

Applications of Corning® BioCoat™ Pre-coated PAMPA Plate System for Studying Human CYP3A4 Inhibition by a Botanical Ingredient of Dietary Supplement, Açai

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Application Note

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Introduction

Natural products are usually ingested for medicinal use either as components of complex extracts in traditional herbal preparations or as phytopharmaceuticals¹. Nutritional supplements available over the counter with health benefits come without specific medicinal claims². Botanical dietary supplement (BDS) from açai (*Euterpe oleracea* Mart) berries are highly consumed globally for potential medicinal use³. In natural product research, especially complex plant mixtures (CPM) such as BDS that contain hundreds of compounds, it has often been difficult to identify the exact compounds responsible for a biological response of a CPM. The Parallel Artificial Membrane Permeability Assay (PAMPA) is a well-accepted screening assay for the early prediction of transcellular passive absorption through biological membranes. Corning BioCoat Pre-coated PAMPA Plate System consists of a lipid-oil-lipid tri-layer artificial membrane that improves the PAMPA model and correctly predicts the permeability of traditionally underpredicted compounds. The lipid-oil-lipid tri-layer structure does not contain excessive amounts of solvent thereby reduces permeation pathway and better mimics the exterior and interior biological membrane of the intestinal barrier⁴⁻⁶.

The human cytochrome P450(CYP) 3A family is of critical importance to drug discovery and development due to its involvement in the metabolism of the majority of drugs on the market. Of the four isozymes within the human CYP3A family, CYP3A4 is the most abundant and is an important enzyme that contributes to the metabolism of about 60% of clinically used drugs⁷. However, the polymorphic member CYP3A5 can also contribute significantly to the metabolism of many drugs, such as midazolam⁸. Here we demonstrate the Corning BioCoat Pre-coated PAMPA Plate System can be used to investigate inhibition potential of açai, an ingredient of BDS, on midazolam 1'-hydroxylation catalyzed by human CYP3A under various conditions.

Materials and Methods

Chemical Reagents, BDS, and Solvents

Açai (*Euterpe oleracea* Mart) berry powder was supplied by Mountain Rose Herbs. All solvents used were HPLC or LC-MS grade and purchased from Thermo Fisher Scientific. DMSO, LC-MS grade formic acid, KH₂PO₄, Na₂HPO₄, MgCl₂, EDTA, midazolam,

and NADPH were purchased from MilliporeSigma. The internal standard Corning Gentest® Hydroxymidazolam-[¹³C₃] (Corning Cat. No. 451010) was purchased from Corning, and Homoeriodictyol (purified compound from açai extract) was purchased from Extrasynthese (Cat. No. 12835).

Preparation of Plant Extracts

Açai berry powder was treated with dichloromethane for the extraction of lipophilic/non-polar compounds. The generated açai residue was further extracted with methanol three times by sonication. The methanol extracts were combined and centrifuged at 4,000 rpm at 4°C for 20 min. The supernatant was filtered through a 0.2 µm polytetrafluoroethylene membrane filter and dried under high vacuum (295 mbar) at 40°C. Açai extract was then further dried by nitrogen evaporation and lyophilization. The yield for açai methanol extract was 7.55% (w/w).

PAMPA Assay

The Corning BioCoat Pre-coated PAMPA Plate (Corning Cat. No. 353015) was warmed to room temperature for at least 30 min. prior to use. The donor compartment of the 96-well microplate system simulated intestinal content pre-absorption, while the acceptor compartment simulated passively absorbed compounds. A serial dilution of açai extract solution (25 µg/µL to 0.195 µg/µL) was prepared in PAMPA assay buffer (0.014 M KH₂PO₄ and 0.054 M Na₂HPO₄, pH 7.4) with an optimized DMSO concentration of 0.417%.

Açai extract solution (300 µL/well) was added in the receiver plate (donor), and PAMPA assay buffer (200 µL/wells) was added to wells in the pre-coated filter plate (acceptor). The filter plate was then coupled with the receiver plate and the plate assembly was incubated at room temperature and/or 37°C for 5 hours without shaking or with shaking at 75 rpm. For shaking, two different shakers were used (Shaker 1: Thermo Scientific MaxQ™ 5000 Floor-Model; Shaker 2: Beckman Coulter Biomek 4000 Automated Liquid Handler using Inheco Single Temperature Control). At the end of the incubation, the plates were separated and the contents in the donor compartment plate were stored directly, whereas contents from the acceptor compartment were transferred to a new 96-well clear microplate for storage and subsequent studies.

Human Liver Microsome Inhibition Assay

CYP3A4 enzymatic reaction matrices contained permeable (acceptor side) and non-permeable diffused (donor side) compounds of açai extracts from PAMPA assays and 0.2 mg/mL single donor human liver microsome (HLM) CYP3A5*3*3 (non-expresser used to assess only CYP3A4 activity) from various manufacturers in inhibition buffer (5 mM MgCl₂ and 1 mM EDTA in 100 mM potassium phosphate buffer, pH 7.4). Ketoconazole (10 µM) was used as a positive control in place of açai extract, while DMSO control from PAMPA plates was used as a negative control to delineate the CYP3A4 inhibition effect of açai extracts from DMSO. Midazolam (gold standard probe for CYP3A4/5 activity) stock solution prepared in methanol:buffer (30:70 v/v) was added at its K_m concentration (3 µM)⁹. The reaction mixtures were pre-incubated at 37°C for 10 min. with shaking (75 rpm), after which the reactions were initiated by the addition of 1 mM NADPH and incubated at 37°C with shaking (75 rpm for 15 min.). Reactions were stopped by the addition of 20 µL water/acetonitrile (ACN)/formic acid (92:5:3, v/v/v) with stable isotope labelled internal standard (13C³ 1'-Hydroxymidazolam, 1.0 µM) to minimize the error generated from dilution bias. Subsequently, reaction mixtures were vortexed for 1 min. and centrifuged at 8,000 x g at 4°C for 15 min. The filtrates were subjected to LC-MS analysis to quantitate the production of metabolite 1'-hydroxymidazolam.

Corning® Supersomes™ Inhibition Assay

The inhibition assay described above was followed with the exception that Corning Supersomes Human CYP3A4 + Oxidoreductase + b5 recombinant enzyme (Corning Cat. No. 456202) (2 pmol final concentration) was used and instead of using the açai methanol extract, a single compound 'homoeriodictyol' obtained from açai methanol extract was used for CYP3A4 inhibition assay, and not the entire açai methanol extract. Homoeriodictyol also went through PAMPA system before adding it to the Supersomes inhibition assay mixture.

LC-MS Method

Liquid chromatography mass spectrometry (LC-MS) was performed exactly as described in detail⁷. Briefly, Agilent 6520 Q-TOF mass spectrometer with a 1220 rapid resolution liquid chromatography system was used for the quantitation of 1'-hydroxymidazolam. The MS conditions were optimized with a positive mode electrospray (ESI)-MS analysis. Nitrogen was used as a nebulizing gas at 25 psi and as a drying gas at 10L/min. LC conditions consisted of a gradient mobile phase with (A) water containing 0.1% formic acid and (B) methanol containing 0.1% formic acid. Detection was performed in positive ESI mode.

The ratio of the peak areas of internal standard and metabolites produced by LC-MS and other calculations were performed using Microsoft® Excel. Log dose-response curves and half maximal inhibitory concentration (IC₅₀) values were calculated using GraphPad Prism 5.02 software (GraphPad Software).

Results and Discussion

As the use of herbal medicines in the form of dietary supplements is universally recognized, concerns on the risk of herb-drug interactions has increased research interest on investigating inhibitory effects on CYPs. PAMPA is a robust method and has been effectively utilized in early stage prediction of drug-botanical screening because of its high throughput capability and correlation with *in vivo* absorption data^{1,10}. Because CYP3A is involved in the metabolism of many medications, in this study we evaluated the CYP3A4 inhibition potentials of passively diffused compounds in the botanical, açai (*Euterpe oleracea*) utilizing the Corning BioCoat™ pre-coated PAMPA plate system. Human liver microsome CYP3A5*3*3 genotype deprived of CYP3A5 was used only for evaluation of CYP3A4 activity from Corning and a comparable brand were screened to determine the inhibition of midazolam metabolism. Using the Corning BioCoat pre-coated PAMPA plate, comparative absorption profiles particularly for passive diffusion of açai plant extract was investigated at recommended conditions (room temperature incubation, without shaking), as well as at 37°C incubation with shaking as a representative of physiological condition.

Compounds in açai may act as both CYP3A4 inducers and inhibitors due to the chemical complexity of the extracts⁷. Data in Table 1 and Figure 1 show inhibitory effect of açai on CYP3A4-catalyzed midazolam 1-hydroxylation in HLM harboring the CYP3A5*3*3 genotype. The lower IC₅₀ and açai extract in the donor chamber than in the acceptor chamber suggests the compounds responsible for CYP3A4 inhibition seem to be more concentrated to the donor compartment, and that they have relatively low passive permeation. As summarized in Table 1, the IC₅₀ values of passively diffused constituents of the açai extract for midazolam 1-hydroxylation are similar between HLMs from different manufacturers. IC₅₀ data for the inhibition potential of açai among CYP3A5*3*3 genotype used showed no significant difference in the bioactivity between the room temperature or 37°C incubation, and static or shaking methods.

To establish methodology for the study of single natural product rather than complex natural product mixtures, passively diffused homoeriodictyol (found in açai) for Corning Supersomes Human CYP3A4 inhibition assay was investigated. The data from IC₅₀ profile screening (Figure 2) demonstrate homoeriodictyol exhibited significant inhibition of CYP3A4 metabolite formation. The similarity of concentration in both the compartments, and both are much higher than açai extract IC₅₀ in donor chamber suggest either high permeability of homoeriodictyol or it may not be the major component that is responsible for the inhibition. The *in vivo* predictions of açai and CYP3A4 inhibition by homoeriodictyol compound are in agreement with previous report⁷ thus validating the predictive value of these *in vitro* screening assays using the pre-coated PAMPA plate system.

Table 1. IC₅₀ values for inhibition of human liver microsome CYP3A5*3*3 expressing only CYP3A4 by compounds in açai extract under various conditions (listed in µg/µL).

Corning	Shaker 1 ^{a,c}	Shaker 2 ^{b,c}	No Shaking ^{c,d}
Acceptor	12.44	11.62	12.05
Donor	0.485	0.553	0.534
Comparable Brand			
Acceptor	16.91	14.64	ND ^e
Donor	0.4393	0.4807	ND

More than one shaker was used to show reproducibility of the method.

^a Thermo Scientific MaxQ™ 5000 Floor-Model Shaker; 37°C incubation.

^b Beckman Coulter Biomek 4000 Automated Liquid Handler using Inheco™ Single Temperature Control; 37°C incubation.

^c T-test with assumed unequal variances determined there to be no statistical significance between HLM from Corning or comparable brand, different shaking methods or temperature conditions.

^d Room temperature incubation.

^e ND (Not determined).

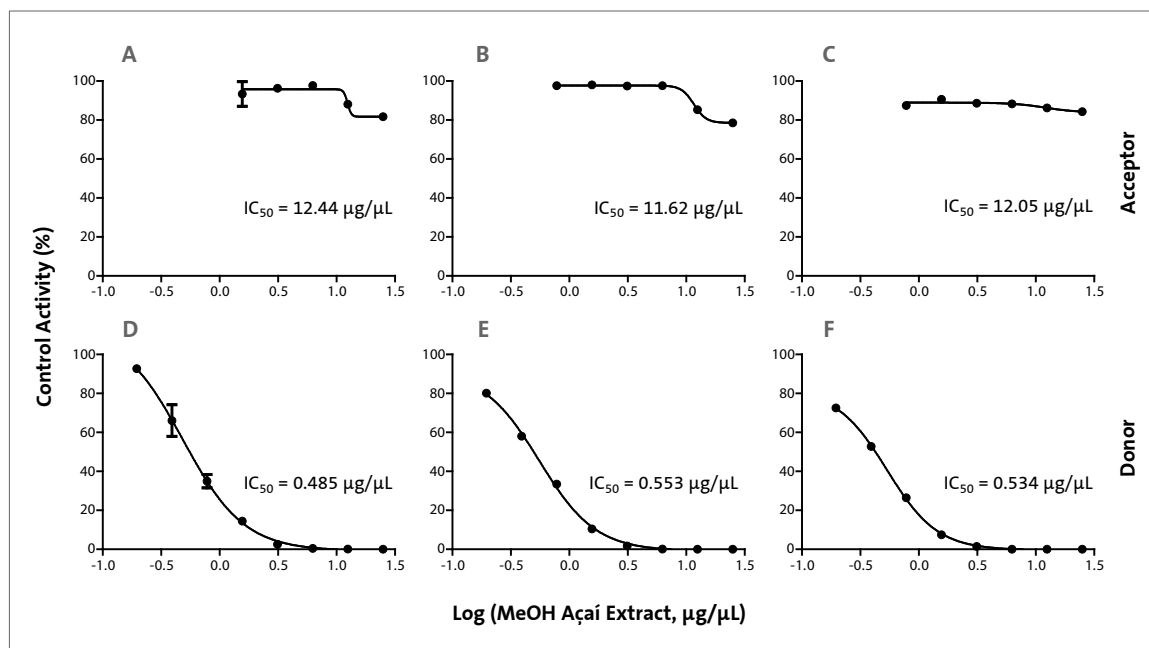


Figure 1. IC₅₀ curves displaying inhibition of Corning human liver microsomes, CYP3A5*3*3 expressing only CYP3A4 by compounds of açai extract obtained from the Corning BioCoat pre-coated PAMPA plate. (A) Acceptor side (passively diffused compounds), Shaker 1, 37°C incubation. (B) Acceptor side, Shaker 2, 37°C. (C) Acceptor side, no shaking, room temperature (RT) incubation. (D) Donor side (non-passively diffused compounds), Shaker 1, 37°C. (E) Donor side, Shaker 2, 37°C. (F) Donor side, no shaking, RT. Activity expressed as the percentage of the remaining activity of 1'-hydroxymidazolam formation in CYP3A5*3*3 in comparison to the control. IC₅₀ values were calculated based on the concentration of the donor compartment at time 0. Data expressed as mean ± SD of three independent experiments.

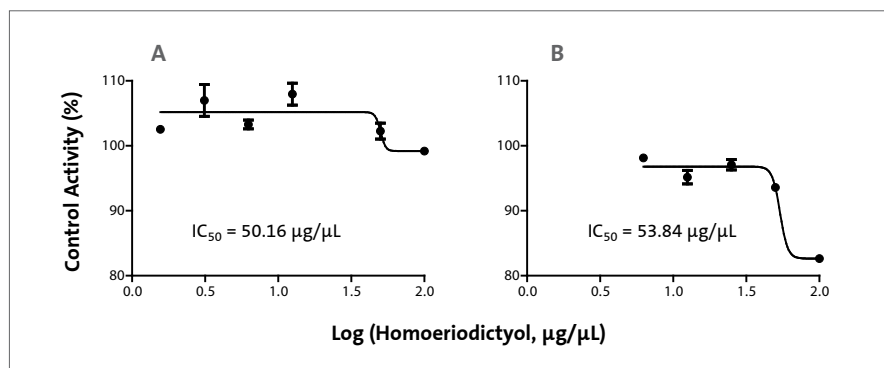


Figure 2. IC₅₀ curves displaying inhibition of Corning Supersomes Human CYP3A4 by Homoeriodictyol; Shaker 2*. (A) Acceptor side. (B) Donor side. Activity expressed as the percentage of the remaining activity of 1'-hydroxymidazolam formation in CYP3A4 in comparison to the control. IC₅₀ were calculated based on the concentration of the donor compartment at time 0. Data expressed as mean ± SD of three independent experiments.

*Beckman Coulter Biomek 4000 Automated Liquid Handler using Inheco™ Single Temperature Control.

Conclusions

The Corning PAMPA system can be used to investigate CYP450 inhibition by compounds in botanical ingredients and botanical dietary supplements. The lipid-oil-lipid tri-layer artificial membrane is ideally suited for conducting studies at room temperature without agitation (recommended), thereby eliminating extra steps but can also be used at 37°C incubation with shaking to mimic physiological conditions.

- ▶ Corning® BioCoat™ Pre-coated PAMPA Plate System is a robust method to predict the transcellular passive absorption of botanical plant extract compounds.
- ▶ The passively absorbable compounds of açai extract exhibited inhibition effects on HLM CYP3A5*3*3 as well as a single compound, homoeriodictyol extracted from açai inhibited CYP3A4, at high concentrations, both suggesting the potential to produce botanical-drug interactions.
- ▶ Comparative data, with and without shaking conditions supports that Corning pre-coated artificial membrane is robust and does not need shaking, but moderate shaking may be used.
- ▶ Room temperature is recommended for PAMPA. However, the pre-coated artificial membrane is robust and higher incubation temperatures such as 37°C can be used to mimic physiological conditions.
- ▶ Data demonstrates that the system used for this study may be a useful methodology to investigate constituents of natural products for future studies.

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