Pre-Clinical Pharmacokinetic and Pharmacodynamic Characterization of Selected Chiral Flavonoids: Pinocembrin and Pinostrobin

Casey L. Sayre[1], Samaa Alrushaid[1], Stephanie E. Martinez[1], Hope D. Anderson[1,2] and Neal M. Davies[1]

[1]College of Pharmacy, Faculty of Health Sciences, University of Manitoba, Winnipeg, MB, Canada.[2] Canadian Centre for Agri-Food Research in Health and Medicine, St. Boniface Hospital Research Centre[3].

Received, June 23, 2015; Revised, July 29, 2015; Accepted, August 10, 2015; Published, September 3, 2015.

ABSTRACT - Purpose: Delineate the stereospecific pharmacokinetics and pharmacodynamics of the chiral flavonoids pinocembrin and pinostrobin. **Objective:** Characterize for the first time the stereoselective pharmacokinetics of two flavonoids, pinocembrin and pinostrobin and their bioactivity in several *in vitro* assays with relevant roles in heart disease, colon cancer, and diabetes etiology and pathophysiology. **Methods:** Chiral flavonoids were intravenously and orally administered to male Sprague-Dawley rats. Concentrations in serum and urine were characterized via stereospecific HPLC or LC/MS. Pure enantiomeric forms of each flavonoid were tested, where possible, to identify the stereospecific contribution to bioactivity in comparision to their racemates. **Results:** Short half-lives (0.2-6 h) in serum were observed, while a better estimation of half-life (3-26 h) and other pharmacokinetic parameters were observed using urinary data. The flavonoids are predominantly excreted via non-renal routes (f_e values of 0.3-4.6 %), and undergo rapid and extensive phase II metabolism. Chiral differences in the chemical structure of these compounds result in significant pharmacodynamic differences. **Conclusion:** The importance of understanding the stereospecific pharmacokinetics and pharmacodynamics of two chiral flavonoids were delineated.

INTRODUCTION

Flavonoids are a group of polyphenolic compounds of low molecular weight (200-600 g/mol) that are present in food and exhibit a common benzo-γ-pyrone structure. They are further sub-categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, anthocyanidins, and catechins. Pinocembrin, pinostrobin, and their corresponding glycosides belong to the flavanone family (1).

Evidence of potential activity against various chronic diseases with flavonoid usage continues to accumulate (2,3). However, the available studies tend to overlook a complete understanding of absorption, distribution, metabolism, and excretion – contributing to the confusion and limitations in the attempts to establish the exact biological activity of flavonoids and translate their transition to safe and effective therapeutic agents (4).

Pre-clinical pharmacokinetic and pharmacodynamic characterization is needed to begin to answer the primary and fundamental concerns of flavonoid efficacy and safety, especially that potentially harmful doses may be present in commercially available nutraceuticals (5) as well possible drug-flavonoid interactions may occur. Our laboratory has published a comprehensive review of the available clinical pharmacokinetic studies in the literature (6). Data from these studies can facilitate the use of relevant concentrations in pre-clinical mechanism of action studies and in-vitro bioactivity studies as well as provide assistance in the choosing of a rational starting point for dosing in larger clinical trials.

Additionally, recent reviews have established the assertion that *in vitro* bioactivity and human intervention studies of flavonoids must be coupled with *in vivo* pharmacokinetic and metabolic data in order to discern their actual potential as preventive and therapeutic agents (4,7).

The importance of stereospecific disposition of racemic flavanones is slowly being recognized and

Corresponding Author: Dr. Neal M. Davies, Faculty of Pharmacy, University of Manitoba, Winnipeg, Manitoba, Canada, Email: neal_davies@umanitoba.ca

reported in the biomedical literature. Most of these preliminary investigations report the quantification of a variety of flavanones in citrus fruit juices and herbs, or report the separation of flavanones on different stationary phases (8). However, no stereospecific information on their pharmacokinetics, pharmacodynamics, in both serum and urine has been reported.

The achiral pharmacokinetics of pinocembrin and pinostrobin, and their corresponding glycosides have been reported (9–12). Differences in the disposition of an individual enantiomer can cause significant or toxic effects in humans.The chiral pharmacokinetics of these compounds have only been preliminarily evaluated by our laboratory (13,14). To our knowledge there are no studies that have comprehensively assessed the serum and urinary pharmacokinetics and/or disposition of these two chiral flavonoids and their glucuronidated metabolites in humans or rodents after intravenous and oral administration.

Previous studies have suggested some pharmacological activity for pinocembrin and pinostrobin including anti-cancer, anti-inflammatory, anti-diabetic, and anti-oxidant activities (15–23). Understanding the role of each flavonoid enantiomer in the pharmacological activity described for the racemic mixtures is important as the combined effect of some enantiomers can be exaggerated or attenuated. Furthermore, assessing the individual activity of enantiomers can be difficult due to the lack of their commercial availability and the degree of purity of the available enantiomer. The methods of stereoseparation usually require high resolution of the assay to obtain stereochemically pure enantiomers.

To more thoroughly understand how these xenobiotics are absorbed, metabolized, distributed, and excreted and to be able to better understand or predict their disposition, pharmacological activity, as well as therapeutic and toxic effects, we have examined for the first time the stereoselective pharmacokinetics of these two racemic flavanones in rat serum and urine after intravenous and oral administration. This study further describes and compares the anti-diabetic, anti-oxidant, cardioprotective COX inhibitory and anti-cancer activities of pinocembrin and pinostrobin, and their enantiomers where possible.

MATERIALS AND METHODS

Chemicals and Reagents

Trans-stilbene, 7-ethoxycoumarin, β-glucuronidase from *Escherichia coli* type IX-A, β -glucuronidase from *Helix pomatia* type HP-2, and halothane were purchased from Sigma (St. Louis, MO, USA). Racemic pinocembrin (50:50, S:R) was purchased from Extrasynthese (France). Racemic pinostrobin (50:50, S:R) was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). HPLC grade acetonitrile and water were purchased from J. T. Baker (Phillipsburg, NJ, USA). Phosphoric acid was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Silastic® laboratory tubing was purchased from Dow Corning Corporation, (Midland, MI, USA). Intramedic® polyethylene tubing was purchased from Becton Dickinson Primary Care Diagnostics, Becton Dickinson and Company (Sparks, MD, USA). Monoject® 23 gauge (0.6 mm × 25 mm) polypropylene hub hypodermic needles were purchased from Sherwood Medical (St. Louis, MO, USA). Synthetic absorbable surgical sutures were purchased from Wilburn Medical US (Kernesville, NC, USA). Rats were obtained from Simonsen Labs (Gilroy, CA, USA). Ethics approval for animal experiments was obtained from University of Manitoba.

Animals and Surgical Procedures

Male Sprague-Dawley pre-jugular vein cannulated rats (200 - 240 g) were obtained from Charles River Labs (Montreal, Canada) and given food (Purina Rat Chow 5001) and water *ad libitum* in the animal facility for at least 5 days before use. Rats were housed in temperature-controlled rooms with a 12 h light/dark cycle. The cannula was flushed with 0.9% saline. The animals were transferred to metabolic cages and were fasted overnight. Animal ethics approval was obtained from University of Manitoba Office of Research Ethics and Compliance.

Pharmacokinetic Studies

Sixteen (16) male Sprague Dawley rats (average weight: 250 g) were purchased pre-cannulated. Each of the animals were placed in separate metabolic cages overnight, and fasted for 12 h before dosing. On the day of experiment, the animals were dosed either intravenously or orally with racemic pinocembrin (10 mg/kg IV, 100

mg/kg PO) or racemic pinostrobin (20 mg/kg IV, 100 mg/kg PO) dissolved in 2% DMSO and 98% PEG-600 (n=4 for each treatment group). Animals received water *ad libitum* pre- and post-dosing, and food (Purina Rat Chow 5001) was provided 2 hour post-dosing. Doses were selected based on previous use in similar pharmacokinetic studies and, in the case of pinostrobin, sensitivity of analytical instrumentation. Serial blood samples (0.30 mL) were collected at 0, 1 min, 10 min, and 30 min, then 1, 2, 4, 6, 12, 24, 48, and 72 h after administration. At 72 h after administration, the animals were euthanized and exsanguinated. Immediately after all the blood collection time points (except the terminal point); the cannula was flushed with the same volume of 0.9% saline to replenish the collected blood volume. The samples were collected into regular polypropylene microcentrifuge tubes, centrifuged at 5,000 RPM for 5 min (Beckman Microfuge centrifuge, Beckman Coulter Inc., Fullerton, CA, USA), and the serum was collected. The serum was divided into two equal fractions into separate regular polypropylene microcentrifuge tubes labeled as free and total serum samples and stored at –80°C until further sample preparation for HPLC analysis and LC/MS validation. Urine samples were also collected at 0, 2, 6, 12, 24, 48, and 72 h following flavonoid administration, the exact volumes were recorded and two equal aliquots were collected into separate regular polypropylene microcentrifuge tubes labeled as free and total urine samples and stored at –80°C until further sample preparation for HPLC analysis and LC/MS validation.

Serum and Urine Sample Preparation for Analysis

Serum and urine samples were run in duplicate with or without the addition of 40 µL of 500 U/mL β-glucuronidase from *Escherichia coli* type IX-A and incubated in a shaking water bath at 37°C for 2 h to liberate any glucuronide conjugates without decomposition of the parent compound.(24) The proteins present in the serum samples (as well as the enzyme in the total samples) were precipitated using 1 mL of cold HPLC-grade acetonitrile, vortexed for 1 min (Vortex Genie-2, VWR Scientific, West Chester, PA, USA), and centrifuged at 15000 rpm for 5 min, the supernatant was transferred to new labeled 2 mL centrifuge tubes. The samples were evaporated to dryness under a constant flow of compressed nitrogen gas. The residue was reconstituted with 200 µL of mobile phase, vortexed for 1 min and centrifuged at 5000 rpm for 5 min, the supernatant was transferred to HPLC vials and 20 µL was injected into the HPLC system, the remaining volume was utilized for LC/MS validation. β-glucuronidase from *Escherichia coli* type IX-A cleaves specifically any glucuronidated metabolites back to the corresponding aglycones (pinocembrin and pinostrobin). Therefore, the samples without enzymatic hydrolysis (free samples) were utilized to determine the concentration of the aglycones, whereas the samples with enzymatic hydrolysis (total samples) were utilized to determine the concentration of the aglycones originally present plus the concentration of the major glucuronidated metabolites converted to their respective aglycones by the cleavage action of the enzyme. Finally, by subtracting the free sample concentration from the total sample, the stereospecific concentration of the glucuronidated metabolites can be calculated.

Pharmacokinetic Analysis

Pharmacokinetic analysis was performed using data from individual rats which the mean and standard error of the mean (SEM) were calculated for each group. The elimination rate constant (k_{el}) was estimated by linear regression of the serum concentrations in the log-linear terminal phase. A non-compartmental model was fitted to the serum concentration versus time data using Phoenix® WinNonlin® software (Ver. 6.3) (Pharsight Corporation, Mountain View, CA) to calculate the pharmacokinetic parameters in the terminal phase, namely mean residence time (MRT by dividing $AUMC_{0-\infty}$ by the $AUC_{0-\infty}$), total clearance (CL_{tot} by dividing dose by $AUC_{0-\infty}$) and volume of distribution (V_{ss} by multiplying dose by the $AUMC_{0-\infty}$- and dividing it by the square of $AUC_{0-\infty}$). Based on the cumulative urinary excretion data, the fraction excreted in urine (f_e by dividing the total cumulative amount of flavonoid excreted in urine (ΣX_u) by the dose), renal clearance (CL_{renal} by multiplying f_e by CL_{tot}), and hepatic clearance ($CL_{hepatic}$ by subtracting CL_{renal} from CL_{tot}, assuming that hepatic clearance is equivalent to non-renal clearance) were calculated.

In order to assess the pharmacokinetic parameters from urinary data, the urinary elimination rate constant (k_e) and half-life were first characterized employing non-compartmental pharmacokinetic methods using Phoenix® WinNonlin® software (Ver. 6.3) (Pharsight

Corporation, Mountain View, CA). The area under the curve from the time of dosing until the last sampling time (AUC_{0-t}) was calculated by dividing the initial concentration (C_0) by the urine elimination rate constant (k_e). The other pharmacokinetic parameters were calculated as described above but instead of employing serum elimination rate constant (k_{el}), urine elimination rate constant (k_e) was utilized.

Pharmacodynamic Studies

Separation and Collection of Pure Enantiomers

Pure S-pinostrobin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pure R-pinocembrin was procured via a custom synthesis by Toronto Research Chemicals (Toronto, ON, Canada). The lack of commercial availability of pure R and S pinocembrin enantiomers made necessary the manual separation and collection of pure enantiomers using the analytical methods described (17,18). The collection of the pure enantiomers occurred following chiral chromatographic separation of multiple injections of racemic pinostrobin and pinocembrin. Racemic mixtures of these flavonoids are readily available from commercial sources. Multiple injections of the racemic mixture of each compound were performed via HPLC. Enantiomeric peaks were collected in sequence using separate 50 mL tubes and were subsequently dried to completion under nitrogen. This labour intensive process combined with the relatively high quantities required for use in pharmacodynamic assays and the need to perform CD spectroscopy on some of the enantiomers also limited the number of stereospecific pharmacological studies completed. CD spectra obtained for pinostrobin and pinocembrin enantiomers was consistent with previously published data (25).

Anti-diabetic Activity

α – Amylase Inhibition Assay

Methanol was used to dissolve racemic pinocembrin and pinostrobin, as well as S-pinocembrin and R-pinocembrin at concentrations between 0 and 200 µg/mL. Subsequent experiments with pinostrobin used methanolic dilutions of the racemates and enantiomers at concentrations from 0 to 1 µg/mL. A colorimetric assay was used to assess inhibition of α-amylase. The assay was adapted and modified from work previously developed by Tadera et al (26,27). A synthetic substrate, non-reducing-end blocked p-nitrophenyl maltoheptaoside (BPNPG7) commercially prepared as Amylase HR Reagent, which is hydrolyzed specifically by α-amylase into p-nitrophenyl maltosaccharide is utilized. The α-glucosidase present in the assay then converts the new substrate into p-nitrophenol which has a yellow colour and absorbance at 410 nm was immediately read at room temperature (23 ± 1ºC) using the Synergy HT Multi-well plate reader and Gen5TM data analysis software (Biotek® Instruments Inc., Winnoski, VT, USA). Inhibition (%) was calculated as $\frac{A-B}{A} \times 100$, where A was the average absorbance of the control wells and B was the absorbance of the wells containing flavonoids.

α– Glucosidase Inhibition Assay

Pinocembrin and pinostrobin were dissolved in DMSO and serially diluted to concentrations between 0 and 200 µg/mL. 160 µL of 100 mM phosphate buffer (pH 6.8), 25 µL of 20 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) in phosphate buffer, and 10 µL of the flavonoids in DMSO were added to a 96-well plate (10 µL DMSO was added to the control wells). The plate was incubated at 30ºC for 5 min and then 10µL of the buffer containing 0.02 mg/mL of enzyme was added to each well. Additional incubation for 5 min followed. 20 µL of 3.25 M sodium hydroxide was added to each well to stop the reaction. α-Glucosidase inhibition was determined through the colorimetric assay mentioned above 26, 27). The assay uses p-nitrophenyl-α-D-glucopyranoside (PNPG), which is hydrolyzed specifically by α-glucosidase into a yellow colored product (p-nitrophenol). The absorbance at 410 nm of liberated p-nitrophenol was measured.

In Vitro Cyclooxygenase-1 and -2 (COX) Inhibition Assay

The COX Inhibitor Screening Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA, catalog No. 560131). Racemic pinocembrin, pinostrobin, and S-pinocembrin were dissolved in DMSO to yield concentrations of 1.0, 10.0, 50.0, and 100.0 µg/ml. Standards and samples used in the assay were prepared two days in advance. On day 1, the reaction buffer, COX-1 (bovine), COX-2 (human recombinant), heme, arachidonic acid, hydrocholic acid, and stannous chloride ($SnCl_2$) were prepared according to the

manufacturer's instructions. Inactivated enzymes (COX-1 and COX-2) were used to generate background values; active enzymes were used to asses 100% initial activity for COX-1 and COX-2. All samples prepared this day were stored at 4ºC. On day 2, the reagents for the assay were prepared and COX inhibitory activity was assessed. The inhibitory activity of the compounds was tested individually for COX-1 and COX-2. The wash buffer, prostaglandin (PG) standard, PG screening AChE tracer, and PG screening antiserum were prepared following the manufacturer's instructions. COX reactions were performed in serial dilutions for the background samples, the 100% initial activity samples, and the COX inhibitor samples. Controls, standards, and samples were placed in 96-well plates and incubated overnight at room temperature. On day 3, the plate was developed using Ellman's reagent for 60 – 90 minutes. The COX inhibitor activity of the compounds was measured using a Synergynt multi-well plate reader (Biotek® Instruments Inc., Winnoski, VT, USA) using Gen 5 software from Biotek®. Absorbance was read at 405 – 420 nm. The COX inhibitor activity of the samples was compared to the percentage of standard bound/ maximum bound ($\%B/B_0$).

The COX Inhibitor Screening Assay Kit directly measures the level of $PGF_{2\alpha}$ produced from PGH_2 after $SnCl_2$ reduction. $PGF_{2\alpha}$ is quantified via enzyme immunoassay (EIA) using a broadly specific antibody that binds PG compounds. This assay includes both ovine COX-1 and human recombinant COX-2 enzymes in order to screen isozyme-specific inhibitors. For more details see the manufacturer's instruction manual. The inhibition of COX – expressed as percentage of COX activity.

In Vitro Anti-oxidant Activity Assay

The Anti-oxidant Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA, catalog No. 709001). Racemic pinocembrin, pinostrobin, and S-pinocembrin were dissolved in DMSO to yield concentrations of 1.0, 10.0, 50.0, and 100.0 µg/ml. The assay buffer, chromogen, Trolox and hydrogen peroxide were prepared on the day of the experiment following the manufacturer's instructions. A Trolox standard curve was constructed using a serial of dilutions. Controls, standards, and treatments at different concentrations (1.0 – 100.0 µg/ml) were placed in 96-well plates and hydrogen peroxide was used to start the oxidative reaction. The anti-oxidant activity of the compounds was measured using a Synergynt multi-well plate reader (Biotek® Instruments Inc., Winnoski, VT, USA) using Gen 5 software from Biotek® after five minutes of exposure to hydrogen peroxide. Absorbance was read at 750 nm to decrease interference. The anti-oxidant capacity of the samples was compared to that of Trolox®.

The Cayman Anti-oxidant Assay Kit measures the total anti-oxidant capacity based on the ability of the anti-oxidants in the sample to inhibit the oxidation of $ABTS^{+}$ to $ABTS^{\bullet+}$. For more details see the manufacturer's instruction manual. The anti-oxidant capacity expressed as Trolox equivalent anti-oxidant capacity (TEAC) of racemic pinocembrin and pinostrobin as well as S-pinocembrin was measured.

Cardiomyocyte Size Assay

Endothelin-1 (ET-1), phosphate buffered saline (PBS), paraformaldehyde (PFA), and alpha-actinin antibody were from Sigma-Aldrich. Alexa 488-conjugated goat anti-mouse antibody was purchased from Life Technologies (Carlsbad, CA, USA). Triton – X was purchased from Omnipur Emscience. Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Thermo Scientific. Cell culture methodology for ventricular cardiomyocyte plating was derived from previous work described by Wu *et al* (28). Isolation of cardiomyocytes from ventricles of 1-day-old neonatal Sprague-Dawley rats was accomplished mechanically via slow pipetting in the presence of 0.05% trypsin. Cell culture conditions included gelatin-coated plates, with DMEM media containing 10% fetal bovine serum as previously published (29). Cells were cultured for 24 hours before treatment. Following the 24-hour culture period, myocytes were serum-deprived for 24 h followed by treatment with racemic pinocembrin, S-pinocembrin, R-pinocembrin, at concentrations between 0 and 100 µg/mL or vehicle for 1 hour. Treatment groups were run in duplicate. To stimulate hypertrophy in the ventricular myocytes; ET-1 (0.1 µM) was then added following incubation for 24 hours. Cells were fixed with 4% paraformaldehyde for 1 hour then permeabilized with 0.1% Triton – X 100 for 5 minutes. A blocking solution consisting of 2% milk in 0.1% Triton X – 100/PBS over 5 minutes was then utilized. Immunostaining was accomplished by incubating the myocytes overnight at 4^0C with

monoclonal anti- α actinin (sarcomeric) clone EA-53 as the primary antibody. Incubation with Alexa 488-conjugated goat anti-mouse as the secondary antibody followed. Using fluorescence microscopy, ventricular myocyte size was measured by computer-assisted planimetry with ImageJ software from images taken randomly from four areas of the culture plate wells as previously described (29,30).

In Vitro Anti-cancer Activity Assay

Trypsin-Ethylenediamenetetraacetic acid (EDTA), trypan blue, phosphate-buffered saline (PBS), 4-methylumbelliferone, resazurin, cell culture tested sodium carbonate, HEPES, β-glucosidase, sodium pyruvate, McCoy's 5A medium, penicillin-streptomycin, and insulin were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 Ham (DMEM/F-12) without phenol red and RMPI 1640 medium were purchased from Gibco Industries Inc. (Langley, OK, USA). Fetal bovine serum (FBS) was purchased from Equitech-Bio Inc. (Kerrville, TX, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemicals (St Louis, MO, USA).

Counted and seeded HT-29 cells were placed on 96-well plates, then incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. On the day of the experiment racemic pinocembrin and pinostrobin were dissolved in DMSO and diluted with the corresponding media to yield concentrations of 250.0, 100.0, 50.0, 10.0, 5.0, and 1.0 µg/ml per enantiomer. Media were aspirated from the wells, and cells were treated with media containing pinocembrin and pinostrobin racemates at different concentrations (1.0 – 250.0 µg/ml); DMSO in media and media alone were used as controls. Treated and control cells were incubated at 37°C in a 5% CO_2 atmosphere for 72 hours. After 72 hours incubation, the 96-well plates were removed from the incubator; 20.0 µl of 10% Alamar blue (resazurin) fluorescent dye was added to the control and treatment groups in the 96-well plates; they were incubated at 37°C in a 5% CO_2 atmosphere for an additional 3 hours. Following 3 hours incubation, the 96-well plates were placed in a darkened environment for 30 minutes at room temperature; then placed into a Synergynt multi-well plate reader using Gen 5 software from Biotek®. Fluorescence was read at an excitation of 530 nm and an emission of 590 nm. The viable cell number (as a percent of control) in each cell line was measured and for each cell line exposed to varying concentrations of racemic pinocembrin and pinostrobin.

CYP 2D6 Inhibition Assay

CYP 2D6 inhibitory activity of racemic pinocembrin and pinostrobin–was assessed using a Vivid P450 CYP2D6 blue screening kit (Life Technologies; Burlington, ON, Canada). Chemicals included P450 reaction buffer, P450 BACULOSOMES reagent, a fluorescent substrate, a fluorescent standard, the regeneration system (333 mmol/L glucose- 6-phosphate and 30 000 U/L glucose-6-phosphate dehydro- genase in 100 mmol/L potassium phosphate, pH 8.0), and 10 mmol/L NADP+ in 100 mmol/L potassium phosphate, pH 8.0. Concentrations of 0.01, 0.1, 1, 10, 50, and 100 µM of racemic pinocembrin and pinostrobin were prepared in methanol. Utilizing a 96-well plate, 40 µL of flavonoid, quinidine (positive CYP 2D6 inhibitor control), or blank methanol (solvent control) was added to each well. 20 minutes of incubation followed with 50 µL of pre-mixture (containing BACULOSOMES® reagent, the regeneration system and reaction buffer) or 50 µL of reaction buffer only as a background control at room temperature (25 °C). To start the reaction 10 µL of a mixture of vivid substrate and NADP+ was added to each well. Fluorescence changes were monitored by immediate reading of the plate on a Synergy HT multiwell plate reader with Gen5 data analysis software (Biotek Instruments Inc., Winooski, VT, USA). Excitation and emission wavelengths were 415 and 460 respectively. Monitoring occurred immediately after the start of the reaction and continued every minute for 60 minutes at room temperature (23 ± 1°C) as stipulated by the Vivid P450 kit protocol. The final methanol volume in the reactions was ≤1%.

STATISTICAL ANALYSIS

Compiled data were presented as mean and standard error of the mean (mean ± SEM). Where possible, the data were analyzed for statistical significance using Excel software. Student's t-test was employed for unpaired samples with a value of $p < 0.05$ being considered statistically significant.

RESULTS

Pharmacokinetics of Pinocembrin and Pinostrobin

Stereospecific Pharmacokinetics of Intravenous Pinocembrin and Pinostrobin

The analytical methods described (17,18) were applied to the stereospecific determination of pinocembrin (LC/MS), and pinostrobin (HPLC). Linearity in the standard curves was demonstrated in the serum samples for the two chiral flavonoids over the concentration range studied, and chromatograms were free of interference from endogenous components. Total samples (incubated with β-glucuronidase from *Escherichia coli* Type IX-A) demonstrated the presence of at least one glucuronidated metabolite based on the increase in the aglycone parent compounds (pinocembrin and pinostrobin) concentrations after the enzymatic hydrolysis, which was assessed as described previously.

The serum disposition profiles observed for pinocembrin and pinostrobin demonstrated some difference in stereoselective disposition. For instance, S-pinocembrin (Figure 1) achieved higher concentrations in serum compared to its enantiomeric counterpart. Independent of the enantiomeric form, the two flavonoids were characterized by a rapid decline in concentrations, representing a distribution phase followed by a rapid elimination phase between 1 and 6 hours (last time point at which flavonoids were detected). The concentration-time profiles of the two flavonoids follow a biexponential pattern clearly indicating that the compounds do not reside within one central compartment. The elimination phase for the parent compounds was characterized with half-lives between 15 minutes and 6 hours. The enantiomers of both pinocembrin and pinostrobin reported similar half-lives in serum, namely 0.262 ± 0.071 h for S-pinocembrin and 0.263 ± 0.027 h for R-pinocembrin; and 6.72 ± 2.19 h for S-pinostrobin and 6.77 ± 2.03 h for R-pinostrobin (Table 1).

a.

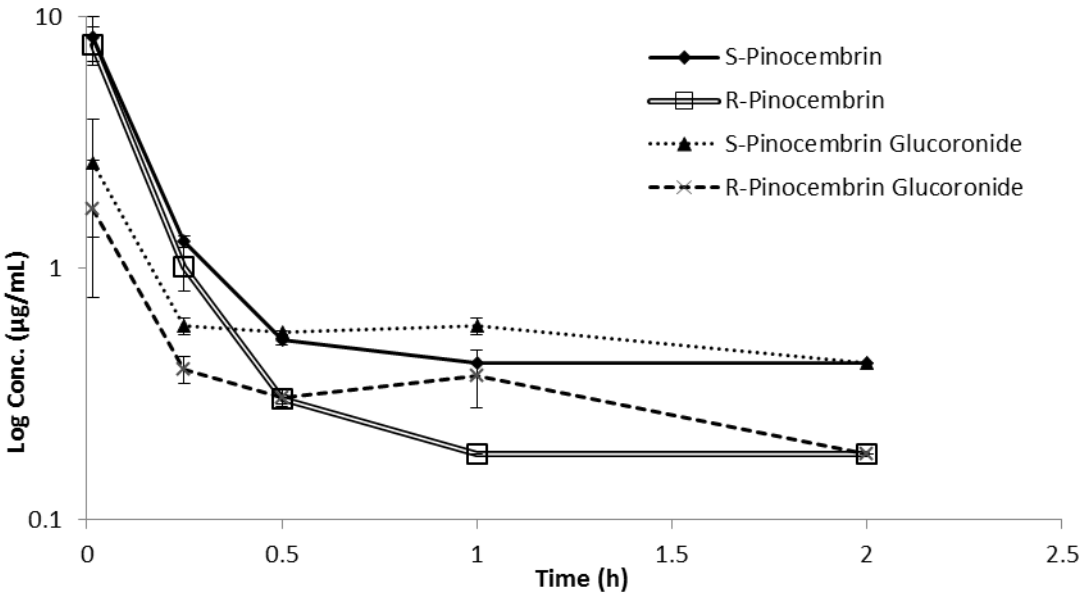
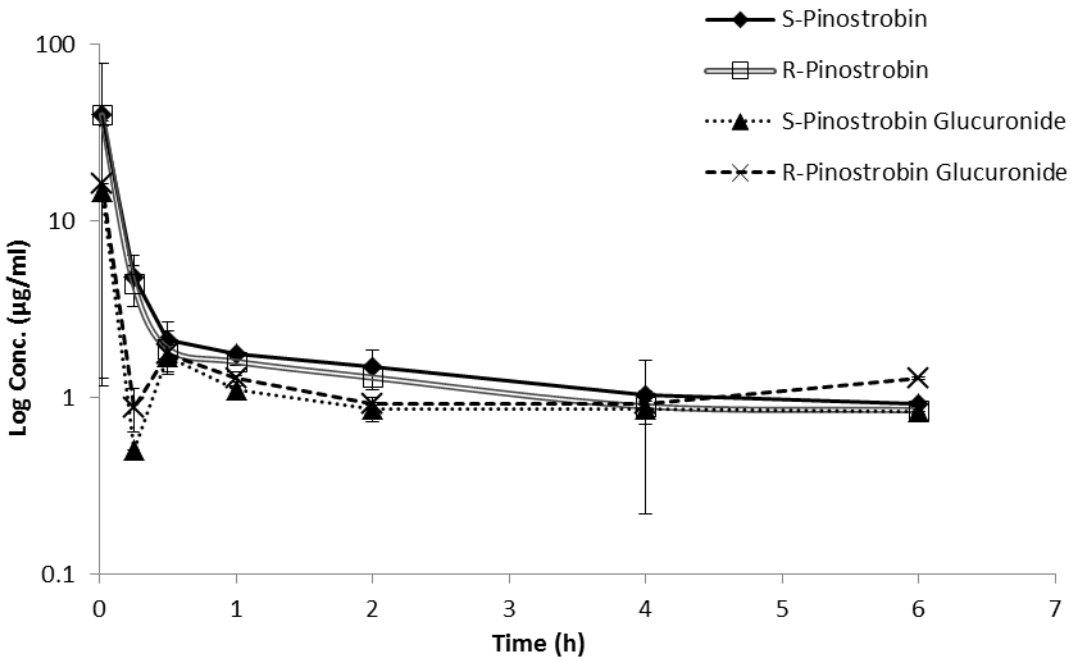


Figure 1. Continued

b.



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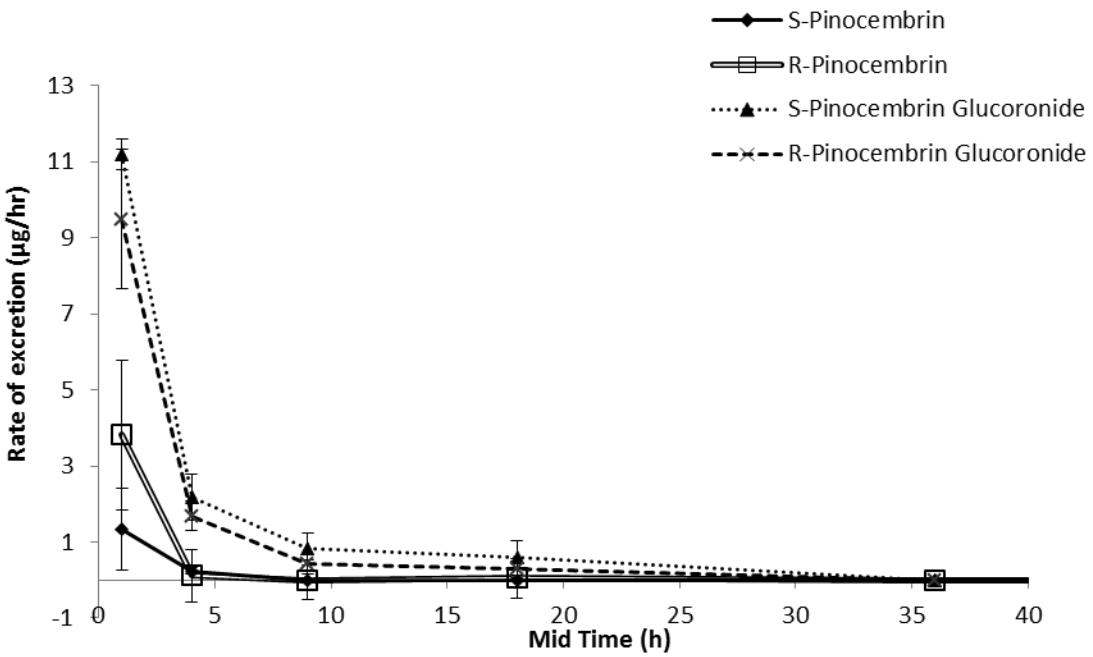


Figure 1. Continued

d.

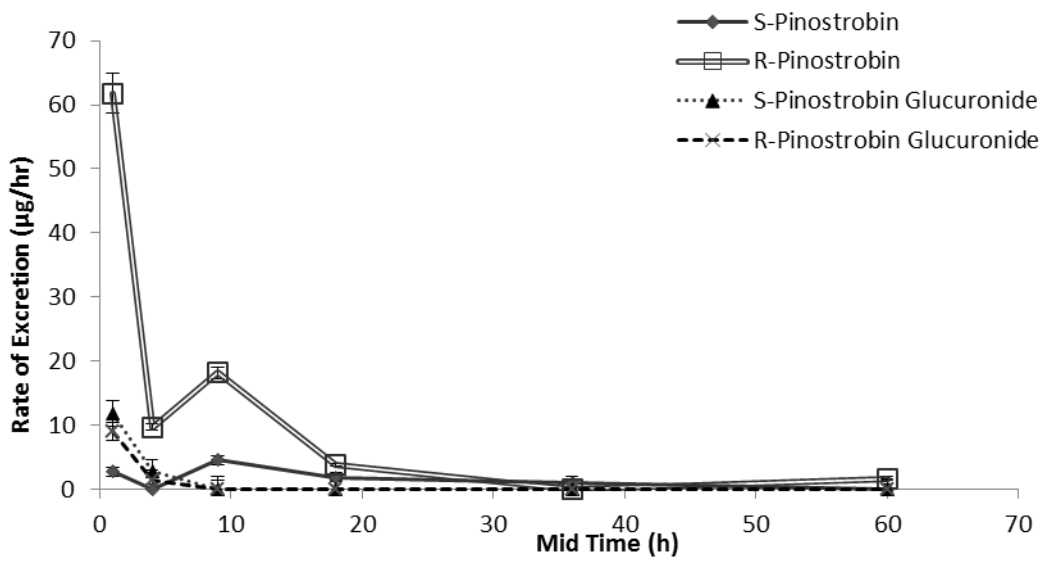


Figure 1. (a) Concentration-time profile in serum of pinocembrin enantiomers following intravenous administration of racemic pinocembrin (10 mg/kg) in rats (n=4, mean ± SEM). (b) Concentration-time profile in serum of pinostrobin enantiomers following intravenous administration of racemic pinostrobin (20 mg/kg) in rats (n=4, mean ± SEM). (c) Rate of urinary excretion of free and glucuronidated pinocembrin enantiomers (µg) excreted in urine following intravenous administration of racemic pinocembrin (20 mg/kg) in rats (n=4, mean ± SEM). (d) Rate of urinary excretion of free and glucuronidated pinostrobin enantiomers (µg) excreted in urine following intravenous administration of racemic pinostrobin (20 mg/kg) in rats (n=4, mean ± SEM).

The glucuronidated metabolites of pinocembrin and pinostrobin exhibited similar concentration-profiles (Figures 1a-b). In the case of pinostrobin glucuronidated metabolites, slight increases in concentration at around 30 minutes were observed, indicating the possibility of secondary peaking enterohepatic recycling likely (Figure 1b). However, with respect to pinocembrin glucuronidated metabolites no indication of enterohepatic recycling was observed.

Non-compartmental analysis of the serum concentrations showed several differential pharmacokinetic parameters between pinocembrin and pinostrobin enantiomers (Table 1). In the case of pinostrobin, renal and total clearance, and f_e were stereoselective showing higher values for the R-pinostrobin enantiomer. R-pinocembrin showed greater values for fraction excreted (f_e), and urine $t_{1/2}$ whereas S-pinocembrin had slightly greater values for the urine k_{el} as well as MRT (Table 1).

The differences in certain pharmacokinetic parameters between pinocembrin and pinostrobin enantiomers demonstrate that these flavonoids may be stereospecifically metabolized. For pinocembrin and pinostrobin, the R-enantiomers showed significantly higher f_e values, slightly higher clearances, and volumes of distribution (V_{ss}). Further, S-pinocembrin showed significantly greater urine k_{el} while R-pinocembrin showed a correspondingly greater urine $t_{1/2}$. These results indicated no significant difference between enantiomers with respect to AUC, serum $t_{1/2}$, and hepatic clearance for the two compounds.

The physiological parameters of a rat of 0.25 kg body weight indicate that it has an average total blood volume of 13.5 mL and a total body water volume of 167 mL (31). This translates in an average total blood volume of 54 mL/kg (0.054 L/kg) and a total water volume of 668 mL/kg (0.668 L/kg).

Table 1. Stereospecific pharmacokinetics of pinocembrin and pinostrobin in serum after IV administration in rats (20 mg/kg) (mean ± SEM, n=4). [a]Denotes statistical significant difference (P<0.05) between enantiomers.

Pharmacokinetic Parameter	S-pinocembrin	R-pinocembrin	S-pinostrobin	R-pinostrobin
AUC_{inf} (μg*h/mL)	1.83 ± 0.092	1.87 ± 0.312	17.8 ± 7.32	16.6 ± 7.07
V_{ss} (L/kg)	1.46 ± 0.591	1.80 ± 0.271	10.02 ± 1.22	10.7 ± 0.904
CL_{renal} (L/h/kg)	0.019 ± 0.004	0.035 ± 0.003	0.016 ± 0.008	0.037 ± 0.033[a]
$CL_{hepatic}$ (L/h/kg)	5.42 ± 0.346	5.79 ± 0.861	1.12 ± 0.311	1.15 ± 0.383
CL_{total} (L/h/kg)	5.44 ± 0.287	5.83 ± 0.865	1.14 ± 0.303	1.19 ± 0.353[a]
f_e (%)	0.346 ± 0.087	0.611 ± 0.046[a]	1.94 ± 1.60	4.55 ± 4.42[a]
k_{el} (h^{-1}) serum	4.58 ± 1.98	3.03 ± 1.15	0.125 ± 0.042	0.117 ± 0.041
k_{el} (h^{-1}) urine	0.255 ± 0.022	0.165 ± 0.027[a]	0.087 ± 0.045	0.073 ± 0.067
$t_{1/2}$ (h) serum	0.262 ± 0.071	0.263 ± 0.027	6.72 ± 2.19	6.77 ± 2.01
$t_{1/2}$ (h) urine	2.73 ± 0.171	4.50 ± 1.13[a]	10.7 ± 4.04	26.4 ± 16.0
MRT (h)	0.326 ± 0.106	0.115 ± 0.094[a]	8.86 ± 2.49	9.41 ± 3.16

For our compounds of interest, it was observed that the volume of distribution (V_{ss}) of R-pinostrobin and S-pinostrobin was 10.7 ± 0.904 L/kg and 10.02 ± 1.22 L/kg (Table 1). These V_{ss} values are significantly higher than the total blood volume (0.054 L/kg) and the total water volume (0.668 L/kg) indicating that both enantiomers are exiting the vasculature and are having a deep penetration into tissues. The same can be stated of pinocembrin enantiomers since they also have significantly large V_{ss} values. R-pinocembrin has an observed V_{ss} of 1.80 ± 0.271 L/kg while S-pinocembrin a V_{ss} of 1.46 ± 0.591 L/kg. These similarly large volume of distribution (V_{ss}) values correlate with the lipophilic nature of these compounds (XLogP values of 2.7 and 3.1 for pinocembrin, and pinostrobin, respectively), which might indicate their preferential binding to tissues and preference to reside in the body.

It was also observed that both flavonoids are predominately cleared via hepatic elimination (fraction excreted in urine, f_e of 0.3-0.6% and 2-4.5%,—for both pinocembrin and pinostrobin enantiomers, respectively) (assuming that hepatic clearance is equivalent to non-renal clearance). The glucuronidated metabolites of pinocembrin and pinostrobin previously identified in serum were also detected in the urine samples (Figure 1c-d).

In the case of these flavonoids it was observed that the terminal urine half-life was not significantly different between enantiomers. However, it was clearly observed that the urine half-life (3- 26 hours among the two flavonoids) was significantly higher than the plasma half-life (0.25-7 hours among the flavonoids). For instance, pinocembrin exhibited serum half-lives of 0.262 ± 0.071 h and 0.263 ± 0.027 h for S- and R-pinocembrin, respectively, while the urinary half-lives for S- and R-pinocembrin were 2.73 ± 0.171 h and 4.50 ± 1.13 h, respectively. In the case of pinostrobin, serum half-lives of 6.72 ± 2.19 h and 6.77 ± 2.01 h were observed for S- and R-pinostrobin, respectively, while the urinary half-lives for S- and R-pinostrobin were 10.7 ± 4.04 h and 26.4 ± 16.0 h, respectively (Table 1).

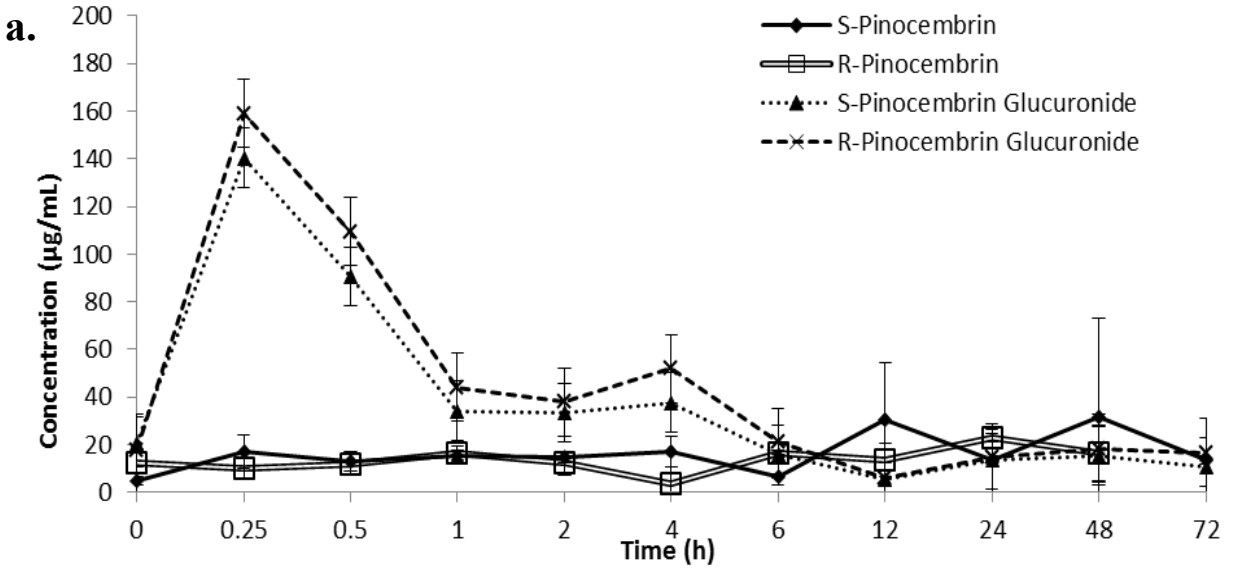
These discrepancies suggest that the serum half-life is likely significantly underestimating the overall half-life of pinocembrin and pinostrobin enantiomers due to assay sensitivity limits in serum. These observations correlate with previous studies that have reported that certain stilbenes also have longer urine half-lives (4.7-fold to 16-fold higher half-lives in urine compared to plasma half-lives) (32,33). Furthermore, it needs to be mentioned that the compounds of interest (pinocembrin and pinostrobin) could be measured only 2-6 hours in serum, while in urine they could be measured up to 60 hours. Urine may be a better biological fluid to utilize in the determination of pharmacokinetic parameters because as it can be observed serum concentrations are at the limit of sensitivity and considerably underestimates the half-life of these compounds.

The pharmacokinetic parameters derived from urine were calculated (Table 1). It can be observed that employing serum significantly underestimates AUC_{inf}, and half-life, while k_{el} was overestimated. Thus, urine may provide more significant pharmacokinetic parameters indicating that these chiral flavonoids have long half-lives up to 26 h) and high volumes of distribution suggesting they considerably distribute out of the vasculature.

In the rat pinocembrin and pinostrobin are rapidly being metabolized to different glucuronides. Despite the differences in solubility and lipophilicity nature of these compounds (parent and glucuronidated compounds) it can be observed that they have similar rates of excretion (Figures 1c-d). This indicates that both parent drug and metabolite undergo similar magnitude of apparent elimination (since their elimination phases are parallel) indicating that the glucuronide conjugates are formation-rate limited and that their half-lives would be a reflection of the elimination of the parent flavonoids.

Stereospecific Pharmacokinetics of Oral Pinocembrin and Pinostrobin

Following oral administration of pinocembrin (100 mg/kg) the serum concentration vs. time curves indicated low absorption of free flavonoid (Figure 2a). Oral administration of pinostrobin (100 mg/kg) showed higher absorption of free flavonoid that was somewhat delayed with a time to maximum concentration of around 2 h (Figure 2b) (Table 2).



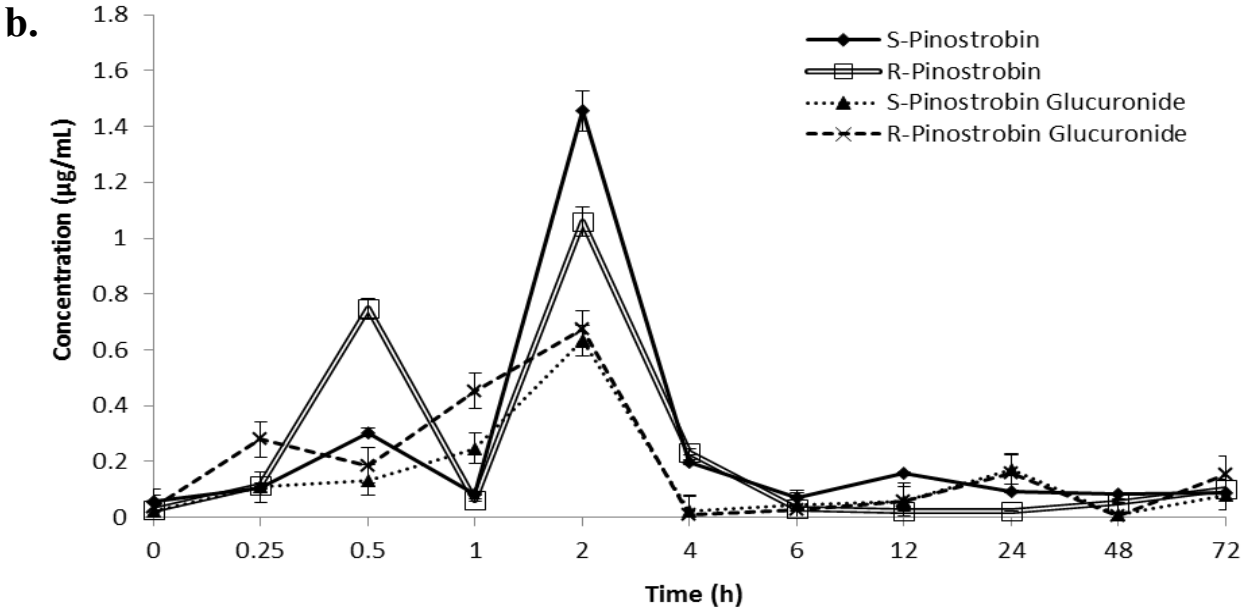


Figure 2. Continued

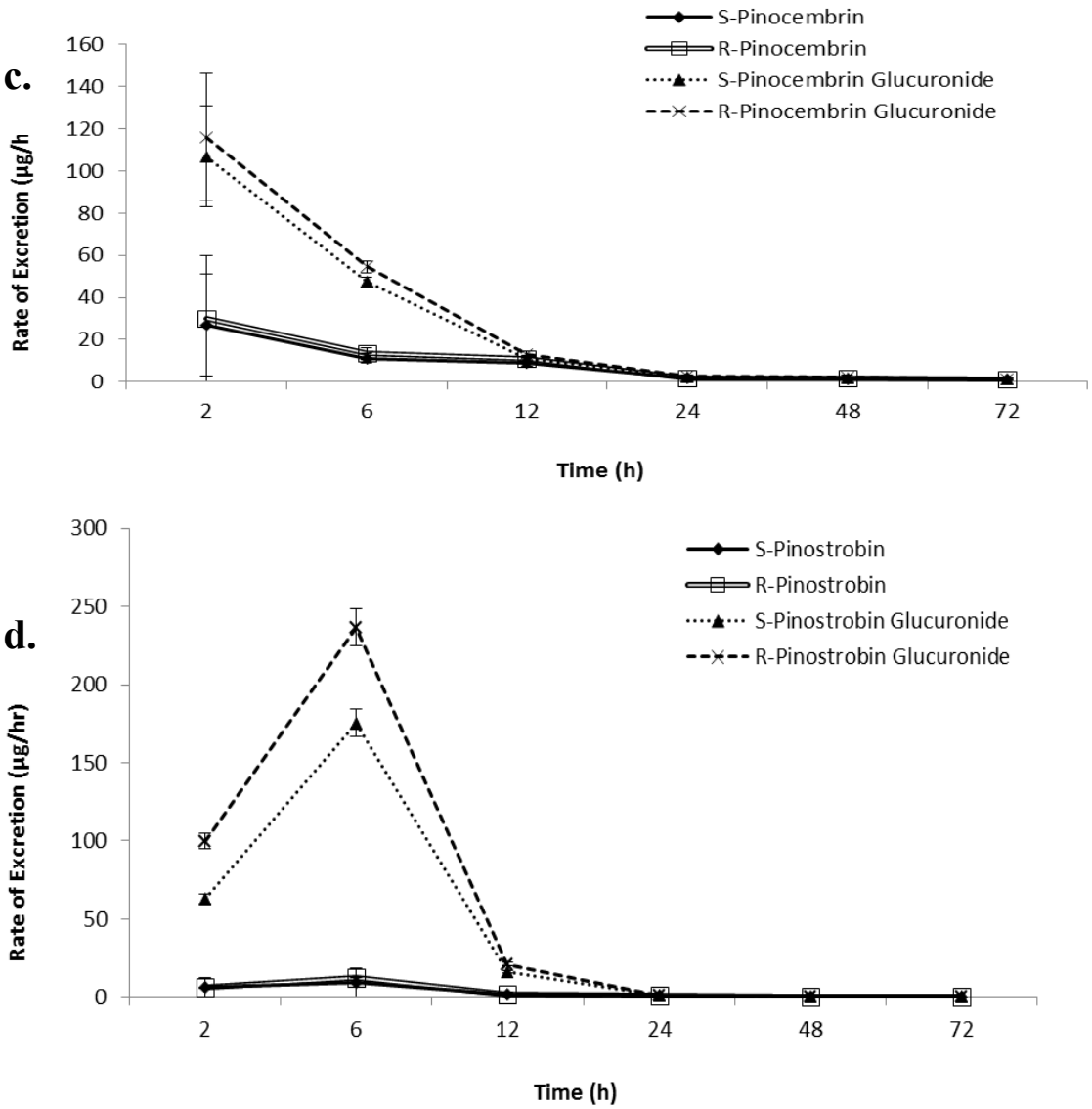


Figure 2. (a) Concentration-time profile in serum of pinocembrin enantiomers following oral administration of racemic pinocembrin (100 mg/kg) in rats (n=4, mean ± SEM). (b) Concentration-time profile in serum of pinostrobin enantiomers following oral administration of racemic pinostrobin (100 mg/kg) in rats (n=4, mean ± SEM). (c) Rate of urinary excretion of free and glucuronidated pinocembrin enantiomers (µg) excreted in urine following oral administration of racemic pinocembrin (100 mg/kg) in rats (n=4, mean ± SEM). (d) Rate of urinary excretion of free and glucuronidated pinostrobin enantiomers (µg) excreted in urine following oral administration of racemic pinostrobin (100 mg/kg) in rats (n=4, mean ± SEM).

Pinocembrin and pinostrobin enantiomers were both glucuronidated but to variable degrees. Pinocembrin was rapidly glucuronidated even at the earliest time points with the conjugated metabolite still detected out to 6 h. The large extent of pinostrobin glucuronidation after oral administration likely results from an extensive first-pass metabolism. The extent of pinostrobin glucuronidation is far less in comparison to pinocembrin enantiomers as indicated by the predominance of pinostrobin in the free form. This may indicate smaller degree of first-pass metabolism or possible poor aqueous gut solubility of pinostrobin enantiomers.

Non-compartmental modeling using WinNonlin® was employed to determine the pharmacokinetic parameters following oral dosing (Table 2). Significant differences between enantiomers following oral administration was apparent. The area under the curve indicating the extent of exposure of these flavonoids was several fold higher for R-pinostrobin (431 ± 21.4) in comparison to S-pinostrobin (33.8 ± 18.1). Bioavailability (F) was calculated by dividing the AUC of oral administration by the AUC of IV administration with dose adjustment. The calculated bioavailability of S-pinostrobin was approximately 2% whereas R-pinostrobin showed a calculated bioavailability of approximately 14% (Table 2). The lower bioavailability of one enatiomeric form over the other may suggest an extensive stereoselective first-pass metabolism. This is supported by the larger amount of R-pinostrobin glucuronides seen following enzyme incubation.

The values for V_d and CL were also calculated for each flavonoid following oral administration. These parameters vary from those calculated from IV studies. This is expected ass these parameters depend on bioavailability. The parameters from IV data will provide the purest pharmacokinetic estimates as they are pure parameters that are not contaminated by bioavailability. The calculated fraction excreted unchanged in the urine (F_e) for pinocembrin and pinostrobin remained low at < 2.5% indicating very little of the administered doses are excreted in the urine. The half-lives of pinocembrin and pinostrobin enantiomers did not differ significantly and ranged from 20 to 44 hours (Table 2). These half-lives following oral administration were longer than those seen after IV administration (0.25-7 h). This may in part be due to the higher dosages given which allowed for extended detection time in the serum. For example, pinocembrin was detected out to 6 h following oral administration in comparison to 2 h following IV administration. This extended detection may have allowed for better estimation of the terminal elimination half-lives of the flavonoids. Another possible explanation of the longer oral half-life may be the presence of flip-flop kinetics. Flip-flop occurs when the rate of absorption is slower than the rate of elimination ($k_a <<< k_{el}$). The physicochemical and physiological mechanisms underlying the occurrence of flip-flop phenomenon are multi-factorial and include but are not limited to solubility-limited absorption, modified release formulations, and alterations in permeability of membranes. It is possible that pinocembrin and pinostrobin are demonstrating solubility-limited absorption where the rate of absorption is slowed due to poor solubility in the gastrointestinal tract.

The rate of urinary excretion plots for pinocembrin (Figure 2c) and pinostrobin (Figure 2d) indicate different rates of urinary excretion for each flavonoid and their corresponding glucuronidated metabolites following oral administration. For pinocembrin, the parent compound and metabolite appear to have similar rates of excretion as indicated by the general parallel lines of elimination, no stereospecific difference is observed. For pinostrobin, the glucuronidated metabolites of the enantiomers differs slightly and appears to be excreted faster than the parent compound as indicated by the metabolite's steeper declining overall elimination rate.

Pharmacodynamics of Pinocembrin and Pinostrobin

CYP 2D6 Inhibition

Pharmacokinetic drug interactions include alterations in drug metabolism. A common culprit of this type of reaction can be a compound which inhibits or induces drug metabolizing enzymes in the CYP 450 family. CYP 2D6 is a CYP 450 enzyme responsible for the metabolism of many commonly used drugs (34). Flavonoids from several subclasses have been shown to inhibit various subtypes of CYP 450 drug metabolizing enzymes (35). Pinocembrin and pinostrobin racemates were assessed for their inhibitory activity of CYP 2D6. Evaluation of the CYP 2D6 inhibition of racemic pinocembrin revealed inhibitory activity at low concentrations. At 0.01 and 0.1 μM around 50% inhibition of CYP 2D6 compared to quinidine, the positive control. As concentrations increased, however, inhibition decreased into apparent CYP 2D6 induction in a dose dependent manner. These results are consistent with other flavonoids tested in our laboratory. Although no studies have looked at the CYP 2D6 inhibitory activity of pinocembrin, one study has looked at honey, a product known to contain pinocembrin (36). Honey was found to have no significant effect on CYP 2D6 activity in this study (36).

Table 2. Stereospecific pharmacokinetics of pinocembrin, and pinostrobin in serum after PO administration in rats (100 mg/kg,) (mean ± SEM, n=4). [a]Denotes statistical significant difference (P<0.05) between enantiomers.

Pharmacokinetic Parameter	S-pinocembrin	R-pinocembrin	S-pinostrobin	R-pinostrobin
AUC_{inf} (μg*h/mL)	570 ± 21.7	531 ± 82.1	33.8 ± 18.1	431 ± 21.4[a]
V_{ss}/F (L/kg)	3.80 ± 1.34	5.14 ± 1.81	67.1 ± 26.1	157 ± 30.4[a]
CL_{total}/F (L/h/kg)	2.82 ± 0.084	2.83 ± 0.844	0.627 ± 0.185	6.27 ± 4.26[a]
F_e (%)	2.20 ± 0.394	2.33 ± 0.565	0.754 ± 0.252	0.883 ± 0.377
F (%)	43.2 ± 1.22	57.8 ± 14.4	1.83 ± 1.43	13.8 ± 3.42[a]
$t_{1/2}$ (h) serum	20.3 ± 8.41	27.1 ± 18.8	31.9 ± 5.62	44.2 ± 8.24

Similar to pinocembrin, the CYP 2D6 inhibition of racemic pinostrobin was weak and only appeared at low concentrations. The greatest inhibition occurred at 0.1 μM showing around 40% relative inhibition compared to the positive control quinidine. At higher concentrations (10 – 100 μM), CYP 2D6 activity appears to be induced. Being structurally similar to pinocembrin, these results could be expected and are consistent with other flavonoids tested in our laboratory. Similarly to pinocembrin, pinostrobin is also largely present in honey. The study citing the CYP 2D6 inhibitory activity of honey may also inform and is consistent with these results, as no significant inhibition was found (36).

After screening racemic pinocembrin and pinostrobin for CYP 2D6 inhibitory activity, the results suggest that pinocembrin and pinostrobin may be weak inhibitors at low concentrations. Similar to other flavonoids tested with this model in our laboratory, apparent induction is seen at higher concentrations for both the pinocembrin and pinostrobin racemates. The results are interesting however, because CYP 2D6 is the only CYP 450 enzyme which is largely non-inducible (37). This lead us to believe that experimental artifacts are apparent at the higher concentrations utilized in this assay. The apparent induction of activity may be from some other cause, including poor solubility. Given these initial results it was decided that CYP 2D6 inhibition would not be a suitable screen to pursue for investigating individual flavonoid enantiomer activities.

In Vitro Anti-cancer Activity

The anti-proliferative activity of racemic pinocembrin and pinostrobin─on HT-29 (human colorectal adenocarcinoma) was assessed**.** This cell line was chosen specifically because pharmacokinetic studies characterization revealed elimination and excretion predominately by non-renal routes (liver and feces) which could present high concentrations in the colon. Additionally, other flavonoids studied in our laboratory have been observed to have particular anti-proliferative activity against liver and colon cancer cell lines. Racemic pinocembrin demonstrated poor anti-proliferative activity, showing only a 68% reduction in HT-29 cell viability at its lowest point (Appendix, figure 1a). Racemic pinostrobin showed a slight decrease in cell viability at the low concentrations, however, cell viability increased as concentrations increased (Appendix, figure 1b).

The screen for anti-cancer activity utilizing an Alamar Blue anti-proliferative assay of HT-29 cells showed moderate to poor decreases in cell viability for racemic pinocembrin and pinostrobin. At increasing concentrations, the pinostrobin racemate began to show increased cell viability. This particular assay is not suitable for pursuit in further investigating the enantiospecific pharmacologic activity of the two flavonoids. As observed in other pharmacologic assays using these compounds, unexpected results occurred at high concentrations with respect to pinostrobin. To explain this dose dependent increase in cell viability, the question of solubility and or dimerization must be addressed.

In Vitro Anti-oxidant Activity

Biomarkers of oxidative stress have been observed at high levels in populations with chronic diseases like diabetes, heart disease, and cancer (38,39). The actual role, if any, of oxidative stress in the pathophysiology of chronic disease remains under active study. However, oxidative stress does appear to play a role in heart failure (40). The assay was also chosen to explore the non-enzymatic pharmacologic activity differences between a

flavonoid racemate (pinocembrin) and the only readily available pure enantiomer (S-pinocembrin, Sigma-Aldrich, St. Louis, MO, USA) at the time this assay was performed.

Racemic pinocembrin showed anti-oxidant activity greater than baseline at 10, 50, and 100 µg/mL in a dose dependent manner (Appendix, figure 2a). S-Pinocembrin showed greater anti-oxidant activity than its racemate at concentrations of 5, 50, and 100 µg/mL. These results were unexpected, as anti-oxidant activity is measured directly by the ability of pinocembrin to prevent the formation of $ABTS^{\bullet+}$. This ability is related to the availability of free hydroxyl moieties, a feature of chemical structure which does not differ between enantiomers. Possible explanations for this observation include the potential presence of impurities, dimerization or differences in physical chemical properties such as solubility, which can differ between racemates and pure enantiomers (41,42). The anti-oxidant activity of the pinostrobin racemate was significantly greater than baseline at the three highest concentrations tested (Appendix, figure 2b). Due to the difficulty in procuring and collecting pure enantiomers as well as the non-enzymatic nature of the anti-oxidant assay further enantiospecific testing was not conducted. However, the disparity in the activity of S-pinocembrin compared to its racemic will merit further investigation into the cause of this observation. The possible presence of impurities and physical chemical characteristic differences between racemates and pure enantiomers or the formation of dimers are possible explanations for these results.

Antidiabetic Activity

α-Amylase and α-glucosidase are enzymes that hydrolyze carbohydrates into glucose. A therapeutic strategy in diabetic patients is to lower the level of blood glucose after meals (postprandial). This can be done with drugs that target and inhibit α-amylase and α-glucosidase to effectively decrease the rate of postprandial glucose absorption.

α-Glucosidase Inhibition

Inhibition of α-glucosidase by racemic pinocembrin and pinostrobin was screened using a colorimetric assay via measurement of p-nitrophenol at 410 nm. In ongoing studies in our laboratory we have determined that this enzyme is inhibited by structurally similar stilbene compounds (30).

α-Glucosidase inhibition was weak for the pinocembrin racemate was variable and weak, with less than 10% inhibition at the lowest concentration and inhibition decreasing as concentrations increased. At 10µg/mL enzyme activity appeared to be induced. Racemic pinostrobin showed a dose dependent moderate increase in α-glucosidase inhibition which looked promising, however, high variability between repetitions, which included potential induction at the concentration of 50 µg/mL. The lack of reproducible concentration dependent α-glucosidase inhibitory activity by the two tested racemic flavonoids made it an unattractive assay for further enantiospecific investigation.

α-Amylase Inhibition

The pinocembrin racemate was screened for α-amylase inhibition with the results presented in figure 3a. Concentration dependent α-amylase inhibition with good reproducibility was demonstrated by racemic pinocembrin. Given these results, the α-amylase inhibition assay was selected to further investigate the pharmacologic contribution of the enantiomers of pinocembrin and pinostrobin to α-amylase inhibition. As previously mentioned, pure S-pinocembrin was purchased from Sigma-Aldrich (St. Louis, MO, USA), while pure R-pinocembrin was subsequently acquired via a custom synthesis from Toronto Research Chemicals (Toronto, ON, Canada). Results for the α-amylase inhibition of pinocembrin enantiomers are also presented in figure 3a.

The contribution of S-pinocembrin and R-pinocembrin to the overall pharmacological activity of racemic pinocembrin appears to be additive, with neither enantiomer showing greater activity than the other and the activity of either enantiomer being about half that of the pinocembrin racemate. Statistically significant difference between activity of racemic pinocembrin and its enantiomers was shown at all concentrations except 1 µg/mL.

Since pinostrobin enantiomers are not commercially available, separation by HPLC was performed as described previously to collect a sufficient quantity of pure enantiomers for the assay. Racemic pinostrobin was assessed for its inhibitory activity of α-amylase (figure 3b). At the concentrations chosen (1 – 200 µg/mL) weak inhibition was observed, accompanied the now characteristic decrease in enzymatic inhibition to apparent enzyme induction as concentrations

increased. As this did not meet the predefined requirements of a reproducible positive dose response curve for further investigation of enantiospecific pharmacologic activity, it was decided to run the assay again using a smaller concentration range (0 – 0.75 µg/mL). By doing this, it was hoped to find a concentration range that demonstrated a positive dose response of α-amylase inhibition on which to test the pure enantiomers and evaluate their contribution to the bioactivity of racemic pinostrobin.

Figure 3b illustrates the α-amylase inhibition of racemic pinostrobin and its enantiomers at 0.01 – 0.75 µg/mL. Here, both racemic pinostrobin and its enantiomers show greater α-amylase inhibition than was seen at the higher concentration range (1 – 200 µg/mL). The decrease in enzyme inhibition is also apparent with respect the pinostrobin racemate whereas this is not observed with the pinostrobin enantiomers. At the two lowest concentration points, the activity of the R-pinostrobin enantiomer was significantly greater than that of S-pinostrobin.

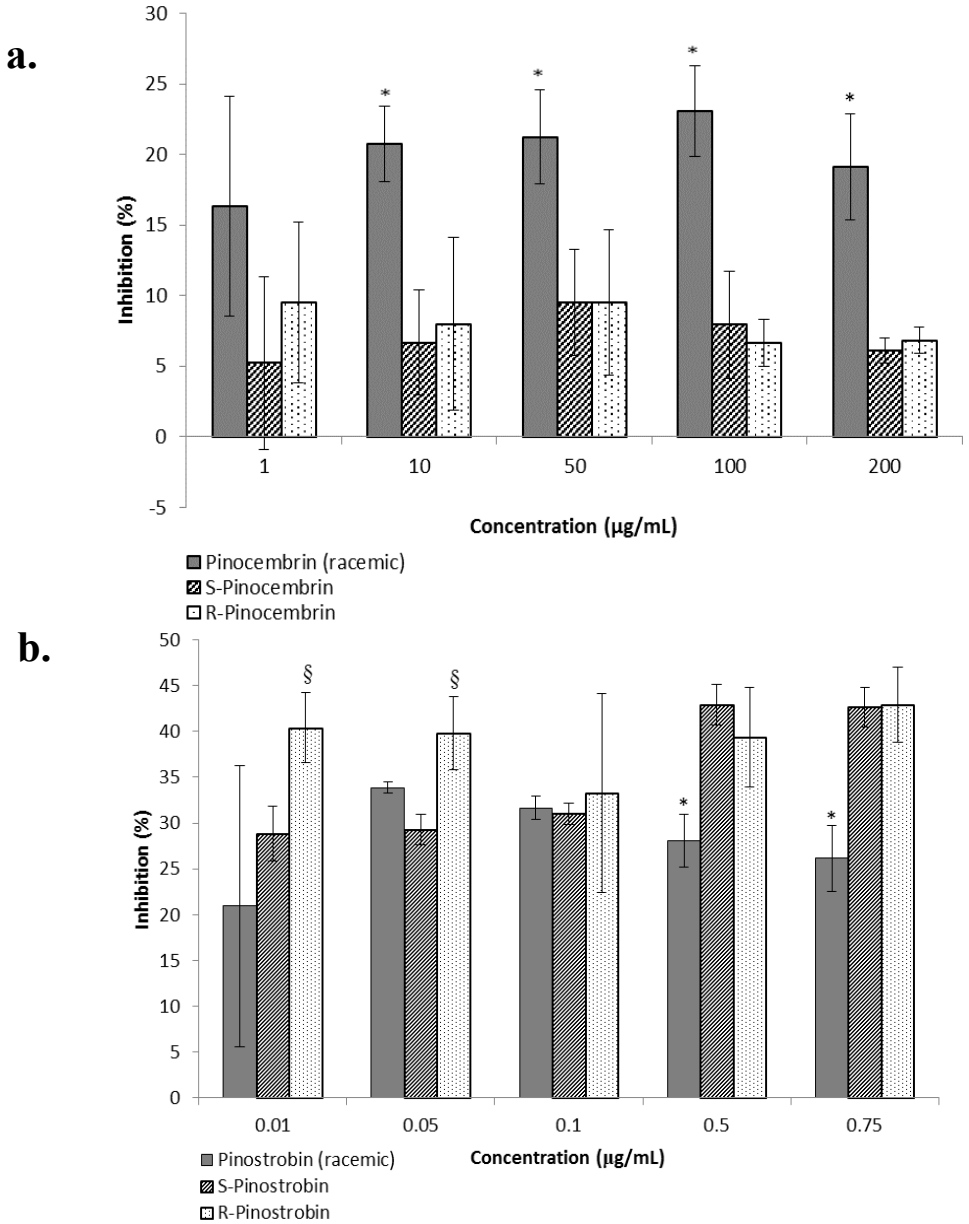


Figure 3. (a) Alpha-amylase percent inhibition of racemic pinocembrin and its pure enantiomers (n = 6 ± SEM). (b) Alpha-amylase percent inhibition of racemic pinostrobin and its pure enantiomers (n = 6 ± SEM). § Represents a significant difference between S-pinostrobin and R-pinostrobin ($P < 0.05$). * Represents a significant difference between racemic pinostrobin and its enantiomers ($P < 0.05$).

In summary, a decreased risk of diabetes has been associated with the ingestion of flavonoids in the diet. Additionally α-amylase inhibitors have been investigated for their potential use in weight loss (43). The combined effect of weight loss and postprandial glucose lowering may contribute to the association of flavonoid intake and decreased risk of diabetes (44,45). α-Amylase inhibition has been demonstrated in products known to contain pinocembrin and pinostrobin, such as honey (44,46). Since performing these experiments, α-glucosidase inhibition was observed by pure S-pinocembrin extracted from a Chinese medicinal plant in one study (47). However, this was not compared to the racemates, which could have different enzyme inhibition characteristics as well as physical chemical properties than the pure enantiomers. Here, we demonstrate moderate to weak α-glucosidase inhibition by racemates of all flavonoids tested. Assessment of α-amylase inhibition of racemic pinocembrin and pinostrobin as well as their pure enantiomers is reported for the first time. α-Amylase inhibition was seen by all racemates with a characteristic decline in enzyme inhibition as concentration increased. Interestingly, the decline in enzyme inhibition and apparent induction in enzyme activity was not seen in the pure enantiomers of each flavonoid. This suggests possible differential pharmacologic behaviour and/or differential physical chemical characteristics of racemate compared to enantiomer.

In Vitro Cyclooxygenase Activity

Cyclooxygenases (COXs) contain cyclooxygenase and peroxidase activities. Two distinct isoforms of COX are known: COX-1, which is constitutively expressed in a variety of cell types and is involved in normal cellular homeostasis; and COX-2, which production is induced by mitogenic stimuli and is responsible for the biosynthesis of prostaglandins under acute inflammatory conditions. The assay measures the inhibition of COX, which catalyzes the conversion of arachidonic acid to PGH_2, by the quantification of $PGF_{2\alpha}$ produced by stannous chloride reduction of PGH_2. Ibuprofen was used as a positive control for the COX-1 assay due to its inhibitory activity towards both COX-1 and COX-2. For the COX-2 assay, ibuprofen and etodolac were employed as positive controls although etodolac is suggested to be a more selective COX-2 inhibitor.

Racemic pinocembrin and its enantiomer S-pinocembrin, were assessed for their COX-1 and COX-2 inhibitory activity using a commercially available ELISA assay. Racemic pinocembrin had COX-1inhibitory activity roughly equivalent to ibuprofen at 1 μg/mL with decreasing inhibition at 10 and 250 μg/mL (Appendix, figure 3). Conversely, S-pinocembrin showed low and variable COX-1 inhibition at 1 and 10 μg/mL with inhibition equivalent to ibuprofen at 250 μg/mL. Additionally, at this highest concentration, S-pinocembrin shows significantly greater COX-1 inhibition than its racemate. Both racemic pinocembrin and S-pinocembrin demonstrated high COX-2 inhibition at all concentrations tested. Interestingly, the COX-2 inhibition demonstrated by racemic pinocembrin and S-pinocembrin exceeded that of the positive control etodolac even at the lowest concentration (1μg/mL) suggesting high potency. The dose response curve was not apparent, but may be elicited with the use of a smaller concentration range. At 10 μg/mL S-pinocembrin demonstrated a significantly higher COX-2 inhibitory activity when compared to racemic pinocembrin. At both the lowest and highest concentrations, however, no difference in activity was observed.

Racemic pinostrobin was screened for its COX-1 and COX-2 inhibitory activity using a commercially available ELISA assay. The pinostrobin racemate showed variable and weak COX-1 inhibitory activity at all concentrations tested (Appendix, figure 4). No positive dose response relationship was noted. However, COX-2 inhibition was equivalent to or exceeding that of etodolac, the positive control. Similar to the results observed with respect to COX-2 inhibition and pinocembrin, racemic pinostrobin demonstrates potency with COX-2 inhibition approaching 100% at the lowest concentration tested (1 μg/mL). These results suggest that racemic pinostrobin may be a more selective COX-2 inhibitor than COX-1 inhibitor.

In summary, racemic pinocembrin and pinostrobin as well as pure S-pinocembrin demonstrated COX-1 and COX-2 inhibitory activity. COX-1 inhibitory activity results were characteristic of decreasing inhibition with increasing concentration of the racemates. S-Pinostrobin showed increasing COX-1 inhibition with increasing concentration, suggesting differential enantiospecific pharmacologic activity.

COX-2 inhibition assay results consistently demonstrated potent and COX-2 selective inhibition over the concentration range tested by all racemates. S-pinocembrin behaved similarly, with a slightly greater inhibition shown at 10 µg/mL. Given the limitations with the procurement and stereoselective separation and collection of the pure enantiomers of pinostrobin as well as the anti-oxidant and COX inhibition results demonstrated by pinocembrin and it's readily available enantiomer S-pinocembrin, pinocembrin was chosen as a focus for further enantiospecific characterization of potential activity with relevance to heart disease.

Cardiomyocyte Size Assay

A common feature of multiple cardiac morbidities including heart failure, arrhythmias and sudden death is cardiac hypertrophy. Additionally, oxidative stress and inflammation seem to play a role. To better understand the potential cardioprotective effects of pinocembrin and its enantiomers the cell size assay was selected. Racemic pinocembrin, S-pinocembrin, and R-pinocembrin were assessed for their effect on their prevention of cardiac myocyte hypertrophy. In figure 4a, neither racemic pinocembrin nor its enantiomers significantly affected cell size compared to control at concentrations 1 – 10 µg/mL with the exception of R-pinocembrin, which had showed an increased effect on cell size at 10 µg/mL. At 50 µg/mL, however, racemic pinocembrin, S-pinocembrin, and R-pinocembrin appeared to have a toxic effect on cardiac myocytes. These results are significant in that they show that pinocembrin and its enantiomers do not appear to have deleterious effects on cell size at concentrations likely to be seen in physiologic conditions.

Figure 4b presents the effect of racemic pinocembrin and its enantiomers on cardiac myocyte size after induction of hypertrophy by endothelin-1. Racemic pinocembrin showed a dose dependent prevention of cardiac myocyte hypertrophy attaining a complete prevention of hypertrophy at 5 and 10 µg/mL. At 50 µg/mL the pinocembrin racemate demonstrated apparent cellular toxicity. R-pinocembrin prevented cardiac hypertrophy similarly to racemic pinostrobin at 1 and 5 µg/mL in a dose dependent manner. However, preventive activity was lost at 10 µg/mL and became apparently toxic at 50 µg/mL. S-pinocembrin showed no hypertrophy prevention at any concentration. These results suggest that pure R-pinostrobin moderately prevents cardiac myocyte hypertrophy in this model whereas pure S-pinostrobin appears not to. When present as a racemate the enantiomers appear to act synergistically to prevent cardiac myocyte hypertrophy.

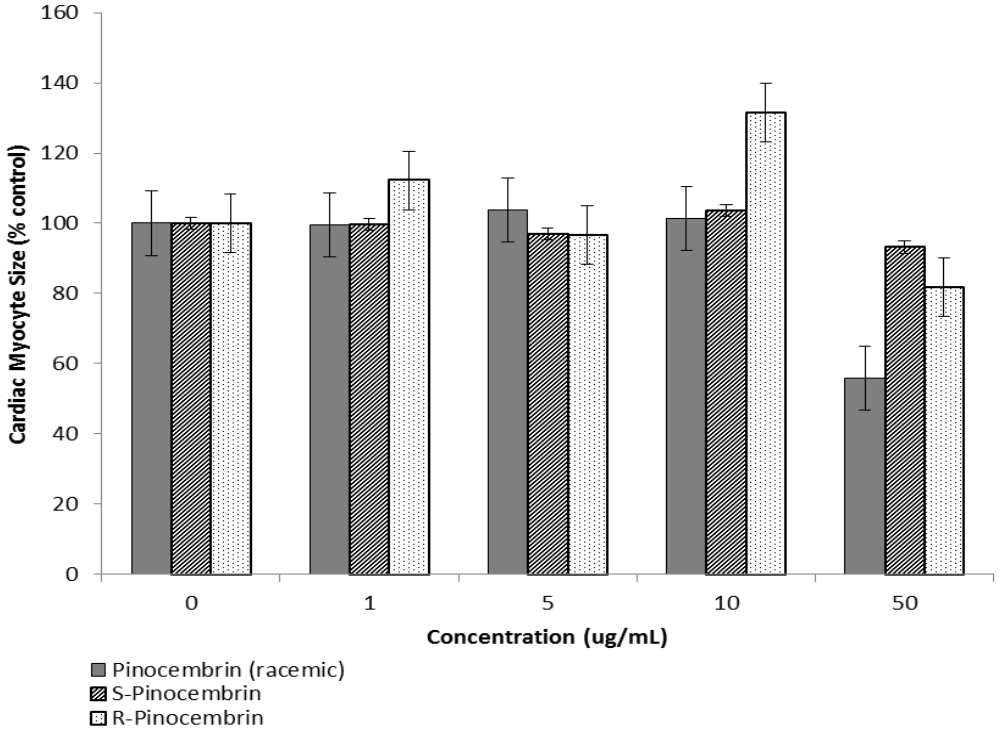
DISCUSSION

As has been stated, pharmacokinetic characterization and analysis of pinocembrin and pinostrobin has been incomplete. Yang, et al. reported an achiral pharmacokinetic study of pinocembrin after IV administration of 22.5 and 67.5 mg/kg in rat serum (15). With a reported half-life of 14.61 ± 3.74 and 13.93 ± 5.02 minutes and clearance of 0.07 ± 0.02 and 0.04 ± 0.01 l/min/kg for the two doses respectively, their results parallel those of this project, which showed a half-life of approximately 15 minutes and total clearance of approximately 5.5 L/h/kg (Table 1). Additionally, Yan et al. have recently assessed the achiral pharmacokinetcs of pinocembrin after a 20 mg IV infusion over 30 minutes in humans (14). The authors report a half-life of 47.4 ± 14.0 minutes, a Vd (L) of 136.6 ± 52.8 and a clearance of 2.0 ± 0.3 L/min. Although the study is done in humans with weight of the subjects not reported, pinocembrin demonstrates a high volume of distribution and a half-life of less than 1 hour. Assuming an average human weight of 75 kg, the value for clearance presented here for both pinocembrin enantiomers (approximately 0.092 L/min/kg) would equal 6.9 L/min which is consistent with the general higher clearance of the rat. A recent study reported by Cao, et al. similarly showed short plasma half-life (40 to 60 minutes) in human participants given 20, 40, 80, 120, or 150 mg intravenously as well as participants given 60 mg twice daily for 5 days (13). $AUC_{0-\infty}$ and T_{max} were dose dependent and there was no evidence of accumulation (13). No stereospecific pharmacokinetic studies of pinocembrin have been reported to date.

Similarly, there are currently no reports on stereospecific pinostrobin pharmacokinetics present in the biomedical literature. Hua et al. report the single achiral study of pinostrobin pharmacokinetics after oral (0.5 mg/kg) administration in rats (48). In this study, the reported half-life was 4.34 ± 0.24 minutes, the

AUC_{0-t} was 3817.80 ± 352.89 ng min/mL, and the mean residence time (MRT) was 6.26 ± 0.31 h. The formulation of the pinostrobin dose is not reported and may represent an explanation for the discrepancy in half-life observed in this project for orally administered pinostrobin (S-pinostrobin 31.90 ± 5.62 h, R-pinostrobin 44.2 ± 8.24 h). Additionally, female Kunming rats were used rather than male Sprague-Dawley which were employed in our studies.

a.



b.

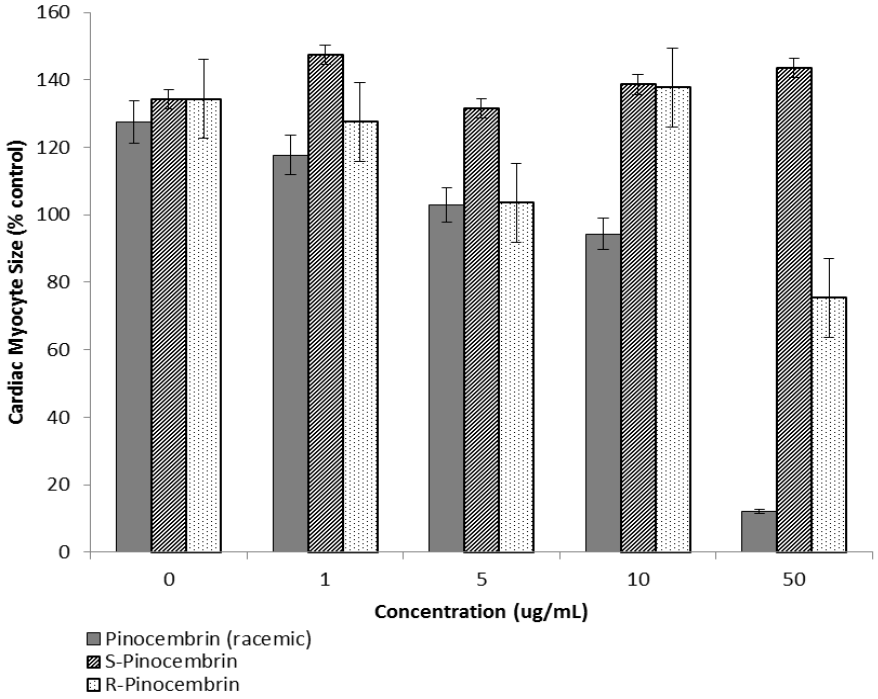


Figure 4. Effect of racemic pinocembrin, S-pinocembrin, and R-pinocembrin on cardiac myocyte size (percent of control) before (n = 3 ± SEM) (a) and after (n = 3 ± SEM) (b) addition of endothelin-1 to induce hypertrophy.

To our knowledge, this is the first literature report that has assessed the stereospecific pharmacokinetics of pinocembrin and pinostrobin after intravenous and oral administration of the pure racemates. Previous studies save one (49) have focused only on the racemic mixtures and utilized achiral analysis after oral ingestion. Our findings indicate that the two flavonoids have relatively short half-lives (0.25-7 h) in serum and long half-lives in urine (3-26 h) after intravenous administration as shown previously with similar compounds (stilbenes) that belong to the same family of polyphenols (33, 34). This discrepancy between plasma and urine half-life in different stilbenes was attributed to assay sensitivity limits that would most likely underestimate the overall half-life of these compounds. Underestimation of plasma half-life due to assay sensitivity limits has been reported before in the case of procainamide (50). Nevertheless, most of the pharmacokinetic studies only collect samples up to 24 hours post-dose, which could underestimate the elimination phase and pharmacokinetic parameters. These discrepancies suggest that the serum half-life is likely significantly underestimating the overall half-life of pinocembrin and pinostrobin enantiomers due to assay sensitivity limits in serum. Thus, the pharmacokinetics and biodisposition of these compounds need to be reconsidered on the basis of their chirality and glucuronidated metabolites in the fact that urine provides higher concentrations of these xenobiotics to assess these parameters.

Furthermore, the large volumes of distribution (1.5-10 L/kg) of pinocembrin and pinostrobin enantiomers are significantly larger than the total blood volume (0.054 L/kg) and the total water volume (0.668 L/kg) in the rat indicating that both enantiomers of these compounds are exiting the blood and penetrating deeply into the tissues. These large volumes of distribution (V_{ss}) values correlate with the lipophilic nature of these compounds (XLogP values of 2.7 and 3.1 for pinocembrin and pinostrobin respectively), which might indicate their preferential binding to tissues and preference to reside in the body. Based on the clearance values it can be observed that these two compounds are mainly excreted via non-renal routes (assuming that hepatic clearance is equivalent to non-renal clearance), which is also verified by their generally low fraction excreted in urine (f_e) values of-0.3-5% (Table 1). Based on these pharmacokinetic data, pinocembrin and pinostrobin appear to be exiting the vasculature and distributing to the different tissues in the body.

In vitro studies of the pharmacological activity of the stereoisomers of pinocembrin and pinostrobin, are necessary because as demonstrated by the results obtained in the pharmacokinetic studies, the differences in the stereochemistry of these compounds can impact their disposition, metabolism, and elimination by the body, and it is also likely that enantiomers produce different pharmacological effects in biological systems depending on their stereochemical configuration. Due to the prohibitive costs of commercially available racemic flavonoids, in vitro studies can be of significant utility in exploratory pharmacology for the screening of the pharmacological activity of these compounds; if the desired pharmacological effect is obtained with the racemate, more in-depth studies can be performed with the active stereoisomers in the future. It has been previously reported that for some racemic xenobiotics, a detrimental effect is apparent when given as a mixture while the pure stereoisomer may produce a desired effect, i.e. the teratogenic effect of thalidomide results from DNA intercalation of the S-enantiomer in the racemate, but R-thalidomide does not produce such an effect.

Therefore, in vitro studies of each stereoisomer of pinocembrin and pinostrobin are needed. Although the isolation of stereoisomers is possible with the validated HPLC methods, the cost of the starting racemic compounds is often a limiting factor to extensive studies in an academic laboratory. In addition, it is a time consuming process, taking between two to six weeks to collect the amount of individual enantiomers necessary for one single assay. Consequently, as many assays were performed with pure compounds as possible within the inherent time and cost constraints. In addition, for the stereoisomers that are commercially available, issues with stereochemical purity must be taken into consideration. Therefore, it is important to consider the commercial sources of the pure stereoisomers to ensure the purity of these compounds. In this study, one in vitro drug interaction assay and six in vitro pharmacologic activity models with relevance to chronic disease pathology were chosen to confirm previously reported bioactivity in pinocembrin, and pinostrobin and explore the contribution of the flavonoid enantiomers to any observed pharmacological

activity. Flavonoid racemates were tested first in each assay to confirm or refute reported activity.

CONCLUSIONS

The stereospecific pharmacokinetics of intravenously and orally administered pinocembrin and pinostrobin, in rats are reported for the first time. These studies indicate that pinocembrin and pinostrobin are highly distributed and rapidly glucuronidated. They likely undergo elimination via non-renal routes, which may indicate high achievable concentrations in the liver and the gastrointestinal tract. These compounds have generally short half-lives and have low bioavailability. The chirality of these flavonoids greatly affected their disposition in serum and urine and their overall pharmacokinetic profile. Moreover, the importance of delineating the disposition of each enantiomer and glucuronidated metabolite in urine as well as serum was shown necessary. Overall, these studies have demonstrated that pinocembrin and pinostrobin have pharmacological activity in a variety of exploratory and well established *in vitro* assays. Interestingly, these investigations have revealed that chiral differences in the chemical structure of these compounds result in significant pharmacodynamic differences.

Pinocembrin and pinostrobin demonstrated concentration-dependent alpha amylase inhibitory activity. Differences in the inhibitory activity of the racemates compared to their individual enantiomers were observed in multiple activity screens. Pinocembrin was singled out as having promise in screens relevant to heart disease, as it demonstrated both anti-oxidant and anti-inflammatory activity in the racemate and pure S-enantiomer. In an assay of cardiac myocyte hypertrophy, the pure enantiomers were seen to have a synergistic contribution to the activity seen in the racemate. Further studies are necessary with the pure stereoisomers of pinostrobin to elucidate its potential differences in anti-inflammatory and anti-oxidant activity.

These studies demonstrated the utility and necessity of developing stereoselective HPLC methods for chiral flavonoids. The validated HPLC methods described were successfully used to isolate the stereoisomers of pinostrobin that are not commercially available. Nonetheless, the expense and time needed to collect enough quantity of the compounds limited the number of investigations able to be conducted. A number of technical issues require further experimental scrutiny including: purity of the compounds obtained from commercial suppliers, and solubility.

ACKNOWLEDGMENTS

The authors would like to acknowledge the University of Manitoba Graduate Fellowship and Manitoba Graduate Scholarship from the University of Manitoba, the Pfizer Canada Centennial Pharmacy Research Award, and the Manitoba Pharmaceutical Association (MPhA)/William G. Eamer Graduate Scholarship in Pharmacy awarded to Dr. Casey L. Sayre. Acknowledgments are also extended to Kuwait University for Graduate Scholarship awarded to Samaa Alrushaid.

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APPENDIX

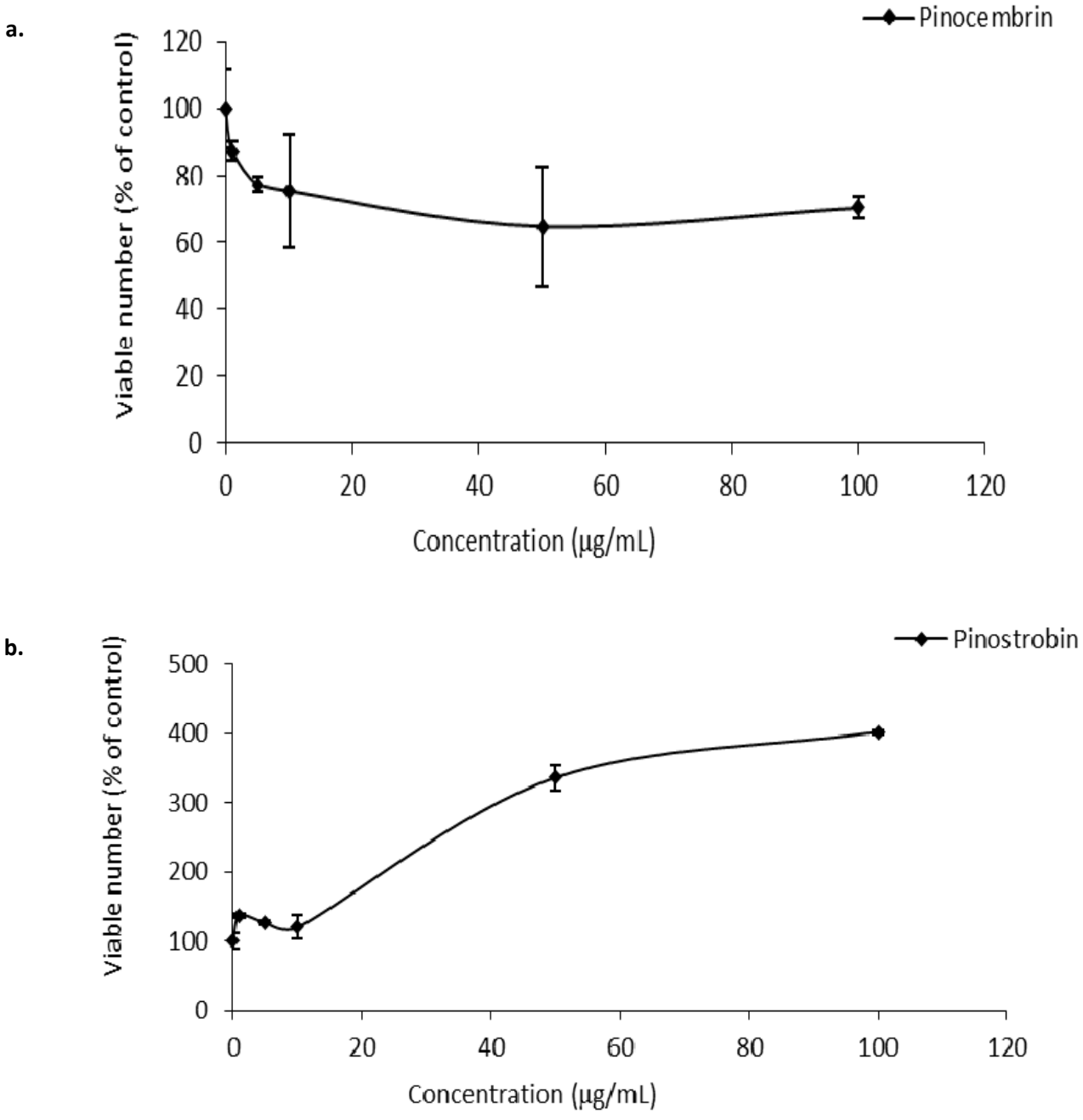
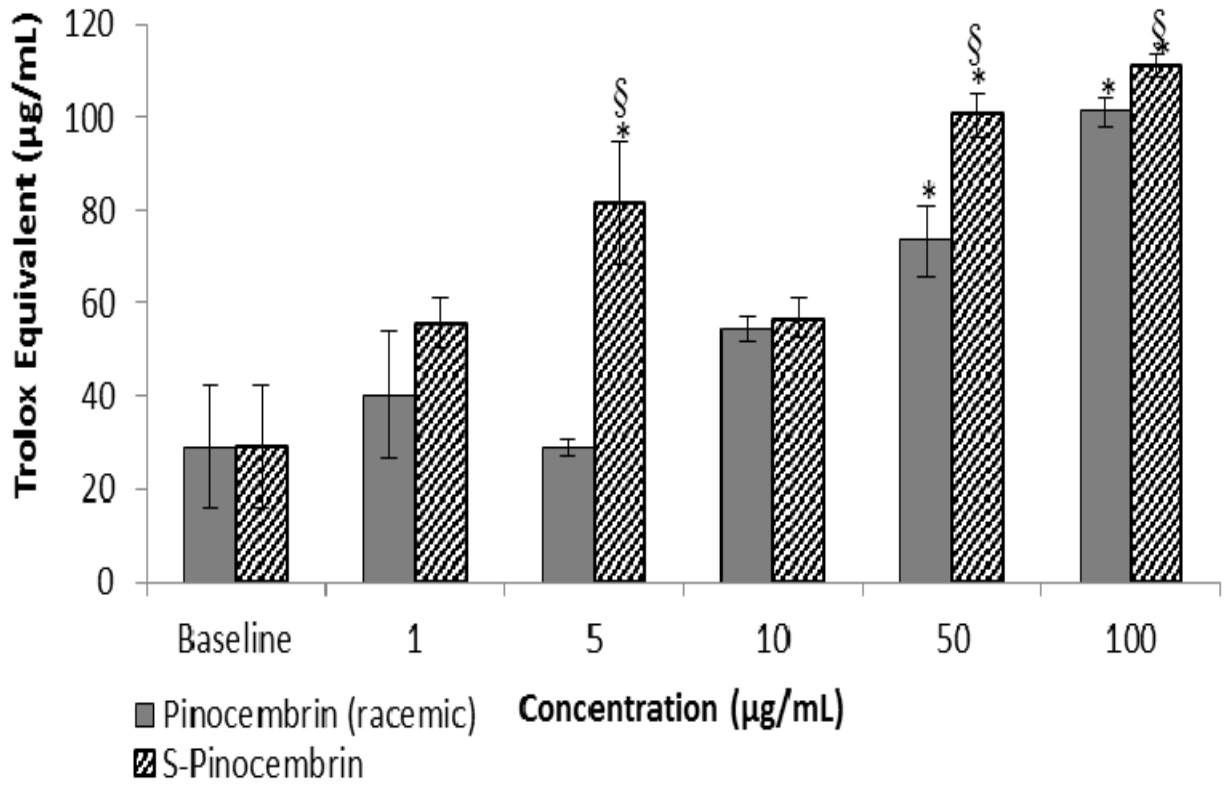


Figure 1. HT-29 Cell viability after administration of racemic pinocembrin (a) and pinostrobin (b) (n = 3, mean ± SEM).

a.



b.

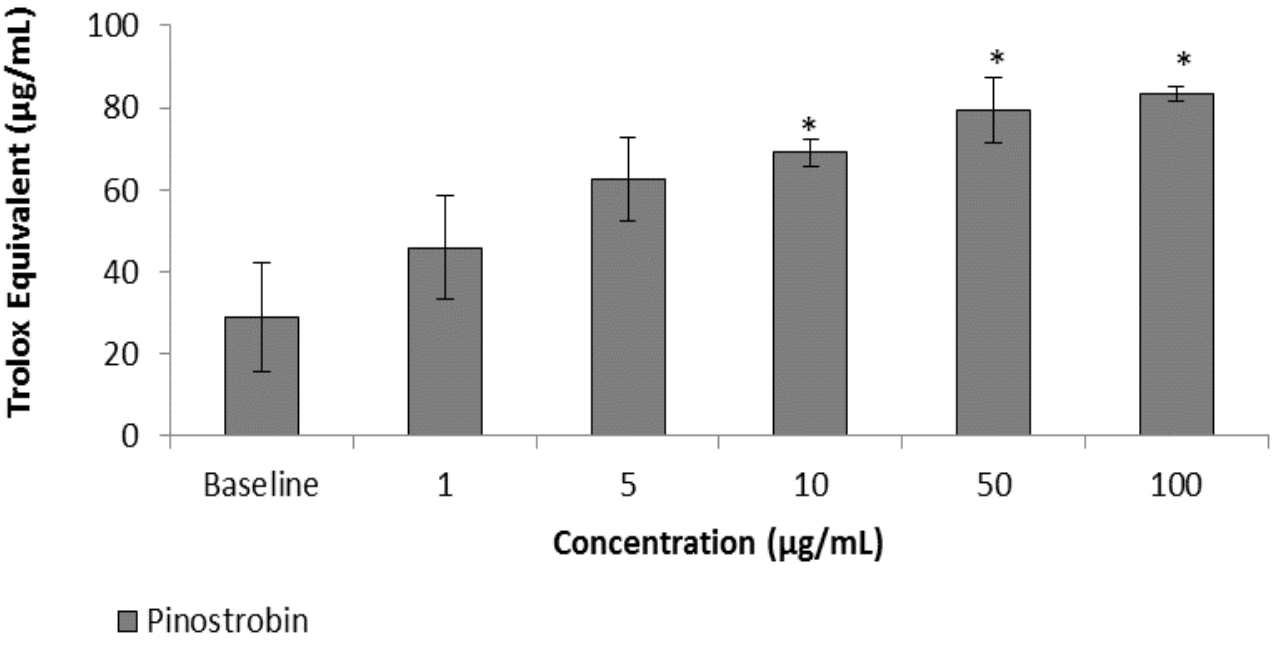
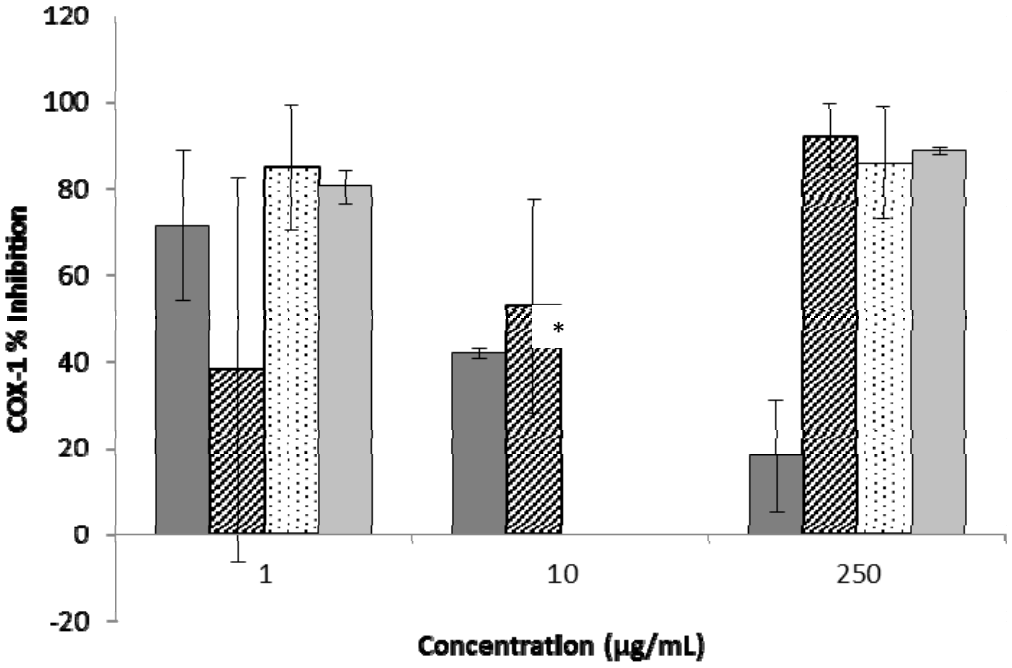


Figure 2. Anti-oxidant capacity (Trolox® equivalent) of racemic pinocembrin and S-pinocembrin (a), and racemic pinostrobin (b) (n = 4, mean ± SEM) compared to DMSO alone (baseline). * Represents a significant difference between racemate and baseline antioxidant activity in DMSO alone ($P < 0.05$). § Represents a significant difference between S-pinocembrin and racemic pinocembrin ($P < 0.05$).

a.



b.

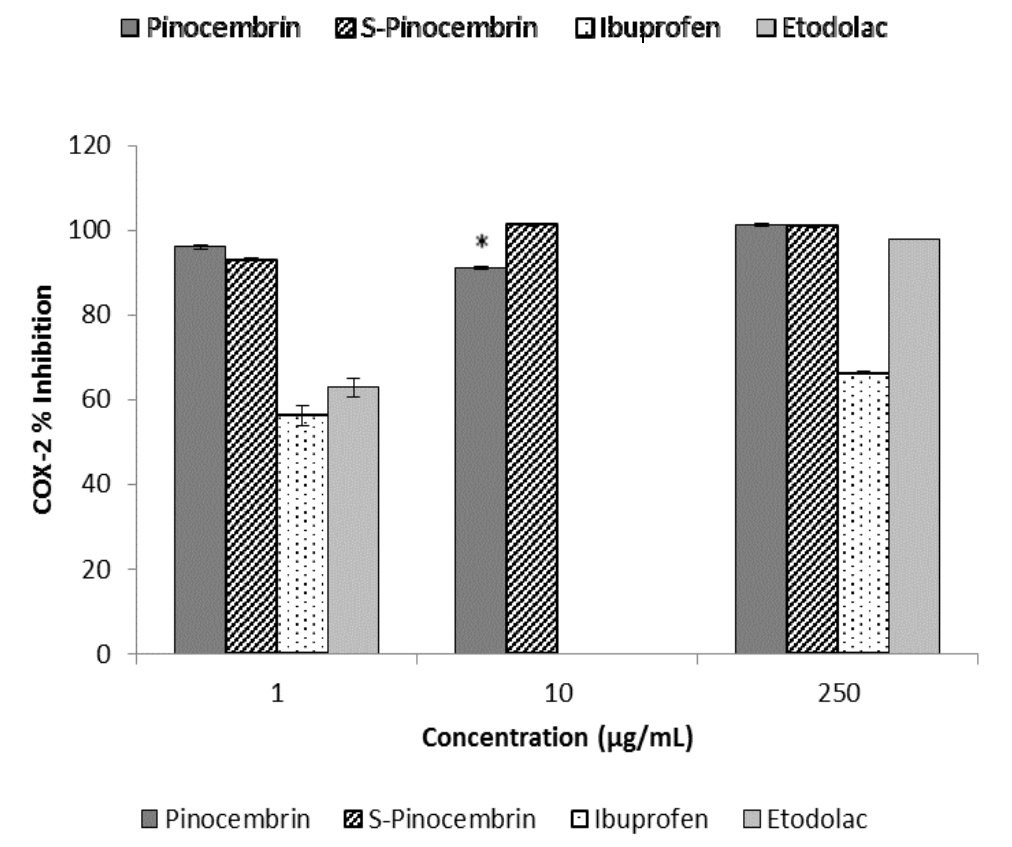
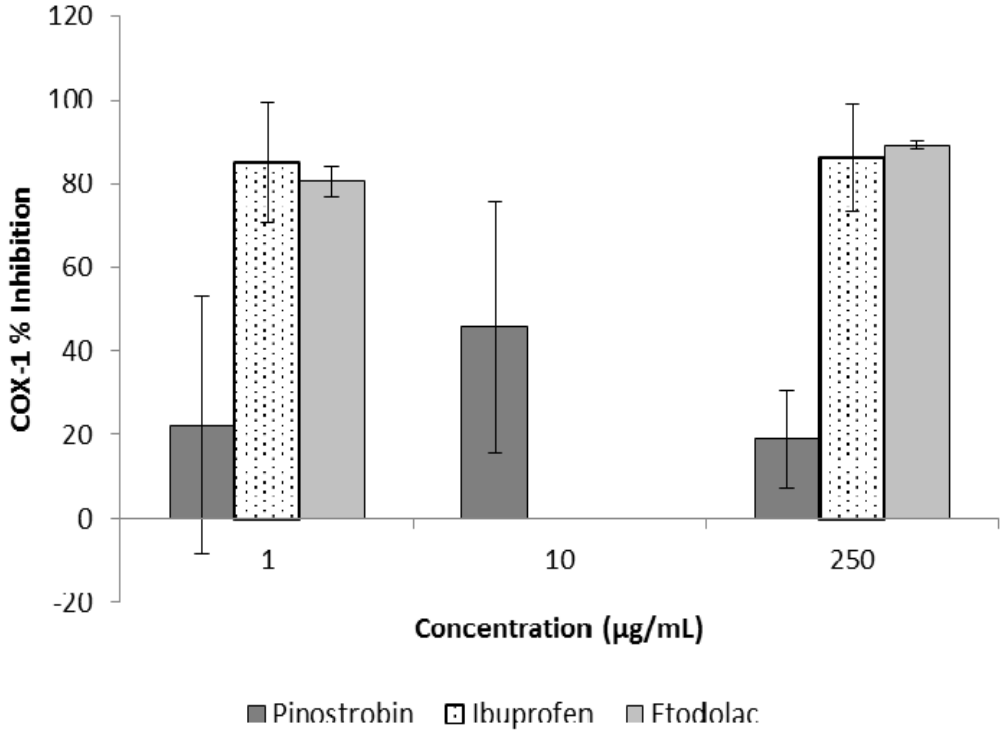


Figure 3. Cyclooxygenase-1 (COX-1) (a) and cyclooxygenase-2 (COX-2)(b) activity after addition of racemic pinocembrin, S-pinocembrin and ibuprofen and etodolac (positive controls) at concentrations 1.0 – 250.0 µg/ml (n = 3, mean ± SEM).* Represents a significant difference between racemic pinocembrin and its enantiomers ($P < 0.05$).

a.



b.

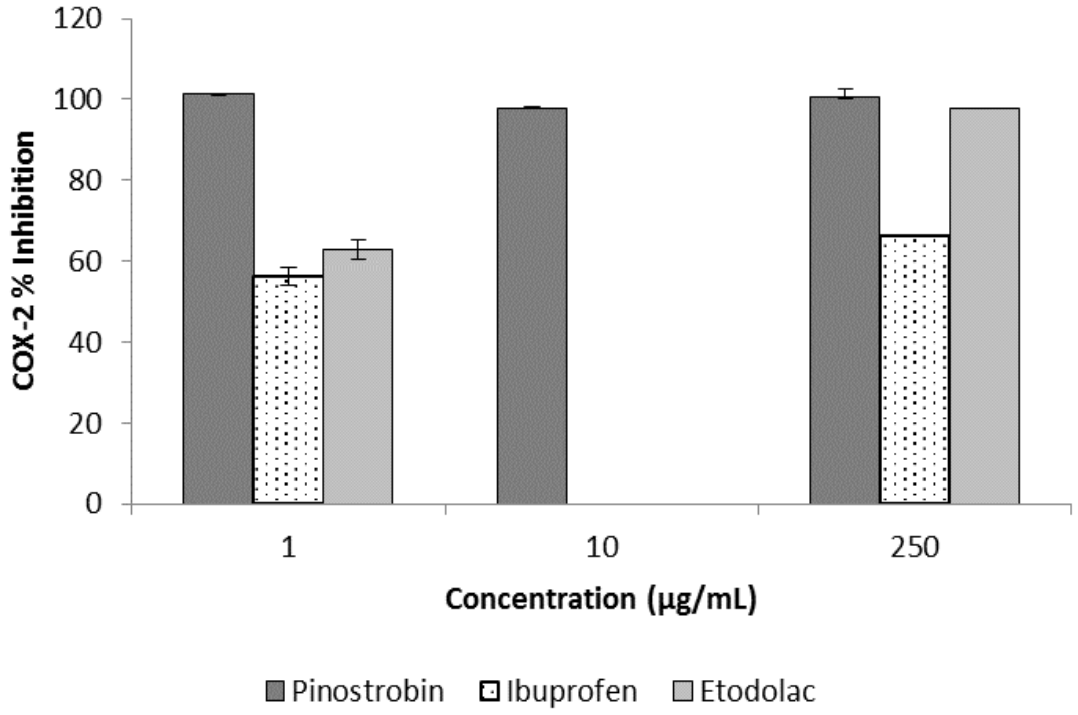


Figure 4. Cyclooxygenase-1 (COX-1)(a) and cyclooxygenase-2 (COX-2)(b) activity after addition of racemic pinostrobin and ibuprofen and etodolac (positive controls) at concentrations 1.0 – 250.0 µg/ml (n = 3, mean ± SEM).