 μg/ml. To primarily determine whether the inhibition by *A. paniculata* followed a mechanism-based inhibition pattern, inhibition studies using time-dependent inhibition assay by pre-incubation of *A. paniculata* crude extract with enzyme mixture for 0 and 30 min were performed in the presence of NADPH before addition of substrate. The assay was based on the principle that time-dependent inhibition exhibits increasing degree of inhibition over time of pre-incubation of enzyme mixture with inhibitor and NADPH to allow generation of reactive metabolite as inhibitor. As shown in Table 1, significant augmentation of inhibition on CYP6AA3 was observed with *A. paniculata* ethanol and hexane extracts when pre-incubation time was increased to 30 min, indicating a time-dependent inhibition pattern, while inhibition of CYP6P7 by these extracts remained the same under co-incubation or pre-incubation conditions. There was no indication of time-dependent inhibition for EtOAc fraction against both enzymes.

Bioassay-guided isolation of inhibitory compounds from *A. paniculata* hexane fraction by silica gel column chromatography, HPLC and TLC analyses acquired four flavones and one flavanone in association with potential inhibition effects against both mosquito enzymes. The common structure of flavone and flavanone is shown in Table 2, with rings (A, B, and C) and carbon positions are labeled. All isolated flavonoids shared C5-hydroxylation and the difference of flavones and flavanones is the presence of 2, 3 double bond in the C ring of flavones (Table 2). Four flavones were identified as 5,7,4′-trihydroxyflavone (apigenin), 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone, while the flavanone compound as 5-hydroxy-7,8-dimethoxyflavanone. In EtOAc fraction, one purified diterpenoid was isolated and identified as 14-deoxy-11,12-didehydroandrographolide (DIAP). We also obtained andrographolide, the major constituent of *A. paniculata* (unreported data) from EtOAc fraction. Preliminary determination of time-dependent inhibition was performed with these purified compounds and results (IC_{50} values) revealed the three methoxylated flavones (5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone) primarily displayed time-dependent inhibition with IC_{50} values decreased by approximately 2-folds against CYP6AA3 at incubation time conducted for 30 min (Table 1). None of the isolated compounds exhibited time-dependent inhibition toward CYP6P7.

Modes and kinetic values of inhibition by purified compounds isolated from *A. paniculata* are summarized in Table 2. There were structure–function relationships of the four flavones and kinetics and modes of inhibition toward both mosquito enzymes. The 5,7,4′-trihydroxyflavone inhibited both enzymes with the least K_i values (1.12±0.02 μM for

Table 1 IC_{50} values of extracts and purified compounds of *A. paniculata* and synthetic compounds in inhibition against BROD of CYP6AA3 and CYP6P7

Samples	IC_{50} (μg/ml)[a]			
	CYP6AA3		CYP6P7	
	Co-incubation	Pre-incubation[b]	Co-incubation	Pre-incubation[b]
Ethanol extract	8.08±0.02[c]	6.05±0.04[c]	10.30±0.02	10.26±0.03
Hexane fraction	5.02±0.02[c]	2.57±0.02[c]	6.20±0.05	5.75±0.21
Ethyl acetate fraction	16.57±0.09	15.72±1.08	42.07±0.24	39.12±1.51
Aqueous fraction	>400	ND[d]	>400	ND[d]
Compounds				
5,7,4′-Trihydroxyflavone (μM)	2.03±0.03	1.98±0.24	2.38±0.16	2.23±0.30
5-Hydroxy-7,8-dimethoxyflavone (μM)	7.24±0.07[c]	2.98±0.03[c]	8.90±0.15	8.69±0.13
5-Hydroxy7,8,2′,3′-tetramethoxyflavone (μM)	6.45±0.02[c]	3.39±0.01[c]	8.35±0.12	8.47±0.18
5,4′-Dihydroxy-7,8,2′,3′-tetramethoxyflavone (μM)	5.91±0.08[c]	2.20±0.02[c]	16.6±0.42	15.9±0.21
5-Hydroxy-7,8-dimethoxyflavanone (μM)	24.8±0.14	23.8±0.58	28.2±0.28	27.8±0.06
14-Deoxy-11,12-didehydroandrographolide (μM)	52.5±0.50	51.8±0.14	62.4±0.56	62.0±0.22
5,7-Dihydroxyflavone (μM)[e]	2.22±0.21	2.10±0.13	2.02±0.06	1.96±0.09
5,7-Dihydroxy-8-methoxyflavone (μM)[e]	14.8±0.13	14.3±0.18	16.0±0.09	15.7±0.26

[a] Each value represents mean±SD of triplicate tests

[b] Pre-incubation for 30 min

[c] Significant differences between co-incubation and pre-incubation, $P < 0.05$

[d] ND, not determined

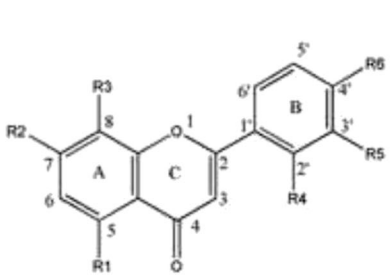
[e] Purchased synthetic compound

CYP6AA3 and 1.35±0.01 μM for CYP6P7). Substitution of 7,8-dimethoxy groups in the A ring and absence of 4′-hydroxy group on 5,7,4′-trihydroxyflavone, resulting 5-hydroxy-7,8-dimethyflavone, decreased inhibition competency (K_i values) by about 5-folds than 5,7,4′-trihydroxyflavone against both enzymes (Table 2). Addition of 2′,3′-dimethoxy groups in the B ring to 5-hydroxy-7,8-dimethoxyflavone, producing 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, slightly decreased the K_i values of CYP6AA3 and CYP6P7. Further 4′-hydroxylation of 5-hydroxy-7,8,2′,3′-tetramethoxyflavone to form 5,4'-dihydroxy-7,8,2′,3′-tetramethoxyflavone decreased the K_i value of CYP6AA3 from 4.73 to 2.32 μM, but significantly increased the K_i value of CYP6P7 from 5.59 to 11.01 μM, indicating an impact of 4′-hydroxy group on inhibition. The flavanone 5-hydroxy-7,8-dimethoxyflavanone was the least potent inhibitor (K_i values of 15.34 and 17.22 μM for CYP6AA3 and CYP6P7, respectively) comparing among five flavonoids. Finally, the diterpenoid DIAP inhibited both mosquito P450s with much less competency than flavonoids.

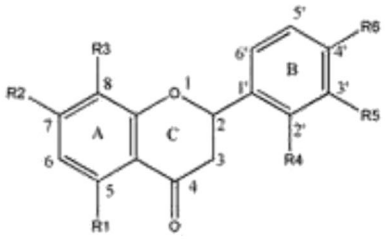
Since 4′-hydroxy and methoxy groups are significant factor for inhibition potential of the four flavones, two commercially available compounds (5,7-dihydroxyflavone (chrysin) and 5,7-dihydroxy-8-methoxyflavone) were examined to see effect on inhibition of methoxy group and with/without the effect of 4'-hydroxy group. The absence of 4′-hydroxy group reduced inhibition effect of CYP6AA3, as illustrated by poorer inhibition of 5,7-dihydroxyflavone than 5,7,4′-trihydroxyflavone (Table 2). In contrast, absence of 4′-hydroxylation in 5,7-dihydroxyflavone improved inhibition against CYP6P7 compared to 5,7,4′-trihydroxyflavone. Addition of 8-methoxy group in A ring onto 5,7-dihydroxyflavone (resulting 5,7-dihydroxy-8-methoxyflavone) significantly decreased inhibition potency (K_i values) against both enzymes compared to 5,7-dihydroxyflavone (Table 2).

All methoxylated flavonoids inhibited CYP6AA3 in a mixed-type (combination of competitive and noncompetitive) mode, while hydroxylated flavones without methoxy groups (5,7,4′-trihydroxyflavone and 5,7-dihydroxyflavone) displayed noncompetitive inhibition (Table 2 and Fig. S1). This is in contrast to modes of inhibition against CYP6P7, of which methoxylated flavonoids were noncompetitive, and hydroxylated flavones without methoxy groups were mixed-type (Table 2 and Fig. S2). Since a 30-min pre-incubation indicated that three methoxylated flavones (5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone) increased inhibition of CYP6AA3 (Table 1), further investigation of inhibition in the presence of NADPH at different pre-incubation times and inhibitor concentrations was performed. Results indicated that inhibition pattern of the three flavones conformed to time- and concentration-dependent inhibition in the presence of NADPH, as inhibition effect was increased

Table 2 Chemical structures of flavonoids and 14-Deoxy-11,12-didehydroandrographolide, K_i values and mode of inhibition



Flavone



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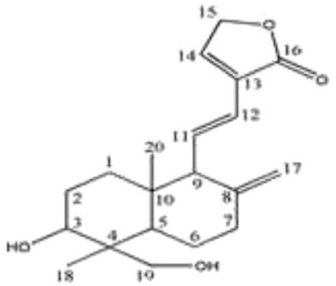

14-deoxy-11,12-didehydroandrographolide

Samples	R1	R2	R3	R4	R5	R6	K_i values (μM)[a] Mode of inhibition	
							CYP6AA3	CYP6P7
Flavones								
5,7,4'-trihydroxyflavone	OH	OH	H	H	H	OH	1.12±0.02 Noncompetitive	1.35±0.01 Mixed-type
5-hydroxy-7,8-dimethoxyflavone	OH	OCH_3	OCH_3	H	H	H	6.50±0.14 Mixed-type	6.39±0.69 Noncompetitive
5-hydroxy7,8,2',3'-tetramethoxyflavone	OH	OCH_3	OCH_3	OCH_3	OCH_3	H	4.73±0.33 Mixed-type	5.59±0.13 Noncompetitive
5,4'-dihydroxy-7,8,2',3'-tetramethoxyflavone	OH	OCH_3	OCH_3	OCH_3	OCH_3	OH	2.32±0.06 Mixed-type	11.01±0.23 Noncompetitive
Flavanone								
5-hydroxy-7,8-dimethoxyflavanone	OH	OCH_3	OCH_3	H	H	H	15.34±0.41 Mixed-type	17.22±0.07 Noncompetitive
Diterpenoid								
14-Deoxy-11,12-didehydroandrographolide	-	-	-	-	-	-	38.75±0.07 Noncompetitive	54.27±0.70 Noncompetitive
Synthetic compounds								
5,7-dihydroxyflavone	OH	OH	H	H	H	H	1.59±0.06 Noncompetitive	1.10±0.16 Mixed-type
5,7-dihydroxy-8-methoxyflavone	OH	OH	OCH_3	H	H	H	9.70±0.12 Mixed-type	10.29±0.41 Noncompetitive

[a] Each value represents the mean±SD of triplicate experiments

together with increased time of pre-incubation and inhibitor concentration (Fig. 1). The absence of NADPH abolished time-dependent inhibition of CYP6AA3 of the three flavones, suggesting that the three compounds are mechanism-based inhibitors of CYP6AA3 through catalysis process (an irreversible inhibition mode). The efficiencies of CYP6AA3 inactivation calculated from the ratio of k_{inact} to K_I by 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone were 10.98, 21.12, and 44.44 $min^{-1}mM^{-1}$, respectively (Fig. 1), in correlation with the order of K_i values shown in Table 2. Molecular docking of previously generated CYP6AA3 and CYP6P7 enzyme homology models (Lertkiatmonkol et al. 2011) with methoxylated flavonoids supported the capacity of the large active site of CYP6AA3 enzyme in accommodating these flavonoids near the heme center during catalytic process (see examples of methoxylated flavones in Fig. 2b, c). The non-methoxylated favones, however, were positioned distantly from CYP6AA3 heme center, in congruence with noncompetitive inhibition (see example of 5,7,4′-trihydroxyflavone in Fig. 2a). In contrast the narrow CYP6P7 cavity selected for non-methoxylated flavones (example of 5,7,4′-trihydroxyflavone is shown in Fig. 2d) and caused structural constraint toward the more bulky methoxylated flavonoids, as these flavonoids situated afar from the CYP6P7 heme center catalytic site (Fig. 2e, f). Finally, considering the K_i values, flavonoids inhibited both enzymes about 2- to 30-folds for CYP6AA3 and 3- to 42-folds for CYP6P7 better than DIAP that non-competitively inhibited both mosquito enzymes.

Crude ethanol extract, hexane and EtOAc fractions, purified compounds of *A. paniculata*, and two synthetic compounds decreased cypermethrin LC_{50} values in P450-expressing cells compared to the uninfected parent cells, due to synergistic action of *A. paniculata* compounds with cypermethrin cytotoxicity (Table 3). Two *A. paniculata*

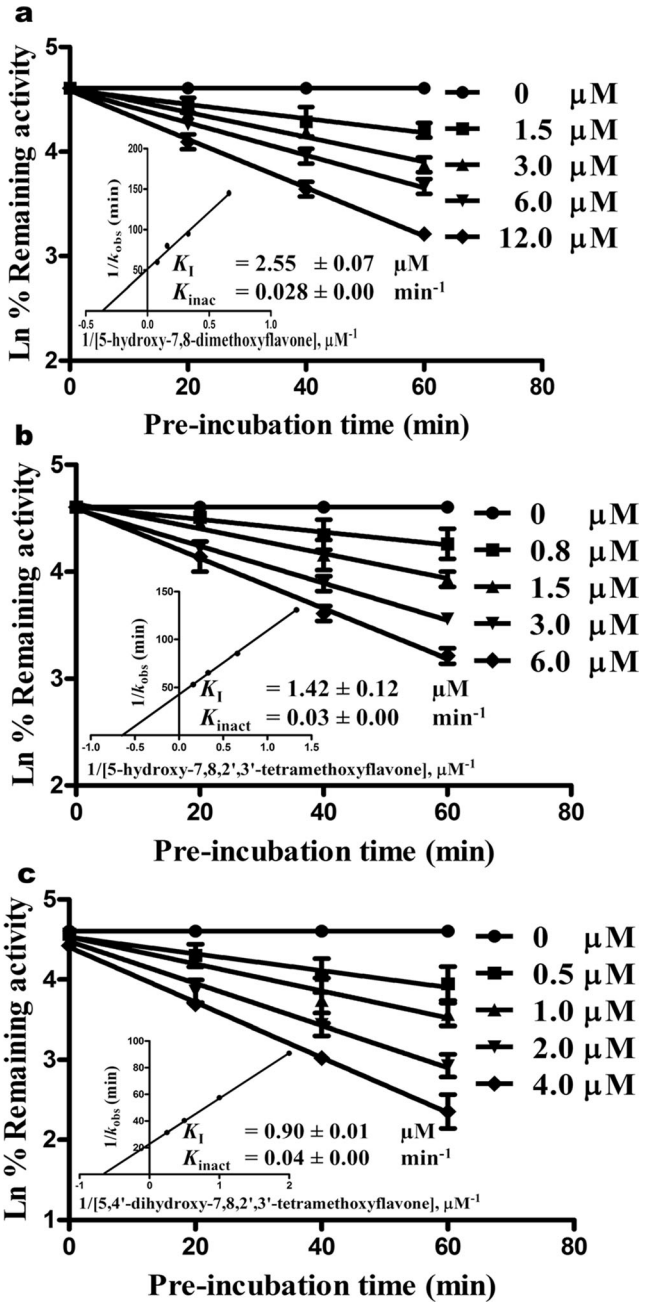

Fig. 1 Time-dependent inhibition and kinetics of inhibition of CYP6AA3-mediated BROD by 5-hydroxy-7,8-dimethoxyflavone (**a**), 5-hydroxy-7,8,2′,3′-tetramethoxyflavone (**b**) and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone (**c**). *Inlets* represent double-reciprocal plots of k_{obs} values against compound concentrations. Each *point* represents the mean±SD of triplicate experiments

compounds, 5,7,4′-trihydroxyflavone and DIAP, and the synthetic 5,7-dihydroxyflavone and 5,7-dihydroxy-8-methoxyflavone, were noted consisting of significant synergistic effect on cypermethrin cytotoxicity in the uninfected Sf9 parent cells. Thus we normalized percent cell viability of P450-expressing cells upon treatment with cypermethrin and each of the fractions or compounds with that of Sf9 parent cells (Table 3).

As displayed in Table 3, the order of synergism of the four *A. paniculata* flavones and two synthetic flavones was in accordance with the order of their enzymatic inhibition potency against both mosquito enzymes in vitro, with highest synergistic effect observed with 5,7,4′-trihydroxyflavone, followed by 5,7-dihydroxyflavone, 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, 5-hydroxy-7,8-dimethoxyflavone, and 5,7-dihydroxy-8-methoxyflavone, in CYP6AA3-expressing cells. Similar order of synergism of flavonoids was observed for CYP6P7-expressing cells, except 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone that showed the least synergistic effect, among flavones. The flavonone 5-hydroxy-7,8-dimethoxyflavanone and DIAP, both of which possessed lower inhibition activity than flavones, comprised lower synergistic effect with cypermethrin toxicity than all flavones. As expected, andrographolide which poorly inhibited both mosquito enzymes in vitro showed the least synergistic effect in cells expressing CYP6AA3 or CYP6P7.

Discussion

We identified four flavones (5,7,4′-trihydroxyflavone, 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′-tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone), one flavanone (5-hydroxy-7,8-dimethoxyflavanone) and a diterpenoid (14-deoxy-11,12-didehydroandrographolide, DIAP) in *A. paniculata* that possessed inhibition effects on BROD mediated by the *An. minimus* CYP6AA3 and CYP6P7. Inhibition of flavonoids against P450 enzymes has been reported in insects including CYP6B1 in the black swallowtail *Papilio polyxenes* (Wen et al. 2006), CYP6D1 in the house fly *Musca domestica* (Scott et al. 2000), CYP6Z2 in the mosquito *An. gambiae* (Mclaughlin et al. 2008), and CYP6AA3 and CYP6P7 in *An. minimus* (Duangkaew et al. 2011b). In this study, rotenone whose structure related to isoflavone displayed inhibitory effect on both CYP6AA3 and CYP6P7, might act together with PBO in inhibition of detoxification system, resulting in effective synergism as previously reported for *Haemonchus contortus* and *Trichostrongylus colubriformis* helminth parasites (Kotze et al. 2006) .

The inhibition kinetics of flavonoids isolated from *A. paniculata* toward CYP6AA3 and CYP6P7 lend information on structure–function relationships for the inhibition of both enzymes by these flavonoids. As noted, the flavone, 5-hydroxy-7,8-dimethoxyflavone, inhibited both enzymes approximately 2.5-fold more potent than flavanone, 5-hydroxy-7,8-dimethoxyflavanone (Table 2) suggesting the 2,3 double bond in the C ring played an important role in inhibition. Similarly the core flavone compound was found more inhibition efficacy than the core flavonone against the pyrethroid

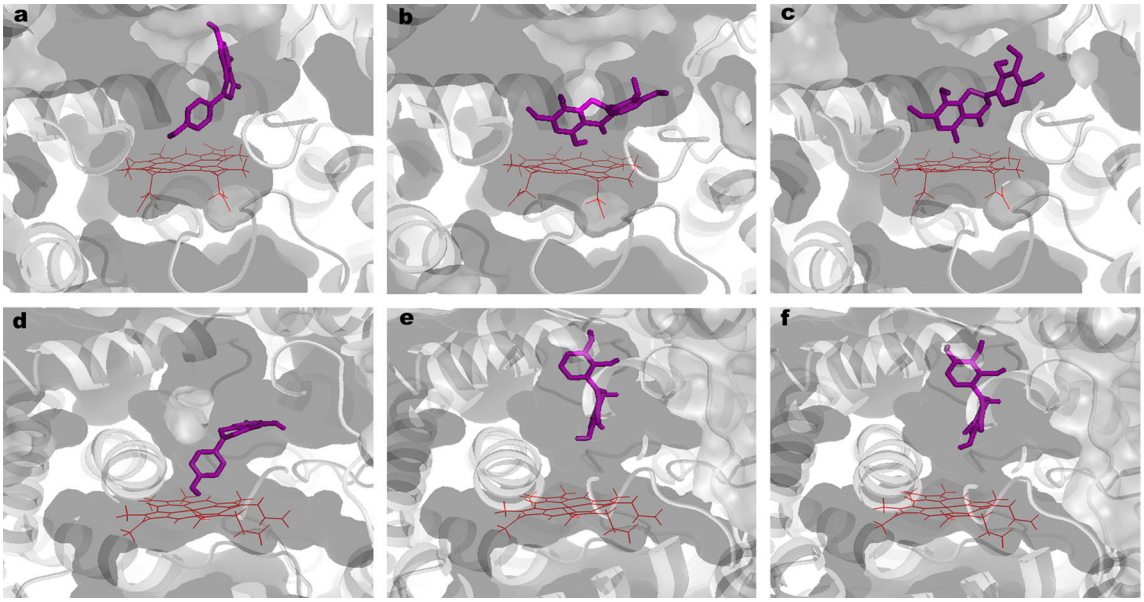

Fig. 2 Binding of 5,7,4′-trihydroxyflavone (**a**, **d**), 5-hydroxy-7,8,2′,3′-tetramethoxyflavone (**b**, **e**) and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone (**c**, **f**) in active sites of CYP6AA3 (**a**–**c**) and CYP6P7 (**d**–**f**). The model structures of CYP6AA3 and CYP6P7 are shown in *white cartoons*. The heme group in the middle of each structure is represented by a *red line*, while flavones are displayed in *magenta sticks*

metabolizing CYP6D1 in housefly *M. domestica* and the allelochemical metabolizing enzyme CYP6B1 in black swallowtail *P. polyxenes* (Scott et al. 2000, Wen et al. 2006).

All flavonoids isolated in the present investigation consisted of 5-hydroxy group, but its role in inhibition capability toward CYP6AA3 and CYP6P7 is not known. Comparing to 5,7,4′-trihydroxyflavone, substitution of 7,8-dimethoxy groups in the A ring and absence of 4′-hydroxy group (resulting 5-hydroxy-7,8-dimethoxyflavone) led to a decrease in inhibition against both CYP6AA3 and CYP6P7, but the presence of 2′,3′-dimethoxy groups of the B ring (5-hydroxy-7,8,2′,3′-tetramethoxyflavone), on the contrary, slightly improved inhibition on both enzymes (Table 2). To better explore the effect on inhibition of a methoxy group in the A ring without the effect by 4′-hydroxy group, two commercially available compounds, 5,7-dihydroxyflavone and 5,7-dihydroxy-8-methoxyflavone, were inspected. The results signified the negative inhibition effect of the 8-methoxy group at the A ring on both enzymes, as 5,7-dihydroxy-8-methoxyflavone (K_i values of CYP6AA3 and CYP6P7 were 9.7 and 10.29 μM, respectively) markedly diminished inhibition than 5,7-dihydroxyflavone (K_i values of CYP6AA3 and CYP6P7 were 1.59 and 1.10 μM, respectively). The effect of 7-methoxy group in A ring could not be concluded. Interestingly, the presence of 4′-hydroxyl group in flavones, comparing between 5,7-dihydroxyflavone and 5,7,4′-trihydroxyflavone, and between 5-hydroxy-7,8,2′,3′-tetramethoxyflavone and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone, augmented inhibition on CYP6AA3 as opposed to a decline in inhibition on CYP6P7 (Table 2). This suggested that different mechanisms of inhibition were acted on CYP6AA3 and CYP6P7 by those flavones with 4′-hydroxyl group and with/without methoxy groups. In human P450s, different inhibition mechanisms for CYP1A1, CYP1A2, CYP1B1, CYP2C9 and CYP3A4 by various flavonoids and that the number and position of hydroxyl and/or methoxy groups have also been found highly impact on inhibition potential of flavonoids (Shimada et al. 2010) .

As shown in Table 2, methoxylated flavonoids inhibited CYP6AA3 with mixed-type mode by which they could compete with substrate for binding with the CYP6AA3 active site and noncompetitively bind to the enzyme–substrate complex, implicating that these flavonoids could fit within the large substrate binding cavity of CYP6AA3 (Lertkiatmongkol et al. 2011). In addition, the ability of 5-hydroxy-7,8-dimethoxyflavone, 5-hydroxy-7,8,2′,3′- tetramethoxyflavone, and 5,4′-dihydroxy-7,8,2′,3′-tetramethoxyflavone to inhibit CYP6AA3 in mechanism-based inhibition (irreversible mode of inhibition) coincides with ability of the large CYP6AA3 active site to accommodate each of the three compounds during catalysis process. In contrast, among flavonoids investigated, non-methoxylated flavones (5,7,4′-trihydroxyflavone and 5,7-dihydroxyflavone) expressed mixed-type inhibition on CYP6P7, while methoxylated flavones were in noncompetitive mode. In this context it could be assumed that the methoxy groups of these flavonoids might be more bulky and be precluded from situating in the narrow active site of CYP6P7 as previously described (Lertkiatmongkol et al. 2011). These assumptions were elucidated by initial molecular docking, of that large CYP6AA3 pocket allowed passage of

Table 3 Effect of extracts and purified compounds of *A. paniculata* and synthetic compounds against cypermethrin susceptibility of Sf9, CYP6AA3-, and CYP6P7-expressing cells

Cells	Treatment	LC_{50} (μM)[a]	SR[b]	Ratio[c]
Sf9 cells	Cypermethrin	150.3±0.57[d,e]	–	–
	+Ethanol extract	138.0±2.88[d,e]	1	NA
	+Hexane extract	99.6±0.23[d,e]	1.5	NA
	+Ethyl acetate extract	60.0±1.15[d]	2.5	NA
	+5,7,4′-Trihydroxyflavone	100.0±1.73[d,e]	1.5	NA
	+5-Hydroxy-7,8-dimethoxyflavone	149.6±0.57[e]	1	NA
	+5-Hydroxy7,8,2′,3′-tetramethoxyflavone	132.3±1.15[d,e]	1.13	NA
	+5,4′-Dihydroxy-7,8,2′,3′-tetramethoxyflavone	120.7±1.30[d,e]	1.24	NA
	+5-Hydroxy-7,8-dimethoxyflavanone	150.0±0.13[e]	1	NA
	+14-Deoxy-11,12-didehydroandrographolide	60.5±0.50[d,e]	2.48	NA
	+Andrographolide[f]	150.0±0.15[e]	1	NA
	+5,7-Dihydroxyflavone[f]	80.9±0.76[d,e]	1.85	NA
	+5,7-Dihydroxy-8-methoxyflavone[f]	50.1±0.76[d,e]	3	NA
CYP6AA3-expressing cells	Cypermethrin	393.3±5.77[d,e]	–	–
	+Ethanol extract	72.66±2.51[d,e]	5.41	5.41
	+Hexane extract	50.0±1.73[d,e]	7.86	5.24
	+Ethyl acetate extract	61.0±1.52[d]	6.44	2.57
	+5,7,4′-Trihydroxyflavone	35.3±1.52[d,e]	11.14	7.42
	+5-Hydroxy-7,8-dimethoxyflavone	75.1±1.04[d,e]	5.23	5.23
	+5-Hydroxy7,8,2′,3′-tetramethoxyflavone	59.0±1.73[d,e]	6.66	5.89
	+5,4′-Dihydroxy-7,8,2′,3′-tetramethoxyflavone	50.0±2.07[d,e]	7.86	6.29
	+5-Hydroxy-7,8-dimethoxyflavanone	180.3±1.23[d,e]	2.18	2.18
	+14-Deoxy-11,12-didehydroandrographolide	54.3±0.57[d,e]	7.24	2.91
	+Andrographolide[f]	205.0±5.00[d,e]	1.91	1.91
	+5,7-Dihydroxyflavone[f]	33.4±0.13[d,e]	11.77	6.36
	+5,7-Dihydroxy-8-methoxyflavone[f]	28.0±3.13[d,e]	14.04	4.68
CYP6P7-expressing cells	Cypermethrin	442.0±2.28[d,e]	–	–
	+Ethanol extract	122.1±2.02[d,e]	3.61	3.61
	+Hexane extract	93.4±0.57[d,e]	4.73	3.15
	+Ethyl acetate extract	90.3±2.88[d,e]	4.89	1.95
	+5,7,4′-Trihydroxyflavone	40.8±0.50[d,e]	10.83	7.22
	+5-Hydroxy-7,8-dimethoxyflavone	93.0±5.19[d,e]	4.75	4.75
	+5-Hydroxy7,8,2′,3′-tetramethoxyflavone	75.3±0.50[d,e]	5.86	5.18
	+5,4′-Dihydroxy-7,8,2′,3′-tetramethoxyflavone	110.22±0.15[d,e]	4.01	3.23
	+5-Hydroxy-7,8-dimethoxyflavanone	141.12±0.63[d,e]	3.13	3.13
	+14-Deoxy-11,12-didehydroandrographolide	62.8±0.76[d,e]	7.03	2.83
	+Andrographolide[f]	254.0±2.64[d,e]	1.74	1.74
	+5,7-Dihydroxyflavone[f]	30.0±0.23[d,e]	14.73	7.96
	+5,7-Dihydroxy-8-methoxyflavone[f]	35.6±1.25[d,e]	12.41	4.13

NA not applicable

[a] Each value is mean±SD of triplicate experiments

[b] Synergism ratio: LC_{50} cypermethrin alone/LC_{50} cypermethrin in the presence of inhibitors

[c] Synergism ratio for P450-expressing cells after normalization with Sf9 cells

[d] Significant difference between cypermethrin alone and with inhibitors, $P < 0.05$

[e] Significant difference of each treatment between Sf9 and P450-expressing cells, $P < 0.05$

[f] Purchased compound

methoxylated flavones to fit in its cavity (see example in Fig. 2b,c), in contrast to the limited access of these flavones to the narrow CYP6P7 cavity (Fig. 2e,f). Moreover, the absence of methoxy groups enabled 5,7,4′-trihydroxyflavone and 5,7-dihydroxyflavone to be orientated within the CYP6P7 cavity (see example in Fig. 2d), supported by their mixed-type inhibition characteristics. However detail of how hydroxyl and/or methoxy groups acting on and inhibiting both mosquito P450 catalytic activities requires further investigation.

By using the MTT cytotoxicity assays on Sf9 cells, the flavonoids we isolated from *A. paniculata* showed a synergistic effect on susceptibility of P450-expressing cells toward cypermethrin cytotoxicity, attributed to their inhibition potential against both enzymes. This is in compliance with the synergistic effect of *A. paniculata* extracts with deltamethrin toxicity against *An. stephensi* (Chenniappan and Kadarkarai 2008). In the current study 5,7,4′-trihydroxyflavone and the synthetic 5,7-dihydroxyflavone and 5,7-dihydroxy-8-methoxyflavone were cytotoxic to the uninfected parent Sf9 cells with LC_{50} values of 27.5, 18 and 13.2 μM, respectively, supporting previous results of their efficient insecticidal activities against the *S. frugiperda* fall armyworm (Romanelli et al. 2010). The *A. paniculata* extracts have been reported insecticidal to *Cx. quinquefasciatus* and *Ae. aegypti* mosquitoes (Govindarajan 2011; Govindarajan and Sivakumar 2012), and the aerial parts of *A. paniculata* that were shown larvicidal to *An. stephensi* mosquitoes was attributed to the flavonoid extract (Guatum et al. 2013). Whether the *A. paniculata* flavonoids we reported herein might be responsible for the insecticidal activity observed against *Anopheles* mosquitoes remain to be examined. In addition oviposition deterrent and ovicidal effects of *A. paniculata* extracts have been shown against *Cx. quinquefasciatus* and *An. stephensi* mosquitoes (Elango et al. 2010; Kuppusamy and Murugan 2010). Further investigation on growth, development, reproduction, and synergistic effects with pyrethroid toxicity toward *An. minimus* mosquitoes of *A. paniculata* flavonoids will be beneficial to mosquito control management.

We previously isolated rhinacanthin-A, -B, and -C whose structures are naphthoquinones from *R. nasutus* (Pethuan et al. 2012). These compounds contained comparable inhibition potency (IC_{50} values of rhinacanthin-A, -B, and -C for CYPAA3 are 9.15, 2.39, and 10.64 μM, respectively, and values for CYP6P7 are 35.14, 3.69, and 9.56 μM, respectively) with flavones reported herein (Table 1). Synergistic effects with cypermethrin toxicity of these flavones on Sf9 cells expressing either CYP6AA3 or CYP6P7 are as effective as rhinacanthins (Pethuan et al. 2012). These results could imply that the phenolic compounds as represented by naphthoquinones or flavones might be important to inhibition against both *An. minimus* P450 enzymes. Taken together flavonoids isolated from *A. paniculata* exhibited high inhibition potency against the *An. minimus* P450s, especially with compounds presented irreversible inhibition mode, and contained synergistic effects on cypermethrin cytotoxicity in cell-based assays. *A. paniculata* has long been used as traditional medicine for treatment of common cold. It exhibits various pharmacological activities including anti-inflammatory, anti-atherosclerotic, anti-infection, and hepato-protective activities and is considering safe (Chao and Lin 2010). Thus considering the potency of *A. paniculata* as insecticidal agents and against growth of mosquitoes and the relatively non-toxicity to rodents (Burgos et al. 1997; Govindarajan and Sivakumar 2012), the *A. paniculata* extract could have an implication for mosquito vector control and insecticide resistance management through prevention of P450 detoxification activity.

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