# Contribution of Selected Flavonoid Enantiomers Implicated in Chronic Disease Prevention to Differential Pharmacokinetic and Pharmacodynamic Behaviour

By

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## Abstract

Decreases in risk of chronic disease associated with diets high in fruits and vegetables have been observed. Difficulty in determining the active ingredients responsible for the beneficial effects of dietary plant intake comes in part from the lack of pre-clinical pharmaceutical characterization of compounds thought to play a role. Flavonoids are a class of low molecular weight secondary metabolites present in plants. Pinocembrin, pinostrobin, and liquiritigenin are three chiral flavonoid implicated in the chronic disease prevention seen in high plant diets. To further the pre-clinical development of novel compounds for the potential prevention of chronic disease, stereospecific analytical methods of detection and quantification were developed for pinocembrin, pinostrobin, and liquiritigenin in biological matrices. These methods were used to elucidate the enantiospecific pharmacokinetics of all three compounds in the rat. Additionally, pinocembrin, pinostrobin, and liquiritigenin enantiomers were quantified in multiple marketed natural health products and dietary supplements. Finally, the compounds were screened for activity in several in vitro pharmacodynamic assays with roles in chronic disease pathology to assess for potential stereopecific pharmacologic behaviour. The methods were successfully used to determine the stereospecific pharmacokinetics, pharmacodynamics, and content analysis of selected marketed products. Stereopecific differences were observed in several instances. Further stereospecific studies are needed, especially in the field of toxicokinetics.

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## Abbreviations and Symbols

| | |
|---|---|
| α | Alpha |
| ABTS | 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulfonic acid) |
| AUC | Area under the curve |
| $AUC_{0\rightarrow\infty}$ or $AUC_{inf}$ | Area under the curve from time zero to infinity |
| β | Beta |
| $C_0$ | Initial concentration |
| line | |
| CDL | Curved desolvation line |
| CL | Clearance |
| $CL_{non\text{-}renal}$ | Non-renal clearance |
| $CL_r$ | Renal clearance |
| $CL_{tot}$ | Total clearance |
| $C_{max}$ | Concentration maximum |
| $CO_2$ | Carbon dioxide |
| Conc. | Concentration |
| COX | Cyclooxygenase |
| CYP/CYP450 | Cytochrome P450 |
| DMEM | Dulbecco's modified Eagle's medium |
| DMEM/F-12 | Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham |
| DMSO | Dimethyl sulfoxide |
| DPPH | 2,2-diphenyl-1-picrylhydrazyl |
| DSHEA | Dietary Supplement Health and Education Act |
| EIA | Enzyme immuno assay |

| | |
|---|---|
| ELISA | Enzyme-linked immunosorbent assay |
| ER | Extraction ratio |
| ESI | Electrospray ionization |
| EtOH | Ethanol |
| F | Bioavailability |
| FBS | Fetal bovine serum |
| FDA | United States Food and Drug Administration |
| Fe | Fraction excreted unchanged in the urine |
| g | gram |
| h | hour |
| HCl | Hydrochloric acid |
| HEPES | N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulfonic acid) |
| HPLC | High performance liquid chromatography |
| HT-29 | Colorectal adenocarcinoma cell line |
| IS | Internal standard |
| IV | Intravenous |
| $k_a$ | Absorption rate constant |
| KE or $k_e$ | Elimination rate constant |
| LC | Liquid chromatography |
| LC-ESI-MS | liquid chromatography – electrospray ionization – mass spectrometry |
| LOD | Limit of detection |
| LOQ | Limit of quantification |

| | |
|---|---|
| min | Minute |
| ml | Milliliter |
| mM | Millimoles per liter |
| MRT | Mean residence time |
| MW | Molecular weight |
| m/z | Mass to charge ratio |
| NA | Not applicable |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| ND | Not detectable |
| OH | Hydroxyl group |
| P | p-value |
| PAR | Peak area ratio |
| PBS | Phosphate buffer saline |
| PEG | Poly(ethylene glycol) |
| PEG-b-PCL | Poly(ethylene glycol)-block-poly(ε-caprolactone) |
| PG | Prostaglandin |
| pH | Power of hydrogen |
| ROS | Reactive oxygen species |
| RSD | Relative standard deviation |
| SD | Standard deviation |
| sec | Second |
| SEM | Standard error of the mean |

| | |
|---|---|
| SIM | Selected ion monitoring |
| $t_{1/2}$ | Half life |
| Tmax | Time of concentration maximum |
| µg | Microgram |
| µM | Micromol per liter |
| µl | Microliter |
| US | United States |
| UV | Ultraviolet |
| $V_d$ | Volume of distribution |
| $V_{ss}$ | Volume of distribution at steady state |
| XLogP | Experimental octanol to water partition coefficient |

# Chapter I: Introduction

## 1.1 Background

Much excitement has been generated surrounding the discovery of anti-oxidant, anti-inflammatory, anti-allergic, anti-bacterial, anti-tumor, and anti-viral activity of flavonoids and other phytochemicals. [1] Indeed, the clinical significance of an essentially food-derived agent with low apparent toxicity, possessing preventive and/or therapeutic activity against chronic disease states cannot be overemphasized. However, recent reviews have established the assertion that *in vitro* bioactivity 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. [2] In other words, without pharmacokinetic and metabolic data, both human and animal, *in vitro* bioactivity studies may be in danger of showcasing irrelevant physiological forms of flavonoids at physiologically irrelevant concentrations. [2,3]

Despite this over sight by the biomedical research community, flavonoids have been and continue to be consumed by a large portion of the Western population via nutraceutical products and plant-based food. [4] Since nutraceuticals are viewed by some regulatory agencies as food rather than drugs, many of these products are developed and marketed without having passed standards of safety or efficacy. [5] In fact, several flavonoids have been shown to exhibit toxicities with effects ranging from anti-thyroidism to alteration of micronutrient absorption and transport. [6] The liberal use of flavonoid containing nutraceuticals presents a potential public health risk that could be ameliorated by flavonoid-specific research generated from a variety of fields. However,

the bulk of *in vitro* and epidemiological evidence in support of the purported health benefits of flavonoids is hard to ignore.

Regardless of the motivation to study flavonoid pharmacokinetics, the ability to detect and quantify flavonoids in various matrices is required. Further complicating this requirement is the fact that some flavonoids exhibit chirality in their molecular structure. Chirality describes two forms of an object whose arrangement in space only differs such that the forms are non-superimposable on their mirror images, like a right and left glove. Accounting for chirality in the detection and quantification of compounds is often more complicated than achiral analysis but is of significant scientific importance, particularly in the pharmaceutical industry. It has long been known that individual stereoisomers can have significantly different pharmacological, toxicological, and pharmacokinetic profiles. [7,8] An infamous example of the toxicological importance of chirality was the racemic drug thalidomide, used as a sedative and anti-nausea medication in the 1960s of which the (-)-stereoisomer was found to cause fetal malformations. [9,10] Data describing the pharmacology and toxicology of single enantiomers is now required for new drug applications by the Food and Drug Administration (FDA), however, no such requirement is yet imposed on manufacturers of nutraceuticals.

## 1.2 Flavonoids and Chirality

Flavonoids are a class of naturally occurring polyphenolic compounds. Several subgroups of flavonoids exist including: isoflavones, flavones, flavanols, flavonols, anthocyanadins, and flavanones. Flavanones exist predominately in citrus fruits and soy products. Flavonoids within this subclass have been the target of many *in vitro*

bioefficacy studies. The chemical structure of all flavanones is based on a $C_6$-$C_3$-$C_6$ configuration consisting of two aromatic rings joined by a three-carbon link. [11,12] Nearly all the flavanones have one chiral center at the carbon in position 2, except for a subclass of flavanones named the 3-hydroxyflavanones (dihydroflavonols) and catechins. These contain two chiral centers at the carbon atoms in position 2 and 3. Some flavanones possess an additional D-configured mono or disaccharide sugar in the C7 position on ring A. Called flavanone-7-O-glycosides, these flavonoids exist as diastereoisomers or epimers; meaning they have the alternate configuration at only one of two or more tetrahedral stereogenic centers present in the respective molecular entities.

It is important to note that there are other classes of flavonoids that can also demonstrate chirality in some of their members. For instance, legumes are a rich source of isoflavones that may have pharmacological properties. The isoflavone reductase enzyme reduces achiral isoflavones to chiral isoflavones during the biosynthesis of chiral pterocarpan phytoalexins. Other examples include the synthesis of (-)-maackiain in red clover, (-)-medicarpin in alfalfa, (+)-pisatin, (-)-maackiain, (-)-sophorol and (+)-6a-hydroxymaackiain in garden pea (*Pisum sativum*). [13-16] A well studied occurrence of enzymatic production of chiral entities are the soy isoflavonoids daidzein and the red clover isoflavonoid formentin, which are converted to the chroman metabolite S-(-)-equol by microbial flora of the gastrointestinal tract. [17-21] As a consequence of the increase in the number of studies involving uncharacterized plant species, various novel flavonoids have been recently isolated from different plant varieties. For example, the dracorupesins (flavanone derivatives) [22] and dracocephins (flavanoidal alkaloids) [23] isolated from *Dracocephalum rupestre*. Another example includes the thearubigins (phenolic pigments)

that have been isolated from black tea and have been reported to maintain the chiral properties of flavanols and theaflavins. [24]

Many chiral flavonoids can be purchased from boutique chemical companies, but they are mainly commercially available only as racemates (equivalent proportions of both enantiomers or epimers). There are several commercial vendors selling flavonoids that do not provide any information on the chiral nature of the molecules.

## 1.3 Separation of Enantiomers through Chromatography

The separation, resolution, and analysis of enantiomers has generally been accomplished through the formation of diastereoisomers either covalently or transiently. This is necessary because enantiomers, other than the direction they rotate polarized light, have identical physicochemical properties in an achiral environment. This makes their separation by traditional chromatographic means fundamentally impossible. However, diastereoisomers do have different physicochemical properties and thus they can be separated on an achiral chromatographic column through differential interaction and retention with the stationary phase. Racemic flavonoid resolution has generally been accomplished by chromatographic enantiospecific resolution through temporary formation of diastereoisomers on a chemically bonded chiral stationary phase (CSP) with an achiral mobile phase.

## 1.4 Chiral Stationary Phases (CSPs) as Direct Methods

A number of different CSPs have been utilized to separate and adequately resolve the enantiomers of chiral flavonoids for subsequent quantification. A commonly used CSP are the chiral polymer phases. These stationary phases can be further sub-divided

into Polysaccharide-Derived Columns, Cyclodextrin Columns, and "Mixed" Cyclodextrin Columns.

## 1.5 Chiral Polymer Phases

### *1.5.1 Polysaccharide Columns*

A variety of chiral columns utilizing synthetic polysaccharides, particularly D-cellulose esters containing a variety of terminal groups, have been employed. Enantioresolution of flavanone enantiomers by HPLC utilizing polysaccharide derivatives such as cellulose trans-tris (4-phenylazaphenylcarbmate) columns was first established in the 1980's. [25] This was followed by separation on cellulose tris (3,5-dimethylphenylcarbamate) columns. [26,27] Unsubstituted flavanones can be easily separated on cellulose mono and disubstituted carbamates including cellulose-4-substituted triphenylcarbamate derivatives as well as cellulose chloro-substituted triphenyl carbamate, and cellulose methyl-substituted triphenylcarbamate supported in silica gel. [28] Hesperetin has been successfully separated in a validated reverse-phase HPLC method and a commercially available Chiralpak AD-RH tris (3,5-dimethylphenylcarbmate) derivative of amylose column. [29]

The chiral recognition of microcrystalline triacetate may involve inclusion complexation. Three commercially available columns of microcrystalline cellulose triacetate were able to resolve several flavanones including naringenin, hesperetin, eriodictyol, homoeriodictyol, pinocembrin, and isosakuranetin. [30] One example of such a column is the Chiralcel OA, a commercially available cellulose triacetate column coated on macroporous silica gel. [31] The seminal work on separation of some racemic flavanones was accomplished on microcrystalline cellulose triacetate supported on non-

macroporous silica gel diol. [31] This CSP employed in normal (apolar) phase using gradient elution was found to be superior to a commercially available cellulose triacetate columns for separation of polyhydroxylated flavanones, particularly the 5,7-dihydroxy substituted on ring A (i.e. pinocembrin, isosakuranetin, naringenin, eriodictyol, homoeriodictyol, and hesperetin). Normal phase chromatography was far superior to reverse (polar) phase for separating flavanone enantiomers. In addition, it is important to note that the flavanone glycosides could also be resolved together with their aglycones and this was applied to analysis of naringenin enantiomers in tomato skin. [31] The substitution of a Chiralcel OA column performed the resolution of 5- and 7-methoxyflavanone as well as naringenin. [32] The Chiralpak IC is a commercially available cellulose tris (3,5-dichlorophenylcarbamate) column coated on macroporous silica gel, which has demonstrated marginal separation of flavanone enantiomers. [33]

The Chiralcel OD column is a macroporous silica gel coated with cellulose tris (3,5-dimethylphenylcarbamate), which has demonstrated an ability to separate a variety of flavonoid derivatives (i.e. flavanone, [33-35]4'- and 6-methoxyflavanone, [34,35] 5-methoxyflavanone; 2'- or 6-hydroxyflavanonone; pinostrobin [34]; 7-methoxyflavanone [32]; and catechin [36]). In the case of catechin, it was observed that there is a better separation with a *n*-hexane/ethanol (Rs = 5.0) or n-hexane/2-propanol (Rs = 5.2) mobile phase, compared to a *n*-hexane/*t*-butanol (Rs = 1.3) mobile phase. [36] The Chiralcel OD column can also effectively separate and resolve naringin epimers during grapefruit maturation [37] and sour orange maturation, [38] as well as flavanone enantiomers. [33,39] In normal phase this column has also been utilized for the direct separation of epimers of the glycosides narirutin, hesperidin, neohesperidin, and naringin, [40] and the aglycones

naringenin, hesperetin, eriodictyol, and pinocembrin. [41] Recently, generic versions of the Chiralcel OD column have been produced and made commercially available such as the RegisCell column, which demonstrated an improved separation of flavanone enantiomers compared to Chirlacel OD at a significantly lower cost to the analyst. [33] The cellulose tris (3,5-dimethylphenylcarbmate) column Chiralpak IB has the advantage of being an immobilized chiral stationary phase instead of a silica gel supported stationary phase enabling a wider range of solvents to be employed as the mobile phase. It has been used to separate naringin enantiomers in pummelo (*Citrus grandis*), [42] as well as naringin and neohesperidin in Japanese Kampo formulations (Kijitsu, Kikoku and Tohi) which are used as crude drugs. [43] Recently, the stereoselective conjugation, transport and *in vitro* bioactivity of hesperetin enantiomers has been assessed by using the novel Chiral AGP-column that is an alpha1-acid glycoprotein (AGP) fixed on silica gel. [44]

A small clinical study involved the administration of the Chinese herbal medicines Daisiko-to and Shosaiko-to to human subjects and analyzed the urine post-administration. Several polysubstituted flavanones including liquiritigenin, naringenin, dihydrowogonin, and dihydrooroxylin A were resolved. [45-47] The Chiralcel OD-RH (tris-3,5-dimethylphenylcarbamate) column has demonstrated the ability to resolve naringenin enantiomers in an isocratic reverse phase in a validated assay in biological matrices. [48] The 2,3,4-tris-O-(3,5-dimethylphenylcarbamoyl) CSP demonstrated the ability to resolve flavanone. [49]The Chiralcel OC column (tris-phenylcarbamate) was shown to resolve flavanone as well as 4'-, 5-, and 6-methoxyflavanone and homoeriodictyol. [32] Finally, the Chiralcel OJ column (tris 4-methylphenyl-benzoate

ester) can resolve flavanone, [39] 4'-, 5-, and 6-methoxyflavanone, eriodictyol, and hesperetin. [32]

In addition, chiral columns employing amylose esters such as amylose tris (3,5-dimethylphenylcarbamate), [28] tris (3,5-dichlorophenylcarbamate) [28]and tris (5-chloro-2-methylphenylcarbamete) [50]supported on silica gel have demonstrated the ability to resolve flavanone. The amylose tris (3,5-dimethylphenylcarbmate) column Chiralpak IA has the advantage of being an immobilized chiral stationary phase instead of a silica gel supported stationary phase enabling a wider range of solvents to be employed as the mobile phase. Furthermore, it has also been shown to have the ability to resolve hesperidin, neohesperidin, narirutin, naringin, [40]flavanone, [51]catechin, [52] and epicatechin. [52] The cellulose tris(3,5-dichlorophenylcarbamate) polymer immobilized on silica column Chiralpak IC has been reported to separate hesperidin and naringin from the butanol extract of *Launeae arborescens* (Asteraceae). [53] Another commercially available column is the Sepapak-3 that is an amylose tris(5-chloro-2-methylphenylcarbamate) column. It has been reported to marginally separate flavanone enantiomers. [33]

Chiralpak OP (+) columns are based on macroporous silica gel coated with poly (diphenyl-2-pyridylmethylmethacrylate). The separation of flavanone, 5-, 6-, and 4'-methoxyflavanone were achieved on this column. [34] ChiraSpher is a small-pore silica gel chiral polymer (poly-N-acryloyl-(S)-phenylalanine ethyl ester). Using this, the separation of flavanone, 2'-, 4'-, and 6-hydroxyflavanone, 4'-, 5-, and 6-methoxyflavanone, and pinostrobin have been described; although naringenin and naringenin tribenzoate were not able to be separated. [34] The use of a Chiralpak AS-H

(tris (S)-1-phenylethylcarbamate) to separate naringenin, eriodictyol, hesperetin, and pinocembrin has recently been reported.[41] Furthermore, a Chiralcel AD column has also been reported to separate naringenin[41] and flavanone.[39]

### *1.5.2 Cyclodextrin Columns*

Cyclodextrins are cyclic oligomers of α-D-glucose bonded through α-(1,4) linkages. Within this group of CSP columns, there is another group that consists of β-cyclodextrin bonded phase type columns, from which the silica-supported cyclodextrin columns are available. Cyclobond I is a β-cyclodextrin column made up of cyclic glucoamyloses that have been found to separate flavanone, 2'- and 6-hydroxyflavanone as well as the 4'- and 6-methoxyflavanone.[34] Acetylating the 3-hydroxylgroups on the mouth of the cyclodextrin molecule introduces further binding sites and an acetylated Cyclobond I column can resolve several flavanones including: flavanone, 2'- and 6-hydroxyflavanone as well as 6-methoxyflavanone.[34] Additionally, the Cyclobond I column can resolve several flavanone glycosides including prunin, naringin, narirutin and neohesperidin. Of those, the flavanones with 7-O-neohesperidose sugars attached were better resolved (i.e. naringin and neohesperidin).[54] Recently, Cyclobond I 2000 has been utilized to baseline separate naringin, neohesperidin, and separate narirutin and hesperidin.[55] Furthermore, the Cyclobond I 2000 column was also used to separate catechin enantiomers from cocoa, as well as chemically synthesized enantiomers of 3'-O-methyl-catechin and 4'-O-methyl-catechin.[56] In another study, this same column was also used to separate catechin and epicatechin enantiomers from cocoa using ulta-performance liquid chromatography (UPLC).[57]

Columns utilizing cyclodextrin bonded silica as well as cellulose-coated silica gel have been successfully employed. Silica coated with a 2-hydroxy-3-methacryloyloxypropyl β-cyclodextrin-co-N-vinylpyrrolidone copolymer has been successfully utilized in reverse phase mode to resolve flavanone and monosubstituted flavanones such as 6- and 7-methoxyflavanones and 6-hydroxyflavanone. [58] Ureido-bonded methylated β-cyclodextrin CSP columns can also separate flavanone; 5-, 6- and 7-methoxyflavanone; hesperetin; naringenin; and taxifolin. [59]

New dichloro-, dimethyl- and chloromethylphenylcarbamate derivatives of α, β, γ-cyclodextrin were prepared as CSPs using normal phase liquid chromatography. Columns utilizing these CSPs resolved flavanone. In particular 2,5- and 3,4-dichlorophenylcarbamates of β-cyclodextrin based CSPs provided better resolution than dimethylphenylcarbamate derivatives. [60] Catechin enantiomers were separated using a PM-γ-cyclodextrin column with a coulometric electrode-array detection (CEAD) system coupled with HPLC. This method was applied to the stereospecific characterization of catechin and its sulfated and glucuronidated metabolites in human plasma after cocoa consumption. [61] Enantioseparation of various flavanones on mono ($6^A$-*N*-allylamino-$6^A$-deoxy) permethylated β-cyclodextrin (MeCD) covalently bonded to silica gel in the reverse phase has been reported. [62] More recently column coupling with achiral reverse phase chromatography has been utilized to separate the flavanone glycosides. This technique involves using a β-cyclodextrin column coupled with negative ion electrospray ionization mass spectrometry. This has been utilized to separate and detect eriocitrin, hesperidin, and neohesperidin enantiomers, and can be applied to their analysis in citrus fruit juices. [63] Recently, a phenyl-carbamate-propyl-β-cyclodextrin stationary phase was

employed for the separation of various flavanones by using nano-liquid chromatography (nano-LC) using various mobile phases of water and acetonitrile or water and methanol. [64] Good separation (Rs = 1.17 – 3.28) was achieved for flavanone, 7-, 6- and 4'-hydroxyflavanone, 7-, 6- and 4'-methoxyflavanone, hesperetin, naringenin and hesperidin [64], while poor resolution was observed for 2'hydroxyflavanone and naringin. [64] This method was applied for the quantification of hesperetin enantiomers in human urine after ingestion of blood orange juice; however, no pharmacokinetic modeling or interpretation was included in the paper. [65] Recent publications have employed Cu (I) catalytic 1,3-dipolar cycloadditions, also called "click" chemistry, in order to make "click" chemistry derived cyclodextrin chiral stationary phases (CSPs) that are tethered to silica via a single triazole linkage. [66] Various CSPs were developed by using either native cyclodextrin (CCN-CSP), [67] mono-6-azido-perphenylcarbamated-β-CD (CCP-CSP) and mono-6-azido-permethylated-β-CD (CCM-CSP), [68] or heptakis(6-deoxy-6-azido)-β-CD (heptakis-$N_3$-CD) and heptakis(6-deoxy-6-azido-phenylcarbamoylated)-β-CD (heptakis-$N_3$-Ph-CD). [66] Flavanone (Rs = 1.31 – 2.26) and 4'-hydroxy-flavanone (Rs = 1.73 – 3.08) were separated effectively by HPLC with the CCN-CSP and various methanol/water and water/acetonitrile mobile phases. [67] Similarly, 7-methoxyflavanone (Rs = 1.52) was separated under a methanol/water mobile phase by using CCM-CSP, while flavanone (Rs = 3.24), 7-methoxyflavanone (Rs = 2.27), 6-methoxyflavanone (Rs = 1.87) and 5-methoxyflavanone (Rs = 1.18), 4'-hydroxyflavanone (Rs = 1.98) and 6-hydroxyflavanone (Rs = 1.37) were separated by using CCP-CSP. [68] On the other hand, 5-methoxyflavanone (Rs = 1.65) was separated using the heptakis-$N_3$-CD column, while flavanone (Rs = 2.54), 6-methoxyflavanone (Rs = 2.12), 7-methoxyflavanone (Rs =

2.91), 4'-hydroxyflavanone (Rs = 1.51) and 6-hydroxyflavanone (Rs = 1.34) were separated using the heptakis-$N_3$-Ph-CD column. [66]

## 1.6 Additives to Chiral Mobile Phases

The addition of an optically active molecule to the mobile phase can facilitate separation of enantiomers on conventional stationary phases. Separation of flavonoids through the addition of cyclodextrins to the mobile phase is a rational approach given the effectiveness of CSP cyclodextrin columns. The interaction of the chiral additive with the enantiomers facilitates the formation of transient diastereomers. This is in contrast to CSP's, where this change happens via the interaction with the column stationary phase. As discussed previously, diastereomeric pairs have different physicochemical properties and thus may distribute differentially between the adsorbing achiral stationary phase and the organic mobile phase. Capillary electrophoresis, a separation technique using small volumes, can be operated in various modes. For example, the separation of several flavanone-7-O-glycosides (naringin, prunin, narirutin, hesperidin, neohesperidin, and eriocitrin) by chiral capillary electrophoresis was accomplished by a variety of cyclodextrin mobile phase additives in borate buffer at a pH range of 8-10. [69] There is no generally applicable cyclodextrin for flavonoid glycoside separation and therefore assays must be developed individually; however, naturally occurring β and γ cyclodextrin and neutral cyclodextrin derivatives such as DM-β-cyclodextrin, HP-β-cyclodextrin, [69] randomly sulfated-substituted β-cyclodextrin (S-β-CD) or dual cyclodextrin (S-β-CD and neutral CD), [70] and charged derivatives CM-β-cyclodextrin and CE-β-cyclodextrin were all successful as chiral selectors. [69] These methods were subsequently applied to examine flavanone-7-O-glycosides in citrus fruit, [71] and more recently have been applied for the

baseline separation of 2'hydroxyflavanone enantiomers from racemic mixtures. [70] In this investigation, 2'hydroxyflavanone enantiomers were separated by using different S-β-CD concentrations and by using a dual cyclodextrin approach, combining S-β-CD and β-CD or S-β-CD and γ-CD. [70] However, 3'- and 4'-hydroxyflavanone were not separated. Catechin and epicatechin enantiomers have also been studied in human plasma following green tea ingestion by using β-CD as the background electrolyte in capillary electrophoresis coupled with a high-sensitivity UV cell. [72] Separation of some chiral flavanones by micellar electrokinetic chromatography (MEKC) has also been accomplished. [73,74] In this study, γ-cyclodextrin (γ-CD) and sodium cholate were used as chiral mobile phase additives. Sodium cholate when used above at critical micelle point concentration forms chiral micelles and was effective at separating flavanone glycosides due to a sugar micelle interaction, while the use of cyclodextrin was more effective in separating flavanone aglycones.

A recent study [75]demonstrated the stereospecific separation of many flavanones and flavanone-7-O-glycosides with capillary electrophoresis by adding cyclodextrins or cyclodextrin derivatives as chiral selectors to the background electrolyte. Separation of several flavanone glycosides and aglycones including eriocitrin, hesperidin, hesperetin, naringin, naringenin, narirutin, neohesperidin, flavanone, 2'- and 6'-hydroxyflavanone, and 6-methoxyflavanone in citrus fruit juices was accomplished by capillary electrophoresis using sulfobutyl ether β-cyclodextrin as the chiral selector. [76]

Using MEKC, the glycoside neohesperidin was baseline separated while naringin was not. For the aglycones examined, the best resolution was for hesperetin although again baseline resolution was not achieved. [73] A more recent investigation demonstrated

that micellar electrokinetic chromatography with a) sodium cholate or b) sodium cholate plus cyclodextrins or cyclodextrin derivatives or c) sodium dodecyl sulfate (SDS) plus cyclodextrin or cyclodextrin derivatives as chiral surfactants/selectors can be employed for the epimeric separation of flavanone 7-O-glycosides. [74]

## 1.7 Chiral Derivatization Techniques and Indirect Analysis

The HPLC separation of flavanone glycosides was initially accomplished 30 years ago in the 1980's. [77] It was suggested that both naringin and narirutin could be acetylated with equal portions of pyridine and acetic anhydride and resolved at low temperatures 0-5˚ C. [77] In the mid-1980's there was some initial separation of prunin (naringenin-7-O-glucoside) using benzoylated derivatives to separate the epimers in *Prunus callus* (sweet cherries), [78] oranges, and grapefruit. [79] The separation of prunin benzoate and naringin benzoate on Cyclobond I columns has also been reported. [54] There is also a mention in the literature of derivatization of naringenin to naringenin tribenzoate and subsequent separation on a Chiralcel OD column. However, naringenin enantiomers were not resolved, suggesting that the hydroxyl groups present in naringenin hinder chiral recognition on this stationary phase. [34] Derivatization of flavonoids is generally not necessary and has not been widely employed. Alternative direct methods of analysis previously discussed have been readily and successfully employed.

## 1.8 Current Methods Pros and Cons

Certainly, all methods of chiral analysis will have certain advantages and disadvantages. Some disadvantages might include long run times that make routine analysis of large volumes of samples impractical. Still another barrier is the fact that many columns and methods that have shown stereospecific separation are not yet

commercially available. The selection of commercial chiral columns is increasing, however, the costs of the columns can also be prohibitive and the mobile phase composition can be rather limited with CSP's. Some CSP columns can only be used with non-aqueous solvents and this requires judicious removal of water from biological samples to retain optimal column efficiency.

Nevertheless, the ultimate advantage of chiral separation methods over achiral methods includes a more thorough understanding of the stereoselective pharmacokinetics of flavanones and the determination of safe and effective dosing regimens. Many publications report the applicability of CSPs to resolve different chiral flavanones; however, there are still comparatively few published and validated chiral assays in biological matrices.

## 1.9 Flavonoid Pharmacokinetic Study Methodology

The use of rats in studying the pharmacokinetic profile of flavonoids has been well established. The following is an example of a typical pharmacokinetic study protocol.

1. After rats have "stabilized" for 3-7 days after arrival, they will undergo surgical placement of an indwelling catheter in the jugular vein. The catheter will be externalized at the nape of the neck to allow for easy access.
2. One day following the catheter placement rats will be moved to metabolic cages and remain housed there till the end of the study (up to 120 hours).
3. Rats are then weighed and the dose calculated.
4. Baseline samples of serum and urine will then be collected.

5. Blood will be drawn (0.25 mL) via the jugular vein catheter and replaced with saline then centrifuged to provide serum for analysis.
6. Urine will be collected via the metabolic cage urine collection cup.
7. A single dose of the study flavonoid is administered, depending on the study group (intravenous pharmacokinetics or oral pharmacokinetics), via intravenous injection through jugular vein catheter, or oral gavage.
8. Sample Collection:
    a. For studies featuring intravenous administration, blood sample times are typically as follows:
    b. 0h, 1min, 1h, 2h, 4h, 6h, 12h, 24h, 48h, 72h
    c. For studies featuring oral administration, blood sample times are as follows:
    d. 0h, 30min, 1h, 2h, 4h, 6h, 12h, 24h, 48h, 72h
    e. Urine sampling times for both intravenous and oral studies are as follows:
    f. 0h, 2h, 6h, 12h, 24h, 48h, 72h

## 1.10 *In Vitro* Pharmacodynamic Screening Assay Methodology

To further characterize flavonoids which may pharmacologically contribute to the health benefits of fruits and vegetables 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, pinostrobin, and liquiritigenin, 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 any reported activity. Assays exhibiting positive dose response curves will be

selected to further investigate the stereospecific contribution of flavonoid enantiomers to pharmacological activity.

## 1.11 Flavonoids of Interest

### *1.11.1 Liquiritigenin*

(+/-) Liquiritigenin (4',7-Dihydroxyflavanone) is a chiral flavonoid present in licorice. It is a potent bioactive compound, demonstrating anti-cancer and hepatoprotective activity as well as attenuation of the acute effects of cocaine administration. [80-82] Still others have shown liquiritigenin to be an estrogen receptor beta agonist. [83] These properties highlight the potential application of liquiritigenin, or products containing liquiritigenin, in human health.

The pharmacokinetics of liquiritigenin via achiral high performance liquid chromatography (HPLC) methods have been studied extensively. [84-88] However, very few studies have been published on the chiral separation of liquiritigenin. [47,89]

One study reported that liquiritigenin was resolved into its respective enantiomers on a Chiralcel OD column in normal phase HPLC and its presence was detected in post-administrative urine of patients administered herbal medicines predominantly as the S enantiomer. [47] Fliegmann, *et al.* reported a reversed-phase HPLC method that successfully separated liquiritigenin using a Chiralpak® IA column. [89] However, no studies applying a developed method of detection to stereospecific quantification in pre-clinical pharmacokinetic analysis exist.

### *1.11.1 Pinocembrin*

Pinocembrin, also known as 5,7-dihydroxyflavanone or dihydrochrysin is also a chiral flavonoid. As with several other flavonoids, it has been shown to have anti-inflammatory, anti-oxidant, neuroprotective, anti-tumor, and anti-microbial activity. [90-95] The compound is found in significant quantities in various plant species used in traditional medicine, including *Cleome droserfolia*, *Alpinia galangal*, and *Boesenbergia pandurata*. [91,96,97] It has also been identified as an active ingredient in the traditional medicine propolis. [98] Propolis is a resinous substance collected by bees from plants. It is used in the beehive as a sealant; both for interior walls and to encase dead intruding organisms, thereby avoiding decomposition. [99] Of the traditional medicines containing pinocembrin currently in use, propolis is by far the most common. Chemical composition of propolis can vary depending on the geographic region from which it is harvested. [99,100] However, pinocembrin has been shown to be the most abundant flavonoid present in propolis. [101] Propolis is also present in many currently marketed dietary supplements consumed by a large portion of the Western population.

Determination of racemic pinocembrin via high performance liquid chromatography (HPLC) methods and its subsequent application in pharmacokinetic studies has recently been shown. [102-104] However, a paucity of studies have been published on the chiral separation of pinocembrin.

Pinocembrin enantiomers were separated on both Chiralcel OD and Chiralpak AS-H columns, although baseline resolution was not obtained. [41] It was also resolved under reverse and normal phase conditions on modified microcrystalline cellulose triacetate (MCCTA). [30] Finally, three commercially available columns of MCCTA were shown to

resolve pinocembrin. [31] Again, no studies exist showing the development of a chiral method of detection in biological matrices or application to pharmacokinetic studies.

### *1.11.2 Pinostrobin*

Pinostrobin also belongs to the growing group of naturally occurring chiral flavonoids being studied for its pharmacological activities. [105-108] Also known as pinocembrin-7-methyl ether or 5-hydroxy-7-methoxyflavanone, pinostrobin has been isolated in many plant species and traditional medicine products, including Thai ginger (Boesenbergia pandurata), propolis, and honey. [109] It is also a prevalent ingredient in many currently marketed botanical nutraceuticals. Determination of pinostrobin via achiral high performance liquid chromatography (HPLC) methods and its subsequent application in pharmacokinetic studies has recently been demonstrated. [110] However, few studies have been published on the chiral separation of pinostrobin.

One group succeeded in stereospecifically resolving pinostrobin with normal phase HPLC utilizing the Chiralcel OD and ChiraSpher column, however baseline resolution was only achieved with the Chiralcel OD column. [34] Another study achieved separation using γ-cyclodextrin as a mobile phase additive and micellar electrokinetic chromatography; however, baseline resolution was not obtained. [55]Finally, the enantiomeric separation of pinostrobin by capillary electrophoresis using various cyclodextrins as selectors demonstrated separation with the best resolution of Rs = 1.44 with methyl-γ-cyclodextrin. [74] Despite these initial attempts there have been no stereospecific analytical methods developed in biological matrices and no application to enantiomeric disposition and quantification of pinostrobin.

## 1.12 Conclusions

Over the last thirty years a number of methods and techniques have been developed for the analysis of chiral flavonoids by scientific researchers from a number of disciplines. The direct chromatographic approach has dominated this field of investigation with resolution being achieved through chiral polymer phases of oligosaccharides and their derivatives. Indirect derivatization methods have been very limited and mostly observational. There has been an increased use of chiral mobile phase additives in recent years often coupled to capillary electrophoresis. Since work began in this field in the 1980's there has been increased awareness and interest in developing the techniques to stereospecifically analyze chiral flavanoids. There remains a lack of stereospecific assays published in the literature for many chiral flavanones. There also remains very few validated stereospecific assays in biological matrices for the majority of compounds in this class. Quality work in these areas is necessary to verify and optimize any beneficial effects found in *in vitro* bioactivity assays. Additionally, it is necessary to identify and prevent any toxicity in the growing population of nutraceutical product consumers.

## 1.13 Organization of Thesis

The hypothesis of the project follows: pinocembrin, pinostrobin, and liquiritigenin may have stereospecific differences in formulation content, pharmacokinetic disposition and pharmacodynamic behavior that may affect their pharmacological activity and suitability as potential therapeutic agents. The following objectives were undertaken to test the hypothesis: develop stereospecific HPLC or LC-MS detection methods for flavonoids of interest, assess stereospecific content of flavonoids of interest in selected

natural health products, perform stereospecific pharmacokinetic studies of intravenously administered flavonoids in the rat, and evaluate stereospecific pharmacodynamics of flavonoids of interest *in vitro.* The thesis is organized into a "sandwich thesis" (manuscripts within a thesis) with chapters 1 and 8 written to introduce and summarize the research projects contained within chapters 2 – 7. Chapter 1 is derived from a book chapter on which I am an author. Chapters 2 – 5 are published papers on which I am the first author. Chapters 6 and 7 are papers on which I am the first author which are about to be published.

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# Chapter II: Chiral Analytical Method Development and Application to Pre-Clinical Pharmacokinetics of Pinocembrin

## 2 Abstract

An analytical method enabling the detection and quantification of the individual enantiomers of racemic (+/-) pinocembrin is required to fully characterize its pharmacokinetic disposition. Direct resolution of the enantiomers of pinocembrin was achieved using a novel and simple reversed-phase high-performance liquid chromatography method with electrospray ionization and detection by mass spectrometry in rat serum. A Chiralcel® AD-RH column was employed to perform baseline separation with electrospray positive-mode ionization with selected ion monitoring detection. The standard curves were linear ranging from 0.5 to 100 µg/ml for each enantiomer. Limit of quantification was 0.5 µg/ml. The assay was applied successfully to stereoselective serum disposition of pinocembrin enantiomers in rats. Pinocembrin enantiomers were detected in serum. Both enantiomers had a serum half-life of ~15 minutes in rats. Similar values of volume of distribution between the enantiomers were also observed (Vd) 1.76 L/kg for S-pinocembrin and 1.79 L/kg R-pinocembrin. Total clearance ($CL_{total}$) was 5.527 L/h/kg for S-pinocembrin and 5.535 L/h/kg for R-pinocembrin, and area under the curve ($AUC_{0\text{-}inf}$) was 1.821 µg h/ml for S-pinocembrin and 1.876 µg h/ml for R-pinocembrin. The large volume of distribution coupled with the short serum half-life suggests extensive distribution of pinocembrin into the tissues.

## 2.1 Introduction

Pinocembrin, also known as 5,7-dihydroxyflavanone or dihydrochrysin, is a chiral flavonoid. As with many other flavonoids it has been shown to have anti-inflammatory, anti-oxidant, neuroprotective, anti-tumor, and anti-microbial activity.[1–4] Pinocembrin is also present in many currently marketed dietary supplements, in particular propolis, which is consumed by a large portion of the Western population.

Achiral determination of pinocembrin via high performance liquid chromatography (HPLC) methods and its subsequent application in pharmacokinetic studies has recently been demonstrated.[5–7] However, an often-overlooked salient feature in the analysis of pinocembrin is its chirality. A paucity of studies has been published on the chiral separation of pinocembrin.[8–11] It has long been known that differences in the disposition and activity of individual stereoisomers exist; and that these differences can cause significant, sometimes harmful effects in humans.[12]

Pinocembrin has been separated on both Chiralcel OD and Chiralpak AS-H columns in normal phase, although baseline resolution was not obtained.[8] It could also be resolved under reversed and normal phase conditions on modified microcrystalline cellulose triacetate (MCCTA).[9] Other work has shown that three commercially available columns of MCCTA were able to resolve pinocembrin.[10] Pinocembrin enantiomers have been successfully synthesized by two groups with one group showing resolution by derivatizaton and separation of the intermediate diasteromers and the other suggesting stereospecific synthesis via a biosynthetic gene cluster.[11,13] There remains a paucity of chiral studies in the literature studying pinocembrin. The previous studies, although valuable, have methodologies that lack in several respects including: no method validation, no baseline resolution, no

presence of an internal standard, and lack of application to biological matrices for pharmacokinetic studies. The current direct method of enantioseparation is uniquely valuable for pharmacokinetic studies and isolation of pure enantiomers for further pharmacological testing as well as large-scale purification.

Importantly, this study is the first to develop a direct stereoselective, isocratic, reversed-phase, validated HPLC assay for detection of pinocembrin in rat biological matrices with an internal standard and application to a pharmacokinetic study.

## 2.2 Experimental

### *2.2.1 Chromatographic system and conditions*

A high performance liquid chromatographic-electrospray ionization-mass spectrometry (LC-ESI-MS) system was employed. The Shimadzu LCMS-2010 EV system (Kyoto, Japan) consisted of two LC-20AD pumps, a SIL-10AD VP auto injector, a SPD-10A VP UV detector, and a SCL-10A VP system controller. Data analysis was accomplished using Shimadzu LCMS Solutions Version 3 software. The analytical column used was a Chiralcel® AD-RH (150mm × 4.6mm i.d., 5-µm particle size, Chiral Technologies Inc. West Chester, PA, USA). The mobile phase consisted of methanol and acetic acid (100:0.1M v:v) filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1°C), and a flow rate of 0.5 ml/min. Pinocembrin enantiomers and the internal standard (7-ethoxycoumarin) were monitored in SIM positive mode at m/z 255 and m/z 191 respectively.

*2.2.2 Stock and working standard solutions*

Twenty-five milligrams of racemic pinocembrin (Apin Chemicals LTD, Abingdon, Oxon, UK) were accurately weighed on an analytical balance (AG245, Mettler) and dissolved in 250 mL of methanol in a volumetric flask to make a stock standard solution with a concentration of 100 μg/ml. These solutions were protected from light and stored at −20°C between uses, for no longer than 3 months. Calibration standards in blank rat serum were prepared daily from the stock solution of pinocembrin by serial dilution, yielding the following concentrations: 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml.

*2.2.3 Sample preparation*

To the working standards or samples (0.1 mL), 25 μL of 7-ethoxycoumarin (internal standard, IS) solution (100 μg/mL) was added into 2.0 ml Eppendorf tubes. Next, 1 mL of cold acetonitrile (−20°C) was added, immediately followed by 1 min of vortexing (Vortex Genie-2, VWR Scientific, West Chester, PA, USA). Samples were then centrifuged at 5,000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was collected into 2.0 ml Eppendorf tubes and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 200 μL of mobile phase, vortexed for 30 seconds and centrifuged at 5,000 rpm for 5 min. The supernatant was then transferred to HPLC vials and 10 μL was injected into the HPLC system.

*2.2.4 Pharmacokinetic disposition of pinocembrin in rats*

Male Sprague-Dawley rats (n=3, average weight of 250 g) were anaesthesized using isoflurane and a silastic catheter was cannulated into the right jugular vein. Rats

were dosed intravenously with 10 mg/kg racemic pinocembrin in 2% DMSO and polyethylene glycol (PEG) 600. A series of blood samples (0.30 ml) was collected at 0, 1, 15, 30 min, and at 1, 2, 4, 6, 24, 48, 72, 96, and 120 h. Following centrifugation of the blood samples in microcentrifuge tubes, serum was collected and stored at –20°C until analysis. Ethics approval for animal experiments was obtained from the University of Manitoba Office of Research Ethics and Compliance.

### *2.2.5 Data analysis*

Quantification was based on calibration curves constructed using peak area ratio (PAR) of pinocembrin to internal standard, against pinocembrin concentrations using unweighted least squares linear regression. Pharmacokinetic analysis was performed using data from individual rats for which the mean and standard error of the mean (S.E.M.) were calculated for each group. Pharmacokinetic modeling was completed using WinNonlin® software (Ver. 5.1). Non-compartmental pharmacokinetic methods were used to calculate half-life ($t_{1/2} = 0.693/ K_E$), clearance (CL by dividing dose by $AUC_{0-\infty}$) and volume of distribution ($Vd_\beta$ by dividing CL by $K_E$). Reported final calculation of $Vd_{ss}$ was found with CL x $MRT_{iv}$.

## 2.3 Results and Discussion

### *2.3.1 Chromatography*

Separation of pinocembrin enantiomers and the internal standard, 7-ethoxycoumarin, in serum was achieved successfully. There were no interfering peaks co-eluting with the peaks of interest (Figure 2.1). The retention times of S and R-pinocembrin were approximately 12 and 17 min respectively. The internal standard 7-ethoxycoumarin eluted at approximately 7.5 minutes.

The performance of the LC/MS assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in serum, linearity, lack of matrix effects and limit of quantitation (LOQ). Various compositions of mobile phase were tested to achieve the best resolution between pinocembrin enantiomers. Optimal separation was achieved with methanol and acetic acid 100:0.1M (v/v) with a flow rate of 0.5 ml/min.

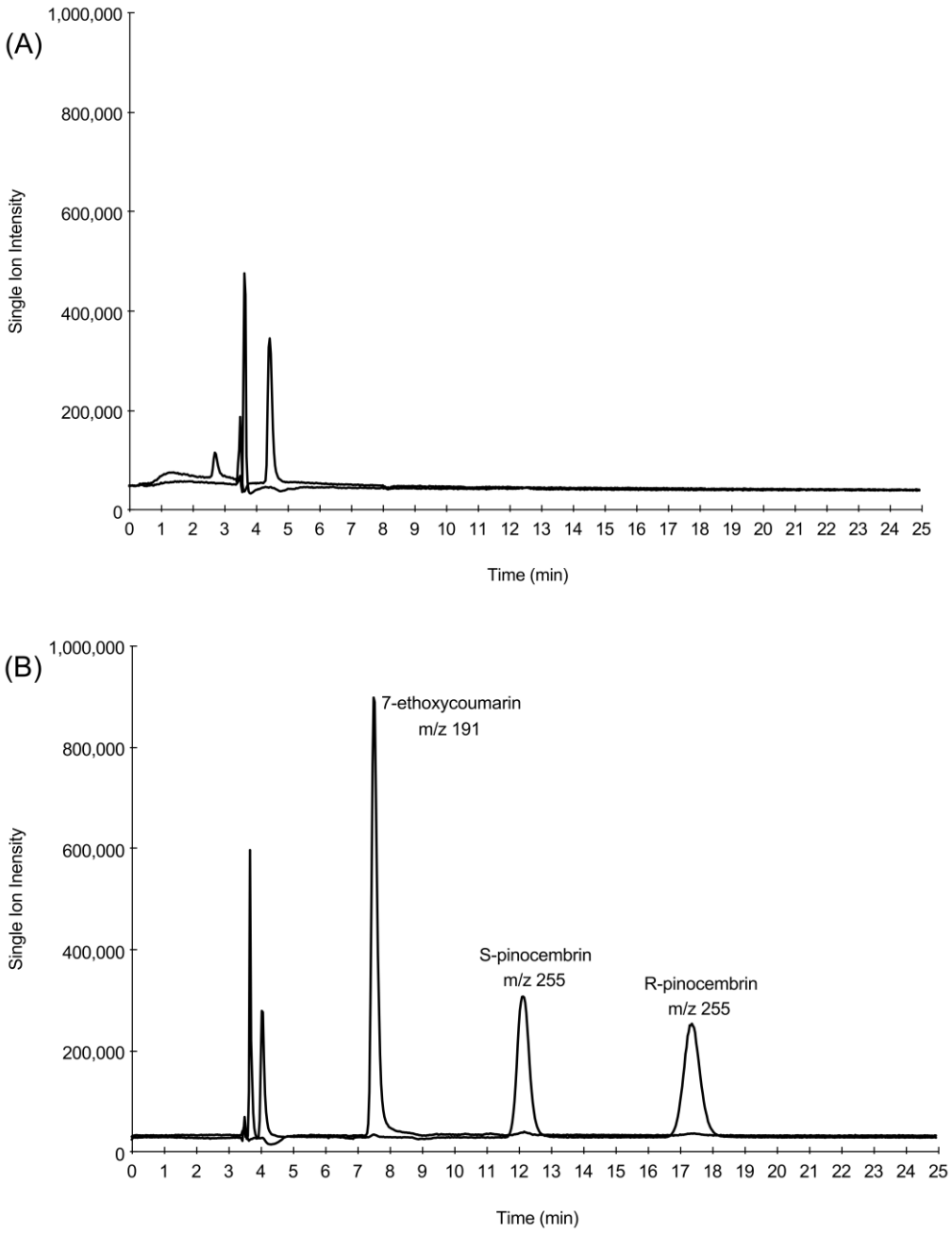


**Figure 2.1.** Representative chromatograms of (A) drug-free serum demonstrating no interfering peaks coeluted with the peaks of interest; (B) serum containing pinocembrin enantiomers each with a concentration of 10 μg/mL and the internal standard, 7-ethoxycoumarin.

*2.3.2 Method validation*

Precision and accuracy of the assay was < 15% (RSD) and was within 15% for all points on the calibration curve in accordance with the US Food and Drug Administration guidelines. Linear relationships ($r^2$ = 0.998) were demonstrated between PAR of S- and R-pinocembrin to the internal standard and the corresponding serum concentrations of pinocembrin enantiomers over a range of 0.5 to 100 μg/ml. The mean regression lines from the validation runs are described as S-pinocembrin (μg/ml) = 0.0393*x* + 0.0072 ($r^2$=0.99996) and R-pinocembrin (μg/ml) = 0.0365*x* + 0.0154 ($r^2$=0.99937). The LOQ of this assay was 0.5 μg/ml in biological fluids.

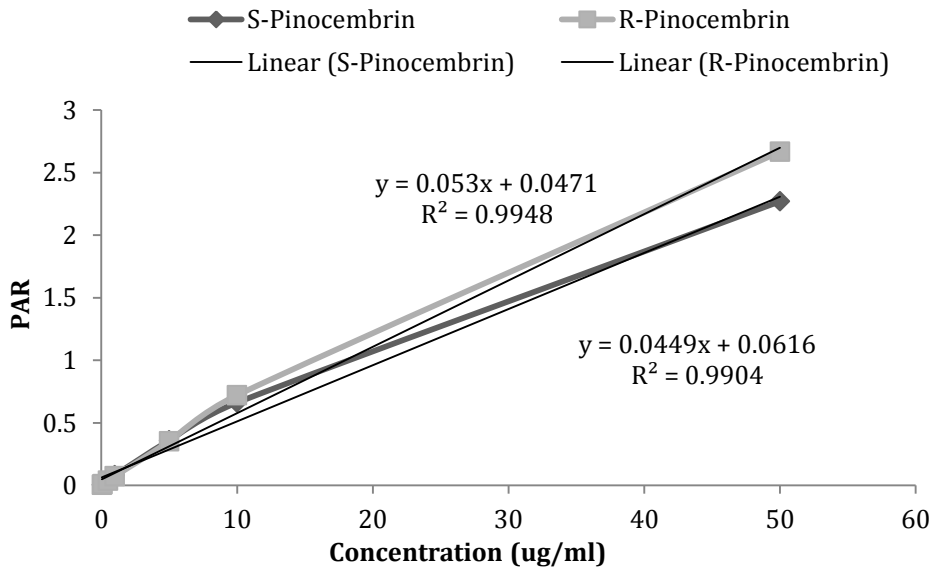


**Figure 2.2.** Standard curve of S-pinocembrin and R-pinocembrin in blank rat serum showing correlation coefficients and trendline.

*2.3.3 Stereospecific pharmacokinetics of pinocembrin in rats*

Pinocembrin enantiomers were detected in serum. Both enantiomers had a serum half-life of ~15 minutes. They also shared similar values of volume of distribution

(Vd; S-pinocembrin = 1.76 L/kg and R-pinocembrin = 1.79 L/kg), total clearance ($CL_{total}$, S-pinocembrin = 5.527 L//h/kg and R-pinocembrin = 5.535 L/h/kg), and area under the curve ($AUC_{0-\infty}$; S-pinocembrin = 1.821 µg h/ml and R-pinocembrin = 1.876 µg h/ml). The large volume of distribution coupled with the short serum half-life suggests extensive distribution of pinocembrin into tissues.

| Parameter | S-pinocembrin (Mean ± SEM) | R-pinocembrin (Mean ± SEM) |
|---|---|---|
| $AUC_{0-\infty}$ (hr*ug/mL) | 1.821 ± 0.21 | 1.876 ± 0.43 |
| Vd (L/kg) | 1.758 ± 1.313 | 1.793 ± 0.81 |
| $Cl_{total}$ (L/h/kg) | 5.527 ± 0.64 | 5.535 ± 1.217 |
| $t_{1/2}$ serum (h) | 0.21 ± 0.14 | 0.223 ± 0.08 |

**Table 2.1.** Pharmacokinetic Properties of Pinocembrin in the Rat

## 2.4 Conclusions

In summary, the developed direct HPLC method for pinocembrin is stereospecific. It has been applied successfully in the study of the pharmacokinetics of pinocembrin in rats for the first time. The HPLC method presented here has also been used in our laboratory in the determination of urinary excretion of pinocembrin as well as stereospecific quantification of pinocembrin in natural health products and for isolation of pure enantiomers for pharmacological testing (unpublished data). Further studies are on going in our laboratory to further characterize the pharmacometric profiles of pinocembrin as well as other flavonoid enantiomers.

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# Chapter III: Stereospecific Analytical Method Development and Preliminary *in vivo* Pharmacokinetic Characterization of Pinostrobin in the Rat

## 3 Abstract

The complete pharmacokinetic disposition of the chiral flavonoid (+/-) pinostrobin remains unknown without the development of an analytical method of detection and quantitation of its individual enantiomers. Resolution of the enantiomers of pinostrobin was achieved using as simple high-performance liquid chromatographic method. A Chiralpak® AD-RH column was employed to perform baseline separation with UV detection at 287 nm. The standard curves were linear ranging from 0.5 to 100 μg/mL for each enantiomer. The limit of quantification was 0.5 μg/mL. Precision and accuracy of the assay was < 15% (RSD) and was with a bias <15% for all points on the calibration curve. The assay was applied successfully to stereoselective serum disposition of pinostrobin enantiomers in rats. Both enantiomers had a serum half-life of ~7 hours. They also shared similar values of volume of distribution ($V_d$ *S*-pinostrobin = 8.2 L/kg; $V_d$ *R*-pinostrobin = 8.9 L/kg), total clearance (*S*-pinostrobin $CL_{total}$ = 0.96 L//h/kg; *R*-pinostrobin $CL_{total}$ = 1.055 L//h/kg), and area under the curve (*S*-pinostrobin $AUC_{inf}$ = 23.16 μg h/mL; *R*-pinostrobin $AUC_{inf}$ = 21.296 μg h/mL). The large volume of distribution suggests extensive distribution of pinostrobin into tissues.

## 3.1 Introduction

Pinostrobin is included in a growing group of naturally occurring flavonoids being studied for their pharmacological activities.[1-4] Also known as pinocembrin-7-methyl ether or 5-hydroxy-7-methoxyflavanone, pinostrobin has been isolated in many plant species and traditional medicine products; including Thai ginger (*Boesenbergia pandurata*), propolis, and honey.[5,6] It is also a prevalent ingredient in many currently marketed botanical nutraceuticals such as *Lindera reflexa* Hemsl, Weitengning tablets and falcate crazyweed.[7,9]

What sets pinostrobin apart from the many bioactive phytochemicals currently being investigated is its chirality. This salient molecular feature has often been overlooked in the field of phytochemical biomedical characterization. Determination of racemic pinostrobin via achiral high performance liquid chromatography (HPLC and LC-MS/MS) methods and its subsequent application in oral pharmacokinetic studies in rats has recently been demonstrated.[10] Other studies have also demonstrated excellent utility of HPLC methodology for pinostrobin determination.[6-9] However, a paucity of studies have been published on the chiral separation of pinostrobin.[11-13]

One group succeeded in stereospecifically resolving pinostrobin with normal phase HPLC utilizing the Chiralcel® OD and ChiraSpher® column. However, baseline resolution was only achieved with the Chiralcel® OD column. No further method development or application has been reported.[11] Another study achieved separation using γ-cyclodextrin as a mobile phase additive and micellar electrokinetic chromatography; however, baseline resolution was not obtained.[12] Finally, the enantiomeric separation of pinostrobin by capillary electrophoresis using various cyclodextrins as selectors demonstrated separation with the best resolution of Rs =

1.44 with methyl-γ-cyclodextrin.[13] Despite these initial chromatographic attempts there have been no stereospecific analytical methods developed in biological matrices, no attempt to quantify the enantiomers, no extraction from biological matrices or use of an internal standard and no application to enantiomeric disposition.

This study is the first to develop a stereoselective, isocratic, reversed-phase HPLC assay of pinostrobin for detection in rat serum and application to a preliminary pharmacokinetic study.

## 3.2 Experimental

### *3.2.1 Chromatographic system and conditions*

The HPLC system used was a Shimadzu LC-2010A (Kyoto, Japan). Data collection and integration were accomplished using Shimadzu EZ Start 7.4 software (Kyoto, Japan). The analytical column used was a Chiralpak® AD-RH (150mm × 4.6mm i.d., 5-µm particle size, Chiral Technologies Inc. West Chester, PA, USA). The mobile phase consisted of acetonitrile and water (80:20 v:v) filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (22 ± 1°C), and a flow rate of 1.0 mL/min**,** with ultraviolet (UV) detection at 287 nm.

### *3.2.2 Stock and working standard solutions*

Twenty-five milligrams of racemic pinostrobin (Apin Chemicals LTD, Abingdon, Oxon, UK) were accurately weighed on an analytical balance (AG245, Mettler) and dissolved in 250 mL of methanol in a volumetric flask to make a stock standard solution with a concentration of 100 µg/ml. These solutions were protected from light

and stored at −20°C between uses, for no longer than 3 months. Calibration standards in blank rat serum were prepared daily from the stock solution of pinostrobin by serial dilution, yielding the following concentrations: 0.05, 0.1, 0.5, 1.0, 5.0, 10.0, 25.0, 50.0 and 100.0 μg/ml.

### *3.2.3 Sample preparation*

To the working standards or samples (0.1 mL), 25 μL of trans-stilbene (internal standard, IS) solution (100 μg/mL) was added into 2.0 ml Eppendorf tubes. Next, 1 mL of cold acetonitrile (−20°C) was added, immediately followed by 1 min of vortexing (Vortex Genie-2, VWR Scientific, West Chester, PA, USA). Samples were then centrifuged at 5,000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was collected into 2.0 ml Eppendorf tubes and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 200 μL of mobile phase, vortexed for 30 seconds and centrifuged at 5,000 rpm for 5 min. The supernatant was then transferred to HPLC vials and 100 μL was injected into the HPLC system.

### *3.2.4 Pharmacokinetic disposition of pinostrobin in rats*

Male Sprague-Dawley rats (*n*=3, ~250 g) were anaesthetized using isoflurane and a silastic catheter was cannulated into the right jugular vein. The animals were placed in metabolic cages where they were allowed to recover and fasted overnight before dosing. On the day of experiment, the rats were dosed intravenously 20mg/kg racemic pinostrobin in 3% DMSO/polyethylene glycol 600. A series of blood samples (0.30 mL) was collected at 0, 1, 15, 30 min, and at 1, 2, 4, 6, 24, 48, 72, 96, and 120 h. The cannula was flushed with 0.30 mL saline after each sample collection. Following

centrifugation of the blood samples in 2.0 mL Eppendorf microcentrifuge tubes, serum was collected and stored at –20°C until analysis. Ethics approval for animal experiments was obtained from the University of Manitoba Office of Research Ethics and Compliance.

### *3.2.5 Data analysis*

Quantification was based on calibration curves constructed using peak area ratio (PAR) of pinostrobin enantiomers to IS, against pinostrobin concentrations using unweighted least squares linear regression. Pharmacokinetic analysis was performed using data from individual rats for which the mean and standard error of the mean (S.E.M.) were calculated for each group. The elimination rate constant ($K_E$) was estimated by linear regression of the serum concentrations in the log-linear terminal phase. In order to estimate the serum concentrations ($C_0$) immediately after racemic pinostrobin IV dosing, a two-compartmental model was fitted to the serum concentration versus time data using WinNonlin® software (Ver. 5.1). The estimated $C_0$ was then used with the actual measured serum concentrations to determine the area under the serum concentration-time curve (AUC). The $AUC_{0-\infty}$ was calculated using the combined log-linear trapezoidal rule for data from time of dosing to the last measured concentration, plus the quotient of the last measured concentration divided by $K_E$. Non-compartmental pharmacokinetic methods were used to calculate clearance (CL by dividing dose by $AUC_{0-\infty}$) and volume of distribution ($Vd_\beta$ by dividing CL by $K_E$). Reported final calculation of $Vd_{ss}$ was found with CL x $MRT_{iv}$.

## 3.3 Results and Discussion

### *3.3.1 Chromatography*

Separation of pinostrobin enantiomers and IS in biological fluids was achieved successfully (Figure 3.1). There were no interfering peaks co-eluted with the peaks of interest (Figs. 3.1 B, and 3.1 C). The retention times of *S*- and *R*-pinostrobin were approximately 8 and 12 minutes, respectively. The IS eluted at approximately 6 minutes (Fig. 3.1 A).

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in biological fluid, linearity, and limit of quantitation (LOQ). Various compositions of mobile phase including methanol, acetonitrile, and water were examined to achieve the best resolution between pinostrobin enantiomers. The optimal separation was achieved when the combination of acetonitrile and water 80:20 (v/v) with the flow rate of 1 mL/min at a UV wavelength maximum of 287 nm was employed. The present assay is practical to use in pre-clinical applications of pinostrobin analysis in which small sample volumes are obtained and has a very short run time (< 15 min). This assay could also be utilized to preparatively scale up and produce and isolate pure enantiomers for further pharmacological testing.

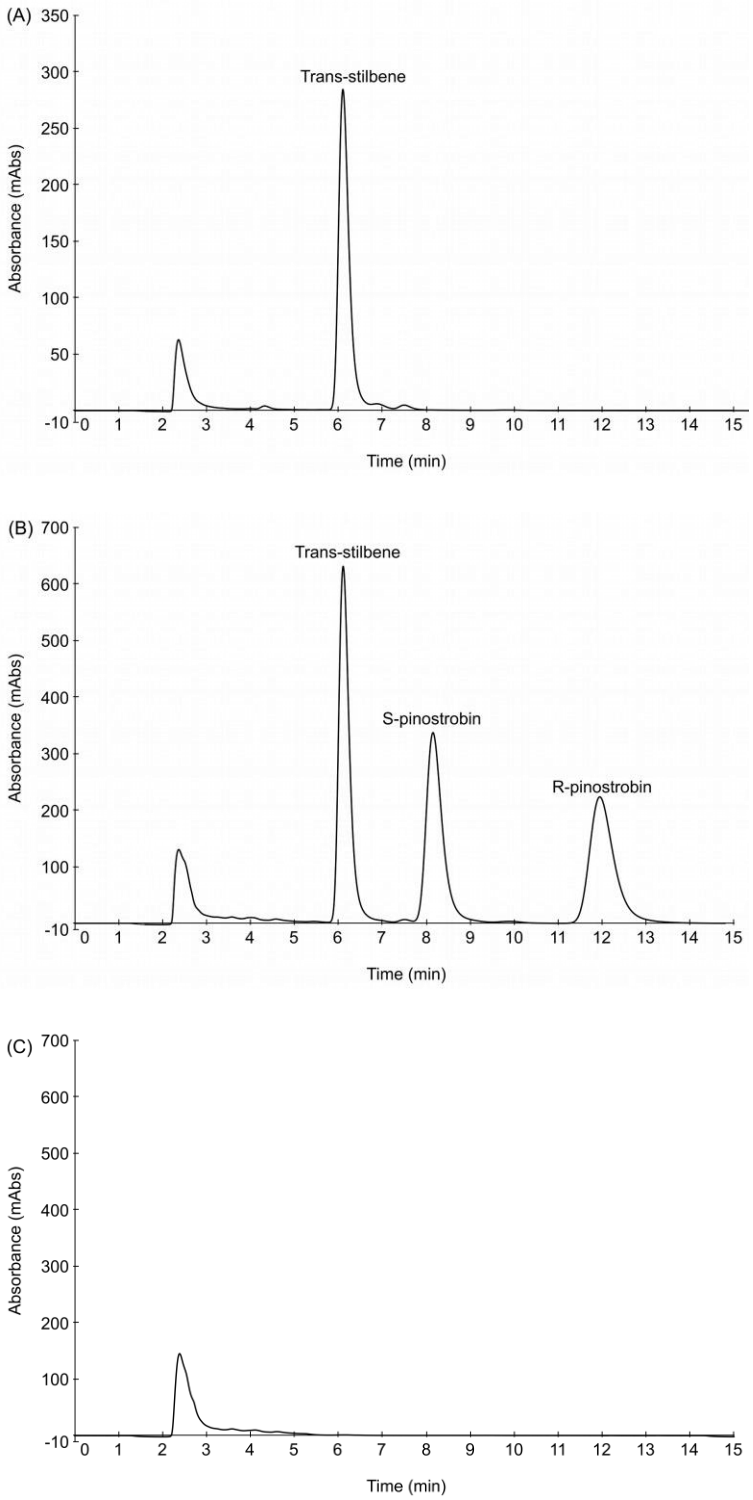


**Figure 3.1.** Representative chromatograms, of (A) drug-free serum and the internal standard, trans-stilbene, (B) serum containing pinostrobin enantiomers each with concentration of 10 μg/ml and the internal standard, trans-stilbene, and (C) drug free serum demonstrating no interfering peaks co-eluted with the peaks of interest and the internal standard.

*3.3.2 Method Validation*

Excellent linear relationships ($r^2$ = 0.998) were demonstrated between PAR of *S*- and *R*-pinostrobin to IS and the corresponding serum concentrations of pinostrobin enantiomers over a range of 0.5 to 100 μg/mL. The LOQ of this assay was 0.5 μg/mL in serum. Precision and accuracy of the assay was < 15% (RSD) and was with a bias <15% for all points on the calibration curve in accordance with the US Food and Drug Administration guidelines. The back-calculated concentrations of quality control samples were within the acceptance criteria.

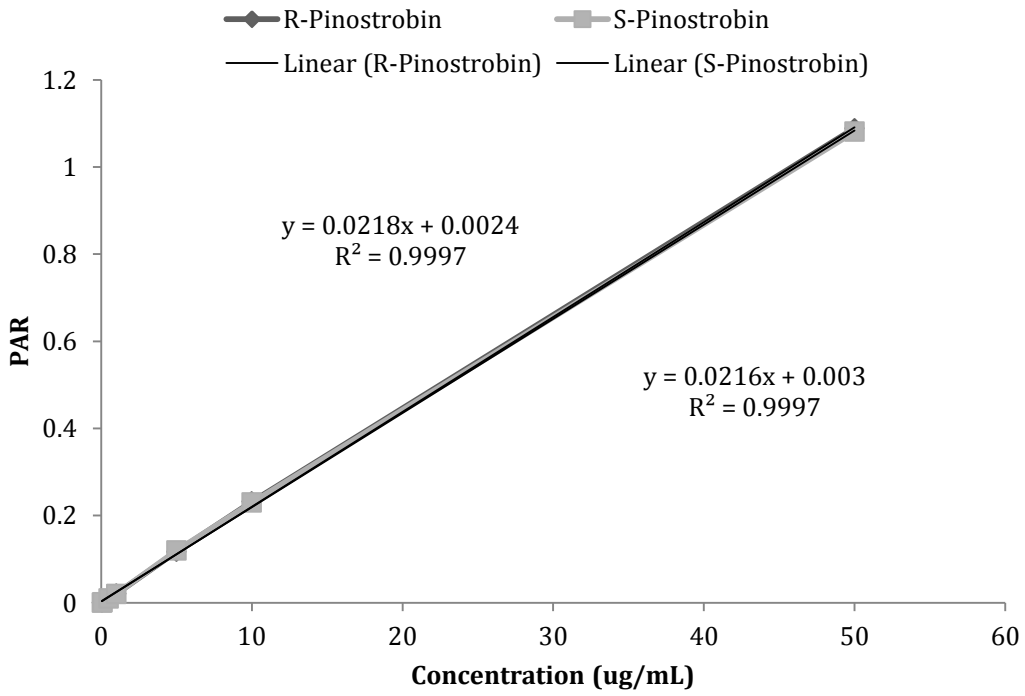


**Figure 3.2.** Standard curve of S-pinostrobin and R-pinostrobin in blank rat serum showing correlation coefficients and trendline.

*3.3.3 Stereospecific pharmacokinetics of pinostrobin in rats*

The HPLC method was applied to the determination of pinostrobin enantiomers in pharmacokinetic studies in rats (*n*=3). Following administration of pinostrobin intravenously, the serum disposition was examined. Pinostrobin enantiomers were

detected in serum. Both enantiomers had a serum half-life of ~7 hours (Table 3.1). They also shared similar values of volume of distribution ($V_d$ *S*-pinostrobin = 8.2 L/kg; $V_d$ *R*-pinostrobin = 8.9 L/kg), total clearance (*S*-pinostrobin $CL_{total}$ = 0.96 L//h/kg; *R*-pinostrobin $CL_{total}$ = 1.055 L//h/kg), and area under the curve (*S*-pinostrobin $AUC_{inf}$ = 23.16 µg h/ml; *R*-pinostrobin $AUC_{inf}$ = 21.296 µg h/ml). The large volume of distribution suggests extensive distribution of pinostrobin into tissues.

| Parameter | S-pinostrobin (Mean ± SEM) | R-pinostrobin (Mean ± SEM) |
|---|---|---|
| $AUC_{0-\infty}$ (hr*ug/mL) | 23.160 ± 7.322 | 21.296 ± 7.066 |
| Vd (L/kg) | 8.259 ± 1.216 | 8.901 ± 0.90 |
| $Cl_{\mathbf{total}}$ (L/h/kg) | 0.96 ± 0.30 | 1.055 ± 0.35 |
| $T_{½}$ serum (h) | 6.938 ± 3.072 | 6.791 ± 2.847 |

**Table 3.1.** Stereospecific Pharmacokinetic Properties of Pinostrobin in the Rat

## 3.4 Conclusions

In summary, the developed HPLC method for pinostrobin is rapid, sensitive, reproducible, accurate, and stereospecific. It has been applied successfully in the preliminary study of the pharmacokinetics of pinostrobin in rats for the first time. Further studies are ongoing in our laboratory to further characterize the pharmacological and toxicological activities of pinostrobin as well as other chiral flavanone and flavonoid enantiomers found in nutraceuticals and plants.

## 3.5 References


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# Chapter IV: Chiral Analytical Method Development of Liquiritigenin with Application to Pharmacokinetic Study

## 4 Abstract

Pharmacometric characterization studies of liquiritigenin have historically overlooked its chiral nature. To achieve complete characterization, an analytical method enabling the detection and quantification of the individual enantiomers of racemic (+/-) liquiritigenin is necessary. Resolution of the enantiomers of liquiritigenin was achieved using a simple high-performance liquid chromatographic method. A Chiralpak® ADRH column was employed to perform baseline separation with UV detection at 210 nm. The standard curves were linear ranging from 0.5 to 100 μg/ml for each enantiomer. Limit of quantification was 0.5 μg/ml. The assay was applied successfully to stereoselective serum disposition of liquiritigenin enantiomers in rats. Liquiritigenin enantiomers were detected in serum and as both aglycones and glucuronidated conjugates. Both unconjugated enantiomers had a serum half-life of ~15 minutes in rats. Volume of distribution (Vd) for S-liquiritigenin and R-liquiritigenin was 1.49 L/kg and 2.21 L/kg respectively. Total clearance ($CL_{total}$) was 5.12 L/h/kg for S-liquiritigenin and 4.79 L/h/kg for R-liquiritigenin, and area under the curve ($AUC_{0\text{-}inf}$) was 3.95 μg h/ml for S-liquiritigenin and 4.23 μg h/ml for R-liquiritigenin. The large volume of distribution coupled with the short serum half-life suggests extensive distribution of liquiritigenin into tissues.

## 4.1 Introduction

(+/-) Liquiritigenin (4',7-Dihydroxyflavanone) is a chiral flavonoid present in liquorice. It is a potent bioactive compound, demonstrating anti-cancer and hepatoprotective activity as well as attenuation of the acute effects of cocaine administration.[1-3] Liquiritgenin has also been shown to be an estrogen receptor beta agonist.[4] These properties highlight the potential therapeutic application of liquiritigenin in human health.

The pharmacokinetics of racemic liquiritigenin via achiral high performance liquid chromatography (HPLC) methods has been studied extensively.[2-5,8] However, chirality is an aspect often overlooked in the pharmacometric characterization of phytochemicals, including flavonoids. Very few studies have been published on the stereospecific separation of liquiritigenin.[9,10]

Differences in the disposition and activity of individual stereoisomers can sometimes cause significant, or harmful effects in humans.[11,12] This study is the first to develop a stereoselective, isocratic, reversed-phase HPLC assay of liquiritigenin for detection in rat biological matrices with application to a pharmacokinetic study.

## 4.2 Experimental

### *4.2.1 Chromatographic system and conditions*

The HPLC system used was a Shimadzu LC-2010A (Kyoto, Japan). Data collection and integration were accomplished using Shimadzu EZ Start 7.4 software (Kyoto, Japan). The analytical column used was a Chiralpak® AD-RH (150mm × 4.6mm i.d., 5-µm particle size, Chiral Technologies Inc. West Chester, PA, USA). The mobile phase consisted of acetonitrile, water, and acetic acid (50:50:0.05 v:v:v)

filtered and degassed under reduced pressure, prior to use. Separation was carried out isocratically at ambient temperature (25 ± 1°C), and a flow rate of 0.6 ml/min**,** with ultraviolet (UV) detection at 210 nm

### *4.2.2 Sample preparation*

To the working standards or samples (0.1 mL), 25 µL of (+/-)-pinocembrin (internal standard) solution (100 µg/ml) was added into 2.0 ml Eppendorf tubes. Next, 1 mL of cold acetonitrile (–20°C) was added, immediately followed by 1 min of vortexing (Vortex Genie-2, VWR Scientific, West Chester, PA, USA) Samples were then centrifuged at 5,000 rpm for 5 min (Beckman Microfuge centrifuge, Beckman Coulter, Inc., Fullerton, CA, USA). The supernatant was collected into 2.0 ml Eppendorf tubes and evaporated to dryness under compressed nitrogen gas. The residue was reconstituted with 200 µL of mobile phase, vortexed for 30 seconds and centrifuged at 5000 rpm for 5 minutes. The supernatant was then transferred to HPLC vials and 100 µL of it was injected into the HPLC system.

### *4.2.3 Pharmacokinetic disposition of liquiritigenin in rats*

Male Sprague-Dawley rats (n=3, average weight of 250 g) were anaesthesized using isoflurane and a silastic catheter was cannulated into the right jugular vein. Rats were dosed intravenously with 20mg/kg racemic (effectively 10mg/kg S-liquiritigenin and 10mg/kg R-liquiritigenin) liquiritigenin (Extrasynthèse, Genay Cedex, France) in saline and polyethylene glycol (PEG) 400 (60:40 v:v). A series of blood samples (0.30 ml) was collected at 0, 1, 15, 30 min, and at 1, 2, 4, 6, 24, 48, 72, 96, and 120 h. Following centrifugation of the blood samples in microcentrifuge tubes, serum was collected and stored at –20°C until analysis. Serum samples (0.1 ml) 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 hours to liberate any glucuronide conjugates.

### *4.2.4 Data analysis*

Quantification was based on calibration curves constructed using the peak area ratio (PAR) of liquiritigenin to internal standard, against liquiritigenin concentrations using unweighted least squares linear regression. Pharmacokinetic analysis was performed using data from individual rats for which the mean and standard error of the mean (S.E.M.) were calculated for each group. Pharmacokinetic modeling was completed using WinNonlin® software (Ver. 5.1). Non-compartmental pharmacokinetic methods were used to calculate clearance (CL by dividing dose by $AUC_{0-\infty}$) and volume of distribution ($Vd_{ss}$ was found with CL x $MRT_{iv}$).

## 4.3 Results and Discussion

### *4.3.1 Chromatography*

Separation of liquiritigenin enantiomers and the internal standard, (+/-)-pinocembrin, in serum (Figure 4.1) and was achieved successfully. There were no interfering peaks co-eluting with the peaks of interest. The retention times of S and R-liquiritigenin were approximately 9 and 12 minutes, respectively. The internal standard (+/-)-pinocembrin eluted at approximately 16 minutes (Figure 4.1).

The performance of the HPLC assay was assessed using the following parameters, namely peak shape and purity, interference from endogenous substances in biological fluid, linearity, and limit of quantitation (LOQ). Various compositions of mobile

phase were tested to achieve the best resolution between liquiritigenin enantiomers. Optimal separation was achieved with acetonitrile, water, and acetic acid (50:50:0.05 v:v:v) with a flow rate of 0.6 ml/min. The present assay is practical to use in pre-clinical applications of liquiritigenin analysis in which small sample volumes are obtained.

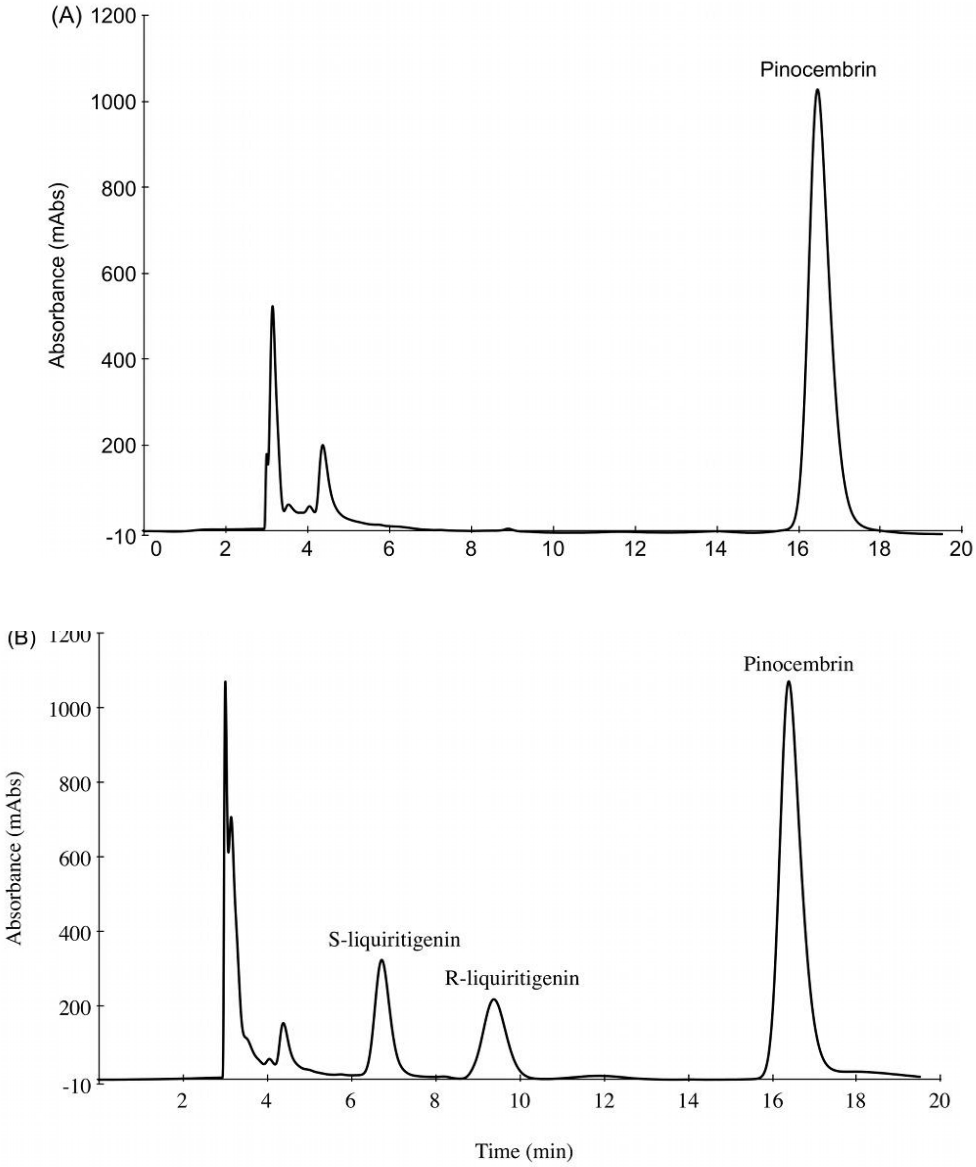


Figure 1. (A) Blank serum with the internal standard, pinocembrin. (B)Liquiritigenin enantiomers in serum (5 µg/ml) and the internal standard, pinocembrin.

**Figure 4.1.** (A) Blank serum with the internal standard, pinocembrin. (B) Liquiritigenin enantiomers in serum (5 µg/mL) and the internal standard, pinocembrin.

*4.3.2 Linearity and LOQ*

Excellent linear relationships ($r^2$ = 0.998) were demonstrated between PAR of S- and R-liquiritigenin to the internal standard and the corresponding serum concentrations of liquiritigenin enantiomers over a range of 0.5 to 100 μg/ml. The LOQ of this assay was 0.5 μg/ml in biological fluids. Precision and accuracy of the assay was < 15% (RSD) and was with a bias <15% for all points on the calibration curve in accordance with the US Food and Drug Administration guidelines. The back-calculated concentrations of quality control samples were within the acceptance criteria.

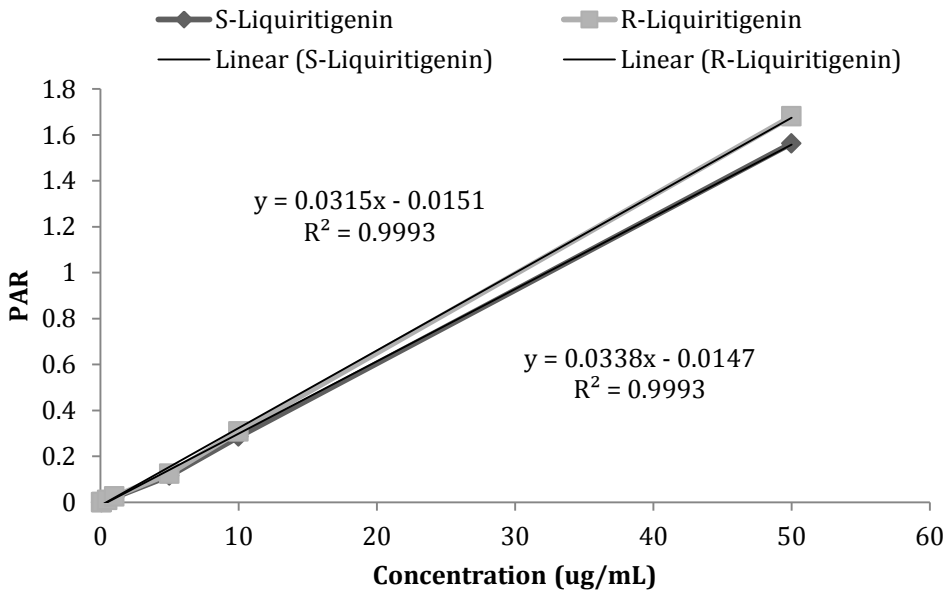


**Figure 4.2.** Standard curve of S-liquiritigenin and R-liquiritigenin in blank rat serum, showing correlation coefficients and trendline.

*4.3.3 Stereospecific pharmacokinetics of liquiritigenin in rats*

The HPLC method was applied to the determination of liquiritigenin enantiomers in pharmacokinetic studies in rats (n=3). Liquiritigenin enantiomers were detected in serum as both aglycones and glucuronidated conjugates. The R and S glucuronidated

conjugates had a serum half-life of around 9 minutes ($T_{1/2}$; S-liquiritigenin-glucuronidate 0.159 h and R-liquiritigenin-glucuronidate 0.141 h) and and area under the curve of 1.202 µg h/ml and 1.589 µg h/ml respectively (Table 4.1). Both unconjugated enantiomers had a serum half-life of ~15 minutes. R-liquiritigenin had a slightly larger volume of distribuition ($V_d$; S-liquiritigenin = 1.492 L/kg and R-liquiritigenin = 2.213 L/kg) than did S-liquiritigenin. Total clearance ($CL_{total}$, S-liquiritigenin = 5.123 L/h/kg and R-liquiritigenin = 4.787 L/h/kg) and area under the curve ($AUC_{0-\infty}$; S-liquiritigenin = 3.951 µg h/ml and R-liquiritigenin = 4.226 µg h/ml) was similar between the two enantiomers. The relatively large volume of distribution coupled with the short serum half-life suggests extensive distribution of liquiritigenin into tissues.

| Parameter | S-liquiritigenin (Mean ± SEM) | R-liquiritigenin (Mean ± SEM) |
|---|---|---|
| $AUC_{0-\infty}$ (hr*ug/mL) | 3.951 ± 0.267 | 4.226 ± 0.278 |
| Vd (L/kg) | 1.492 ± 0.103 | 2.213 ± 0.340 |
| $Cl_{\mathbf{total}}$ (L/h/kg) | 5.123 ± 0.349 | 4.787 ± 0.316 |
| $f_e$ (%) | 31.49 ±1.00 | 5.02 ± 0.97 |
| $T_{½}$ serum (h) | 0.20 ± 0.00 | 0.33 ± 0.07 |

**Table 4.1.** Stereospecific pharmacokinetic parameters of unconjugated liquiritigenin.

## 4.4 Conclusions

In summary, the developed HPLC method for liquiritigenin is stereospecific. It has been applied successfully in the study of the pharmacokinetics of liquiritigenin in rats for the first time. Further studies are on going in our laboratory to further characterize the pharmacometric profiles of liquiritigenin as well as other flavonoid enantiomers.

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# Chapter V: Quantification of Three Chiral Flavonoids with Reported Bioactivity in Selected Licensed Canadian Natural Health Products and US Marketed Dietary Supplements.

**Abstract:** PURPOSE: Research indicating potentially beneficial bioactivity of flavonoids has produced a market and demand for natural health products and dietary supplements containing flavonoids. Implementation of the Canadian natural health product (NHP) regulations in January of 2004 increased regulation and oversight of NHP manufacture and marketing leading many consumers and clinicians to assume a similar pathway of development and approval to over-the-counter or prescription drugs. METHODS: Three stereospecific liquid chromatograph/mass spectrometry (LC/MS) methods were used to assess the flavonoids, liquiritigenin, pinocembrin, and pinostrobin, in selected Canadian licensed NHP's and US marketed dietary supplements. RESULTS: The present study quantifies bioactive flavonoids in these products and notes variability in flavonoid content. CONCLUSIONS: Efficacy and safety of NHP's and dietary supplements should not be assumed due to differences in criteria for NHP licensure by Health Canada as well as variation of flavonoid content between manufacturers and products with similar indications for use.

## 5.1 Introduction

Since the implementation of the Canadian Natural Health Product (NHP) regulations in January of 2004, regulation and oversight of NHP manufacture and marketing has increased. Consumers practicing self-care have benefited from the increased information present on the label of these Health Canada licensed products. However, licensure by Health Canada of an NHP can lead to the incorrect assumption that NHP's pass through a development and approval pathway similar to other consumer health care products, like over-the-counter (OTC) or prescription drugs. This is can be confusing and potentially problematic for consumers attempting to self-treat conditions with products containing flavonoids.

Evidence of the cardiovascular benefit of diets high in flavonoids continues to accumulate.[1-3] In addition, investigation of the bioactivity of individual flavonoids has also increased. These pre-clinical bioactivity studies often seek to define the pharmacological role of a specific flavonoid, whether isolated by itself or present within a complex botanical mixture, in various models of disease. Liquiritigenin, pinocembrin, and pinostrobin are three flavonoids found in an array of natural sources. Liquiritigenin (Figure 5.1) has recently been shown to be a highly selective estrogen receptor ß agonist.[4] It is present in licorice species (*Glycyrrhizae uralensis and Glycyrrhizae glabra*) and has been identified as one of multiple active components in a proprietary botanical mixture called MF101 currently being tested in clinical trials for activity against menopausal vasomotor symptoms.[5] Pinocembrin (Figure 5.2) is present in the traditional medicinal plant *Alpinia galangal* and is notably recognized as the flavonoid of highest concentration in propolis, the resinous glue collected from plants by bees for use in hive

building, and also a traditional medicine.[6,7] Pinocembrin (Figure 5.3) has exhibited multiple

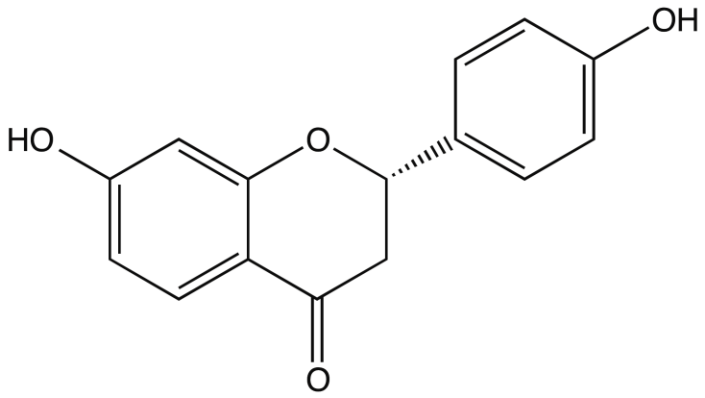


**Figure 5.1.** Structure of S-liquiritigenin

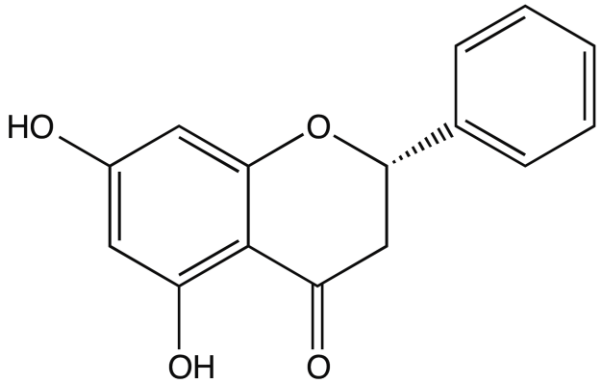


**Figure 5.2.** Structure of S-pinocembrin

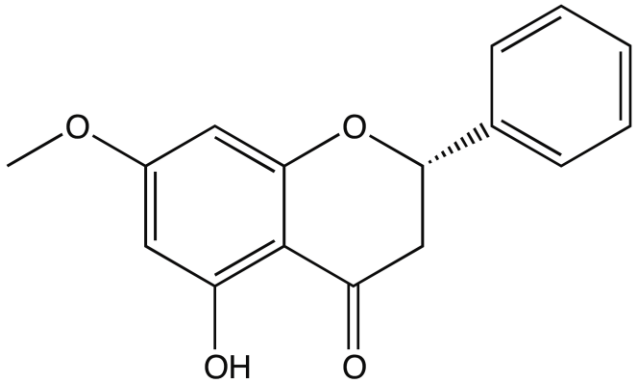


**Figure 5.3.** Structure of S-pinostrobin

bioactivities including neuroprotection and anti-inflammatory activity.[8,9] Finally, pinostrobin has been isolated in Thai ginger (*Boesenbergia pandurata*), propolis, and

honey.[10] Pinostrobin has recently been found to possess activity against herpes simplex virus-1 (HSV-1).[11] Chirality and the possibility of glycosides are common structural features of liquiritigenin, pinocembrin, and pinostrobin. The contribution of each of their two enantiomeric forms to their bioactivity has yet to be studied. In general, despite the conclusive evidence that diets high in flavonoids seem to produce a clear and significant health benefit, scientists and clinicians have yet to agree exactly how consumers should use this information.[12] The question of flavonoid self-treatment through dietary means or concentrated supplementation needs further research. However, NHP's and dietary supplements claiming to contain plant material or other products known to contain the specific flavonoid, or claiming to contain specific quantities of flavonoids have been and are currently available in Canadian and US markets.

The number of consumers utilizing NHP's and dietary supplements has been steadily increasing in North America and their use has moved from the fringes to the mainstream of society.[13,14] However, conclusions that consumers or clinicians may draw from pre-clinical or clinical evidence demonstrating the benefit of specific flavonoids may be incorrect. The expected benefit from using or recommending a marketed product claiming to contain plant material or other products known to contain a specific flavonoid may or may not occur simply because the criteria for efficacy and safety of NHP licensure and dietary supplement marketing is unlike that of OTC or prescription drugs. Additionally, variability in the quantity and recommended dose of a purported active ingredient between manufacturers and formulations could prevent reliable replication of therapeutic results obtained from clinical trials, or even anecdotal reports.

Furthermore, the quality, stability, purity, and activity of many of these flavonoids are not yet fully understood. And the apparent "stamp of approval" by Health Canada via licensure of NHP's may cause confusion to consumers and clinicians accustomed to a much more rigorous pathway of development and approval. In addition, many assays to reproducibly measure flavonoid concentrations including their enantiomeric forms and glycosides in either plant material or marketed NHP's and dietary supplements have not been developed or validated.

In the present study, we assess the content of selected Canadian NHP's and US marketed dietary supplements claiming to contain plant material or other products known to contain liquiritigenin, pinocembrin, or pinostrobin. We have previously validated a stereospecific liquid chromatography/mass spectrometry (LC/MS) method for the quantification of pinocembrin and HPLC methods for stereospecific quantification of liquiritgenin, and pinostrobin. These methods can be adapted for LC/MS use to further increase detection limits. Herein we describe three LC/MS methods for the stereospecific detection and quantification of liquiritigenin, pinocembrin, and pinostrobin in selected licensed Canadian natural health products and US marketed dietary supplements.

## 5.2 Methods

Nineteen products were selected based on claims of containing plant material or other natural products (propolis or honey) known to contain a specific flavonoid or claims of containing a specific flavonoid. Selection was also guided by an attempt to match indications for use with positive bioactivity results previously reported for a specific flavonoid, namely: products indicated for menopausal symptoms were analysed for liquiritigenin content, those indicated for immune system support were analysed for

pinocembrin, and products indicated or used for topical anti-infective support were analysed for pinostrobin. Since pinocembrin and pinostrobin often appear in natural sources together, the 9 products purported to contain one or the other were analysed for both. Products analysed and labelled indications for use are reported in Table 1. Products were purchased on the open market through retail stores, and on-line retailers. Several products were donated directly from the manufacturer. All lots were within one year of the noted expiry date.

**Table 5.1:** Label claims of licensed Canadian NHP's and US marketed dietary supplements claiming to contain plant material or other products known to contain the specific flavonoid. † Indicates a product claiming an amount of flavonoid. • Indicates a licensed Canadian NHP.

| **NHP's Containing Liquiritigenin** | **Labeled Indication for Use** |
|---|---|
| Clef des Champs® Hormonatop capsules | Used in Western herbalism to relieve symptoms associated with premenstrual syndrome and menopause. |
| Clef des Champs® Hormonix tincture | Traditionally used in Western herbalism to normalize the female reproductive system in the relief of symptoms associated with premenstrual syndrome and menopause. |
| Clef des Champs® Mamaboost tincture | Traditionally used in Western herbalism as a nerve tonic and antispasmodic to help relieve nervous tension and premenstrual symptoms. |
| Clef des Champs® Purifytop capsules | Traditionally used in Western herbalism as an alternative to help alleviate skin conditions. |
| Clef des Champs® Licorice tincture | Traditionally used to treat dry cough, lung disorders, sore throat and laryngitis, as well as urinary and intestinal inflammation. |
| Vadik® Herbs Licorice powder | None |
| Himalaya® Licorice 250 mg tablets | Aids in normal stomach function, supports normal bowel function in occasional sluggishness, supports |

| | |
|---|---|
| | normal digestive system function |
| Biosnacky® Alfalfa Seeds | None |
| Swiss® Alfalfa Leaf 1000 mg tablets | Traditionally used in Herbal Medicine as a nutritive tonic. |
| Natures Harmony® Alfalfa Leaf 500 mg capsules | Traditionally used in Herbal Medicine as a nutritive tonic. |
| **NHP's Containing Pinocembrin and or Pinostrobin** | **Labeled Indication for Use** |
| Organika® Bee Propolis liquid 167 mg/ml | Used in herbal medicine to help relieve sore throat and/or other mouth and throat infections. |
| T.C. Unicorn Ltd.® Bee Propolis 120 mg/ml | None |
| †Quantum Nutrition Labs® Propolis Complex 290 mg capsules | Supports healthy immunity, thymus and skin health, high energy levels and healthy histamine response. |
| †Bee Healthy Farms LLC® Propolis capsules 500 mg | Immune system booster. |
| Wedderspoon® Wild Dandelion Honey | None |
| Himalaya® Soliga Forest Honey | None |
| Comvita® Manuka Honey | None |
| Amazon Herbs® Boesenbergia pandurata tincture | Relieves flatulence and indigestion, strengthen energy, stimulates nerves. |
| Amazon Herbs® Alpinia galangal tincture | Powerful inflammatory support. |

### *5.2.1 Sample extraction and preparation*

Of the 10 products analyzed for liquiritigenin content, 3 were capsules 2 were tablets, 3 were liquids, one was powder, and 1 was raw plant material. Of the 9 products analysed for pinocembrin and pinostrobin content 2 were capsules, 3 were honeys, and 4 were liquids. For flavonoid extraction and preparation, 1 tablet, the contents of 1 capsule, or 1 gram of honey or powder were weighed. Each tablet was ground in a mortar to a fine powder. Powders of capsules/tablets as well as honeys were extracted with 4 ml of methanol by placing on a rotating shaker for 3 h. 1 ml of the liquids were sampled without extraction. Samples were centrifuged at 2,000 rpm for 5 min. The supernatant was collected and then divided into two groups with 100 µl aliquots for each group. The

first group was enzymatically incubated to cleave the glycoside form of the polyphenol into its aglycone form. For this, the 100 µl aliquot was dried to completion under a stream of nitrogen gas and reconstituted in 200 µl phosphate buffer saline (PBS). To this, 20 µl of β-glucosidase from almonds was added and then incubated at 37ºC for 48 h in a hot water bath. Internal standard (25 µl) and 1 ml of cold acetonitrile was added to each sample. Samples were again centrifuged at 2,000 rpm for 5 min and the supernatant dried under a stream of nitrogen gas. The residues were reconstituted with 200 µl mobile phase and 10 µl was injected into the LC/MS system. For the second group, samples were prepared without enzymatic incubation thereby allowing quantification of the aglycone alone. Specifically, to the 100 µl aliquots, 25 µl of internal standard was added. Samples were dried under a stream of nitrogen gas, residues reconstituted in 200 µl mobile phase, and 10 µl was injected into the LC/MC system. By subtracting of the concentration of the non-enzymatic incubated sample from the incubated sample, the amount of glycoside can be determined.

### *5.2.2 Analysis methods*

To assess flavonoid content, a liquid chromatographic-electrospray ionization-mass spectrometry (LC-ESI-MS) system was employed. A Shimadzu LCMS-2010 EV liquid chromatograph mass spectrometer system (Kyoto, Japan) connected to the LC portion consisting of two LC-10AD pumps, a SIL-10AD VP auto injector, a SPD-10A VP UV detector, and a SCL-10A VP system controller was used. Data analysis was accomplished using Shimadzu LCMS Solutions Version 3 software. The mass spectrometer conditions consisted of a curved desolvation line (CDL) temperature of 200ºC and a block temperature of 200ºC. The CDL, interface, and detector voltages were

-20.0 V, 4.5 kV, and 1.2 kV, respectively. Vacuum was maintained by an Edwards® E2M30 rotary vacuum pump (Edwards, UK). Liquid nitrogen was used as a source of nebulizer gas (1.5 L/min). Each polyphenol was monitored in selected ion monitoring (SIM) negative mode. Standard curves for each compound were linear over the concentration ranges of 0.05-100 µg/ml. The LOQ was 50 ng/ml for each compound. E-stilbene and 7-ethoxycoumarin standards were purchased from Sigma Chemicals (St. Louis, Mo, USA). Pinocembrin, pinostrobin, and liquiritigenin standards were purchased from Extrasynthese (Cedex, Genay, France).

To assess liquiritigenin content, a previously validated HPLC method was adapted for LC/MS use.[15] The analytical column used was a Chiralpak ® AD-RH (150mm × 4.6mm i.d., 5-µm particle size, Chiral Technologies Inc. West Chester, PA, USA). The mobile phase consisted of acetonitrile and HPLC water and formic acid (50:50:0.01 v/v/v) at a flow rate of 0.6 ml/min. (±)-Pinocembrin was used as an internal standard. S(-)liquiritigenin, R(+)liquiritigenin, and (±)-pinocembrin were monitored in selected ion monitoring (SIM) negative mode with the single plot transition at m/z 255.15.

For assessment of pinocembrin content, a previously validated LC/MS method was utilized using a Chiralpak ® AD-RH (150mm × 4.6mm i.d., 5 µm particle size, Chiral Technologies Inc. West Chester, PA, USA).[16] The mobile phase consisted of methanol and ammonium acetate (100:0.1M, v/v) at a flow rate of 0.5 ml/min. 7-Ethoxycoumarin was used as an internal standard. S(+)pinocembin and R(-)pinocembrin were monitored in SIM negative mode with the single plot transitions at *m/z* 255. 7-Ethoxycoumarin was monitored in SIM positive mode with the single plot transitions at *m/z* 191.

To quantify pinostrobin, a second LC/MS method was developed from a previously validated HPLC method.[17] Separation was achieved using a Chiralpak ® AD-RH (150mm × 4.6mm i.d., 5-µm particle size, Chiral Technologies Inc. West Chester, PA, USA). The mobile phase consisted of acetonitrile, water, and formic acid (80:20:0.01, v/v/v) at a flow rate of 1 ml/min. Daidzein was used as an internal standard and monitored in SIM negative mode with the single plot transitions at *m/z* 255. S(+)pinostrobin and R(-)pinostrobin were monitored in SIM negative mode with the single plot transitions at *m/z* 271.

## 5.3 Results

Two novel and one previously validated LC/MS assays were successfully applied to the quantification of liquiritigenin, pinocembrin, and pinostrobin in selected licensed Canadian NHP's and US marketed dietary supplements as shown in Table 5.2, 5.3, and 5.4. When assessing content, both the S and R enantiomers as well as the aglycone and glycoside forms were measured. Previous reports have indicated that many flavonoids exist as glycosides with a smaller amount existing as the aglycone or parent compound. It is hypothesized that the glycoside form is cleaved in the gastrointestinal tract and liver during the first pass to the more bioactive aglycone.[18] In the present study, flavonoids were found in both the glycoside and aglycone forms.

To assess label claims of flavonoid, appropriate labeling was defined as a product that contained a least 100% and no more than 120% of its claimed amount. If the label claimed a plant extract or other product known to contain a specific flavonoid, appropriate labeling was attained if any amount of the flavonoid was quantifiable. Of the 17 products claiming a plant extract or other product known to contain a particular

flavonoid, 5 did not contain the purported flavonoid constituent. Of the two products that claimed specific flavonoid amounts, one did not approach stated label claims; however the label claims did not discriminate between flavonoid enantiomers and are assumed to reflect the S and R forms additively. Furthermore, the label claims did not discriminate between aglycone and glycosidic forms of the flavonoid. Several products which claimed to contain plant material or other products known to contain certain flavonoids did not contain any detectable amount of the purported flavonoid.

| **Number** | **S(-)liquiritigenin** | | | **R(+)liquiritigenin** | | |
|---|---|---|---|---|---|---|
| | Claimed | Aglycone | Glycoside | Claimed | Aglycone | Glycoside |
| 1 | † | 0.08 | 0.01 | † | 0.09 | 0 |
| 2 | † | 0.11 | 0.04 | † | 0.17 | 0 |
| 3 | † | 0.36 | 0 | † | 0.45 | 0 |
| 4 | † | 4.119 | 0 | † | 5.943 | 0 |
| 5 | † | 1.244 | 0.718 | † | 1.866 | 0.763 |
| 6 | † | 10.771 | 8.325 | † | 13.193 | 9.164 |
| 7 | † | 0.43 | 0 | † | 0.93 | 0 |
| 8 | † | 4.152 | 0 | † | 4.836 | 0 |
| 9 | † | 4.034 | 0 | † | 4.524 | 0 |
| 10 | † | 53.161 | 0 | † | 56.689 | 0 |

**Table 5.2.** Stereospecific liquiritigenin content in selected licensed Canadian NHP's and US marketed dietary supplements (μg per tablet/capsule/mL). † indicates a product claiming to contain plant material or other products known to contain the specific flavonoid.

| Number | S(+)pinocembrin | | | R(-)pinocembrin | | |
|---|---|---|---|---|---|---|
| | Claimed | Aglycone | Glycoside | Claimed | Aglycone | Glycoside |
| 1 | † | 1.623 | 0 | † | 0.887 | 0 |
| 2 | † | 1046.004 | 124.142 | † | 274.122 | 62.889 |
| 3 | † | 0 | 0 | † | 0 | 0 |
| 4 | † | 7.851 | 13.111 | † | 5.205 | 7.067 |
| 5 | † | 1.138 | 10.091 | † | 0.95 | 0 |
| 6 | † | 1.174 | 2.193 | † | 0.71 | 1.015 |
| 7 | † | 0 | 0 | † | 0 | 0 |
| 8 | 6000 | 649.179 | 8027.806 | 6000 | 471.452 | 5509.727 |
| 9 | 2.19% | 530.828 | 5370.094 | 2.19% | 424.072 | 3430.607 |

**Table 5.3.** Stereospecific pinocembrin content in selected licensed Canadian NHP's and US marketed dietary supplements (µg per tablet/capsule/mL). † indicates a product claiming to contain plant material or other products known to contain the specific flavonoid. If a specific pinocembrin concentration was claimed, the percent or amount in µg is indicated below, claimed amounts did not discriminate between enantiomers or aglycone or glycosidic forms.

| Number | S(+)pinostrobin | | | R(-)pinostrobin | | |
|---|---|---|---|---|---|---|
| | Claimed | Aglycone | Glycoside | Claimed | Aglycone | Glycoside |
| 1 | † | 15.061 | 0 | † | 12.888 | 0 |
| 2 | | 0 | 0 | | 0 | 0 |
| 3 | † | 42.618 | 33.691 | † | 54.745 | 0 |
| 4 | | 0 | 0 | | 0 | 0 |
| 5 | † | 0 | 0 | † | 0 | 0 |
| 6 | | 61.848 | 0 | | 77.394 | 0 |
| 7 | | 0 | 0 | | 0 | 0 |
| 8 | † | 0 | 0 | † | 0 | 0 |
| 9 | † | 28.869 | 0 | † | 35.182 | 0 |

**Table 5.4.** Stereospecific pinocembrin content in selected licensed Canadian NHP's and US marketed dietary supplements (µg per tablet/capsule/mL). † indicates a product claiming to contain plant material or other products known to contain the specific flavonoid.

There was significant variability of flavonoid content between manufacturers and products. This is not surprising given the nature of plant variability in flavonoid production as well as the lack of standards for testing the uniformity of botanical NHP's and dietary supplements. This lack of manufacturer analysis is also likely in part due to a limited number of validated analysis techniques such as HPLC and LC/MS detection assays. Here we evaluate three LC/MS methods for measuring the flavonoid content of selected NHP's and dietary supplements. Not only were these methods successfully able to assess claims of a set amount of flavonoid, but they were also used to assess claims of particular plant material or other products known to contain a particular flavonoid. These applied methods indicate the presence of bioactive flavonoids in NHP's and dietary supplements marketed in Canadian and US markets. Many of these products carry indications for use that are generally in line with the pre-clinical studies for specific flavonoid bioactivity. However, a lack in uniformity of these botanical products warrants further assessment of flavonoid content uniformity and standardization of botanical products and claims. The developed methods have potential to assess the content of a wide range of botanical NHP's and dietary supplements. The detection methods for liquiritigenin, pinocembrin, and pinostrobin can be used to evaluate label claims of these flavonoids. Additionally, the developed detection methods have potential for evaluating the validity of certain plant extract claims. For instance, pinostrobin can be a marker of *Boesenbergia pandurata*; pinocembrin for propolis extracts; and liquiritigenin for licorice and alfalfa extracts. Measuring flavonoid content may provide an appropriate means to monitor botanical product claims and their content uniformity.

## 5.4 Discussion

Herein, we present the analysis of flavonoid containing botanical supplements using three LC/MS methods for stereospecific detection of liquiritigenin, pinocembrin, and pinostrobin. Results indicate that stereospecific quantitation of all three flavonoids is attainable in various botanical NHP formulations. Analysis of flavonoid content indicated the presence of expected flavonoids in 14 of the 19 products. One of the products claiming a specific quantity of flavonoid passed strict evaluation criteria of content with 100-120% of label claims.

The results indicate that variability exists between products and manufacturers, even for products with similar licensed indications for use. As mentioned before, variability in the quantity and recommended dose of a purported active ingredient between manufacturers and formulations could prevent reliable replication of therapeutic results obtained from clinical trials, or even anecdotal reports. This variability is present, even for NHP's with similar Health Canada approved indications for use. This variability may result in large part from a deficit of detection methods that can allow reasonable analysis and quantification. Studies such as this one may allow development of new methods that can be easily applied to the assessment of botanical NHP's and dietary supplements. Given the pharmacological effects and potential health-benefits of flavonoids it is recommended that NHP's and dietary supplements that incorporate them be correctly and accurately labeled and regulatory procedures be adhered to for standardizing manufacturing and content uniformity of these nutraceutical supplements.

A comprehensive analysis of flavonoid pharmacokinetics, methods of analysis, pre-clinical and clinical pharmacokinetics, safety, and toxicology facilitates a better understanding of their reported health-benefits.[19] A further understanding of flavonoid

enantiomeric and glycosidic forms would also provide more comprehensive scientific regulation of products containing them.

## 5.5 References


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# Chapter VI: Pre-clinical Pharmacokinetic Characterization of Selected Chiral Flavonoids: Pinocembrin, Pinostrobin, and Liquiritigenin

## 6 Abstract

The majority of pharmacokinetic studies with administration of individual flavonoids have overlooked the chirality of some of these xenobiotics. In order to characterize for the first time the stereoselective pharmacokinetics of three flavonoids, pinocembrin, pinostrobin, and liquiritigenin were intravenously and orally administered to male Sprague-Dawley rats. Concentrations in serum and urine were characterized via stereospecific HPLC or LC/MS. Short half-lives (0.2-6 h) in serum were observed, while a better estimation of half-life (3-27 h) and other pharmacokinetic parameters was observed using urinary data. Renal exrection of the three flavonoids is very low ($f_e$ values of 0.3-24%), thus they are predominantly excreted via non-renal routes. All three flavonoids undergo rapid and extensive phase II metabolism. This study reports for the first time the stereospecific pharmacokinetics of three chiral flavonoids administered IV and PO in rats.

## 6.1 Introduction

Flavonoids are a group of polyphenolic compounds of low molecular weight (200-600 g/mol)[1] that present a common benzo-γ-pyrone structure.[2] They are further sub-categorized into various subclasses including flavones, flavonols, flavanones, isoflavanones, anthocyanidins, and catechins. The average human diet contains a considerable amount of flavonoids mainly consumed through ingestion of fruits (i.e. orange, grapefruit, apple, and strawberry), vegetables (i.e. onion, broccoli, green pepper, and tomato), soybeans and different herbs.[3,4]

Evidence of potential activity against various chronic diseases with flavonoid usage continues to accumulate.[5-16] This published body of short duration human intervention literature has succeeded in defining the bioactivity of a limited number of flavonoids in chronic disease. 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.[17]

Adding to this, the nutraceutical industry has produced a myriad of flavonoid containing formulations, many of which contain doses that greatly exceed the amount found normally in plant based foods.[18] This fact, coupled with public assumptions of safety, efficacy and cost effectiveness of nutraceuticals has, in a very real sense, created a therapeutic case of "jumping the gun" of pandemic proportions. Pre-clinical *in vivo* pharmacokinetic characterization is needed to begin to answer the primary and fundamental concerns of flavonoid efficacy and safety, especially as potentially harmful doses may be in current use as well as possible drug-flavonoid interactions.

Though flavonoids have many beneficial properties that continue to be investigated, understanding clinical flavonoid pharmacokinetics is important to the optimization and safe use of flavonoids as potential chronic disease preventive and therapeutic agents. Clinical pharmacokinetics also provides metabolic profiles, allowing the identification of metabolites with potentially differing bioactivity from the parent compound. Flavonoids can also affect the metabolism of many other drugs, most commonly through the manipulation of cytochrome P450 iso-enzymes. I have published a comprehensive review of the available clinical pharmacokinetic studies in the literature.[19] 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. However, the overall expense and time consuming nature of human studies limits their ability to address the need for basic characterization of such a large and diverse group of compounds as flavonoids with the efficiency and rapidity that is needed to bridge this knowledge gap.

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.[17,20] In other words, without pharmacokinetic and metabolic data, both human and animal, human intervention and *in vitro* bioactivity studies may be in danger of using irrelevant physiological forms of the flavonoid at physiologically irrelevant concentrations.[17,20,21] In fact, the concentrations of the popular stilbene resveratrol used to demonstrate bioactivity in many *in vitro* experiments are

suspected to be unattainable *in vivo*.[22-24] This has been termed the "bioavailability conundrum"[22] and has given researchers difficulty in explaining the beneficial effects of resveratrol observed in interventional and epidemiological studies.[25] One explanation put forward for the low concentration of resveratrol and other flavonoids in serum is its lipophilicity. This physicochemical property can cause extensive distribution into tissues of resveratrol and other flavonoids after administration, leaving little to nothing for detection in the serum.

Among the classes of flavonoids, flavanones have been defined as citrus flavonoids due to their almost unique presence in citrus fruits.[26-28] However, flavanones have also been reported in other plant based foods[29-34] as well as multiple nutraceuticals, natural health products, and traditional medicines in widely varying concentrations.[18,20,35]

In addition, some flavanones present a unique structural feature known as chirality, which distinguishes them from other classes of flavonoids. All the flavanones have a chemical structure based on a $C_6$-$C_3$-$C_6$ configuration consisting of two aromatic rings joined by a three-carbon link.[36] Almost all the flavanones have one chiral carbon atom in position $C_2$, except for a subclass of flavanones named the 3-hydroxyflavanones or dihydroflavonols that have two chiral carbon atoms in position $C_2$ and $C_3$.[37] Some flavanones possess an additional D-configured mono or disaccharide sugar in the $C_7$ position on ring A. These flavanone-7-O-glycosides exist as diastereoisomers or epimers that have the opposite configuration at only one of two or more tetrahedral stereogenic centers present in the respective molecular entities.[37]

The importance of stereospecific disposition of racemic flavanones is slowly being recognized and reported in the biomedical literature. Most of these preliminary

investigations report the quantification of a variety of flavanones in citrus fruit juices and herbs,[34,38-41] or report the separation of flavanones on different stationary phases.[37] There is a paucity of investigations detailing the importance of stereospecific pharmacokinetics and pharmacodynamics of chiral flavanones. Pinocembrin, pinostrobin, liquiritigenin and their corresponding glycosides (pinocembrin, pinostrobin, and liquiritigenin) belong to this flavanone family,[42] but no stereospecific information on their pharmacokinetics, pharmacodynamics, in both serum and urine have been reported.

Non-stereospecific assay methods cannot interpret the time-course of an individual enantiomer and hence any interpretation based on achiral assay analysis could be misleading in determining concentration dependence of each enantiomer of a racemic flavonoid xenobiotic in terms of pharmacokinetics, toxicity, or health benefits.

The achiral pharmacokinetics of pinocembrin, pinostrobin, liquiritigenin, and their corresponding glycosides have been reported.[43-55] The chiral pharmacokinetics have of these compounds have only been preliminarily evaluated by our laboratory and one other group.[56-59] However, to our knowledge there are no studies that have comprehensively assessed the serum and urinary pharmacokinetics and/or disposition of these three chiral flavonoids and their glucuronidated metabolites in humans or rodents after intravenous and oral administration. Various pharmacokinetic parameters can also be derived from urinary data.[60,61] However, most of the studies of these three compounds use only serum measurements. Furthermore, multiple studies have reported that flavonoids have relatively short serum half-lives.[62,63] Interestingly, several studies have reported that certain polyphenols (stilbenes) have longer urine half-lives (4.7-fold to 16-fold higher half-lives in urine compared to plasma half-lives).[64,65] This discrepancy

between serum 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.[66] In the report by Jamali *et al.*[66] an improved HPLC method was employed and it was observed that there was a significant 2-fold increase in plasma half-life compared to previous reports. Additionally, the lipophilicity of many flavanones could account for the discrepancy in serum and urine half-lives. Therefore, it is necessary to have improved analytical methods with higher detection limits allowing the detection of the compound(s) of interest for longer periods of time in both serum and, perhaps most importantly, in urine. This would allow the assessment of the pharmacokinetics of pinocembrin, pinostrobin, and liquiritigenin enantiomers and/or pinocembrin, pinostrobin, and liquiritigenin epimers as well as the determination of the stereospecific content in nuraceuticals. 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, especially in light of their lipophilicity, we have examined for the first time the stereoselective pharmacokinetics of three racemic flavanones in rat serum and urine after intravenous and oral administration.

## 6.2 Materials and Methods

### *6.2.1 Materials*

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 and liquiritigenin were

purchased from Extrasynthese (France). Racemic pinostrobin 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.

### *6.2.2 Animals and Surgical Procedures*

Male Sprague-Dawley rats (200 - 240 g) were obtained from Simonsen Labs (Gilroy, CA, USA) 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 day before the pharmacokinetic experiment the right jugular veins of the rats were catherized with sterile silastic cannula (Dow Corning, Midland, MI, USA) under halothane anesthesia. This involved exposure of the vessel prior to cannula insertion. After cannulation, the Intramedic PE-50 polyethylene tubing (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) connected to the cannula was exteriorized through the dorsal skin. 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.

### *6.2.3 Pharmacokinetic Study*

Twenty four (24) male Sprague Dawley rats (average weight: 250 g) were cannulated as described in the previous section. Each of the animals were placed in separate metabolic cages, allowed to recover 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), racemic pinostrobin (20 mg/kg IV, 100 mg/kg PO), or racemic liquiritigenin (20 mg/kg IV, 50 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, 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.

### *6.2.4 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.[108] 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, pinostrobin, and liquiritigenin). 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.

### *6.2.5 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 sofware. Student's t-test was employed for unpaired samples with a value of $p < 0.05$ being considered statistically significant.

### *6.2.6 Pharmacokinetic Analysis*

Pharmacokinetic analysis was performed using data from individual rats for which the mean and standard error of the mean (SEM) were calculated for each group. The elimination rate constant ($\lambda$) was estimated by linear regression of the serum concentrations in the log-linear terminal phase. In order to estimate the serum concentrations ($C_0$) immediately after IV dosing, a two-compartmental model was fitted to the serum concentration versus time data using Phoenix® WinNonlin® software (Ver. 6.3) (Pharsight Corporation, Mountain View, CA). The estimated $C_0$ was then used with the actual measured serum concentrations to determine the area under the serum concentration-time curve (AUC). The $AUC_{0-\infty}$ was calculated using the combined log-linear trapezoidal rule for data from time of dosing to the last measured concentration, plus the quotient of the last measured concentration divided by $\lambda$. Non-compartmental pharmacokinetic methods were used to calculate the different 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, the fraction excreted in urine ($f_e$ by dividing the total cumulative amount of flavonoid excreted in urine ($\Sigma X_u$) by the dose), renal clearance ($CL_{renal}$ by multiplying $f_e$ by $CL_{tot}$), non-renal clearance ($CL_{non-renal}$ by subtracting $CL_{renal}$ from $CL_{tot}$).

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 ($\lambda$), urine elimination rate constant ($k_e$) was utilized. Sigma minus plots were inputed into the modeling software which used the terminal phase to calculate urinary half life.

## 6.3 Results

### *6.3.1 Stereospecific Pharmacokinetics of Intravenous Pinocembrin, Pinostrobin, and Liquiritigenin*

The analytical methods described in Chapters II, III, and IV were applied to the stereospecific determination of pinocembrin (LC/MS), pinostrobin (HPLC), and liquiritigenin (HPLC). Linearity in the standard curves was demonstrated in the serum samples for the three 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, pinostrobin and liquiritigenin) concentrations after the enzymatic hydrolysis, which was assessed as described previously.

The serum disposition profiles observed for pinocembrin, pinostrobin, and liquiritigenin demonstrated some difference in stereoselective disposition. For instance, S-pinocembrin (Figure 6.1) achieved higher concentrations in serum compared to its enantiomeric counterpart. Independent of the enantiomeric form, the three 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 three 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. S-liquiritigenin was the only enantiomer to exhibit a significantly shorter $t_{1/2}$ (0.53 ± 2.846 h) than its counterpart (1.548 ± 0.07 h) in serum (Table 6.1). In the case of pinocembrin and pinostrobin both enantiomers reported similar half-lives in serum, namely 0.26 ± 0.07 h for S-pinocembrin and 0.26 ± 0.03 h for R-pinocembrin; and 6.72 ± 2.189 h for S-pinostrobin and 6.77 ± 2.013 h for R-pinostrobin (Table 6.1).

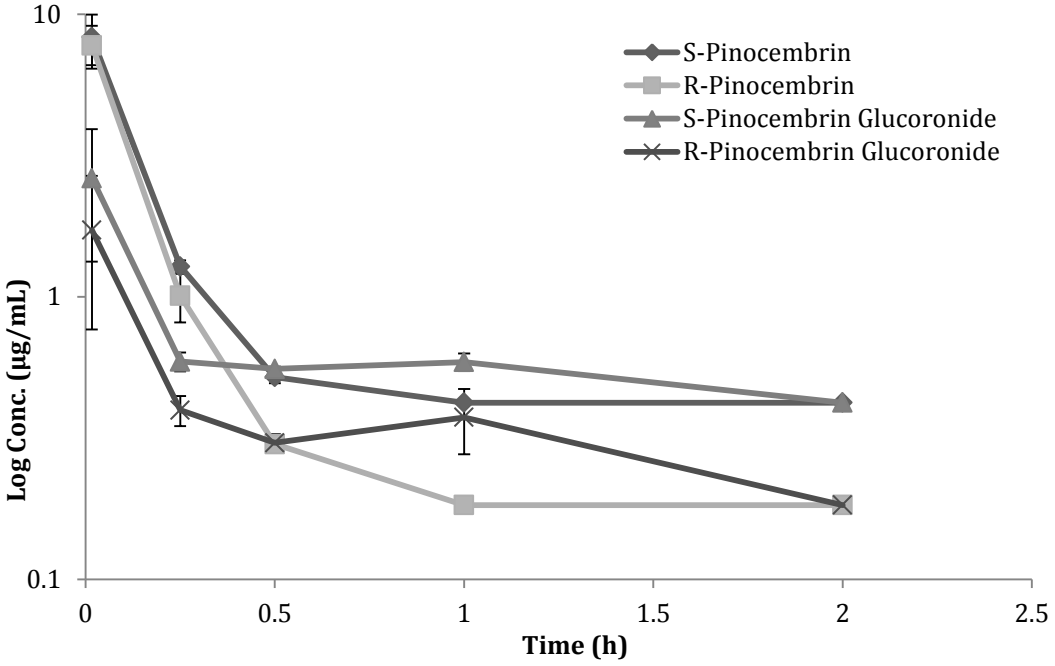


**Figure 6.1.** Concentration-time profile in serum of pinocembrin enantiomers following intravenous administration of racemic pinocembrin (10 mg/kg) in rats (n=4, mean ± SEM).

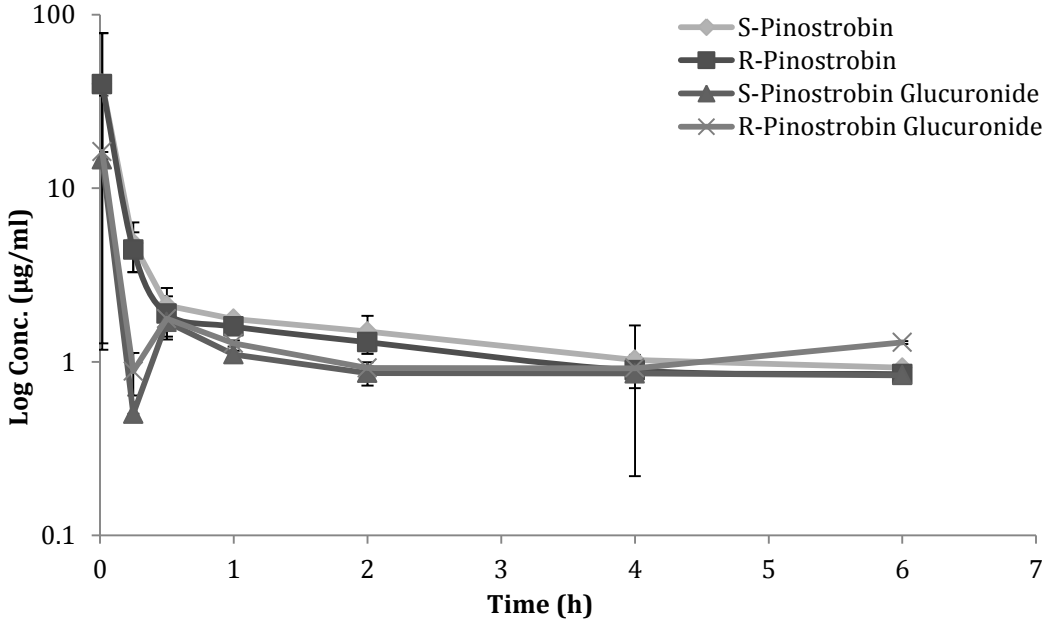


**Figure 6.2.** Concentration-time profile in serum of pinostrobin enantiomers following intravenous administration of racemic pinostrobin (20 mg/kg) in rats (n=4, mean ± SEM).

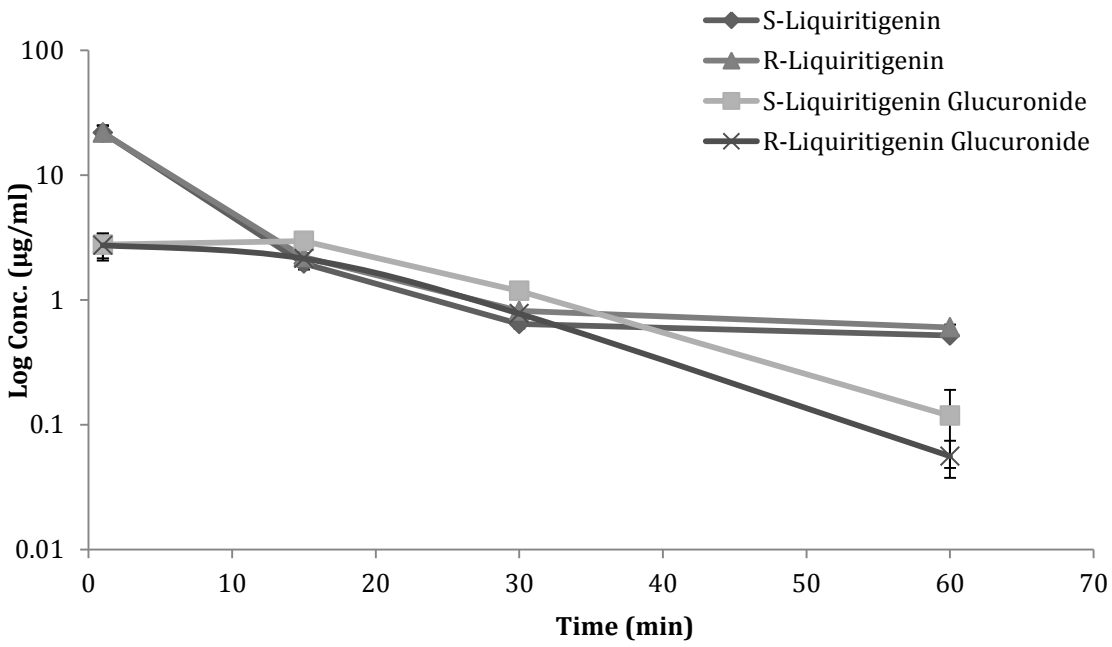


**Figure 6.3.** Concentration-time profile in serum of liquiritigenin enantiomers following intravenous administration of racemic liquiritigenin (20 mg/kg) in rats (n=4, mean ± SEM).

The glucuronidated metabolites of pinocembrin and pinostrobin and liquiritigenin enantiomers exhibited similar concentration-profiles (Figures 6.1, 6.2, and 6.3). 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 6.2). However, with respect to pinocembrin and liquiritigenin glucuronidated metabolites no indication of enterohepatic recycling was observed.

Non-compartmental analysis of the serum concentrations showed several differential pharmacokinetic parameters between pinocembrin, pinostrobin, and liquiritigenin enantiomers (Table 6.1). For instance, R-liquiritigenin showed significantly higher values for volume of distribution ($V_{ss}$) while, fraction excreted unchanged in the urine ($f_e$), and serum elimination rate constant ($\lambda$) had larger values for the S-enantiomer. 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 $\lambda$ as well as MRT (Table 6.1).

**Table 6.1.** Stereospecific pharmacokinetics of pinocembrin, pinostrobin, and liquiritigenin 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 | S-liquiritigenin | R-liquiritigenin |
|---|---|---|---|---|---|---|
| $AUC_{inf}$ (μg*h/mL) | 1.831 ± 0.09 | 1.872 ± 0.31 | 17.782 ± 7.322 | 16.591 ± 7.066 | 4.67 ± 7.816 | 6.43 ± 0.009 |
| $V_{ss}$ (L/kg) | 1.456 ± 0.59 | 1.797 ± 0.2 | 10.018 ± 1.216 | 10.662 ± 0.904 | 2.675 ± 0.724 | 4.637 ± 0.344[a] |
| $CL_{renal}$ (L/h/kg) | 0.019 ± 0.01 | 0.035 ± 0.00 | 0.02 ± 0.01 | 0.04 ± 0.03[a] | 0.01 ± 0.17 | 0.01 ± 0.00 |
| $CL_{non\text{-}renal}$ (L/h/kg) | 5.419 ± 0.3 | 5.790 ± 0.861 | 1.122 ± 0.31 | 1.154 ± 0.38 | 2.835 ± 1.201 | 2.655 ± 0.318 |
| $CL_{total}$ (L/h/kg) | 5.438 ± 0.29 | 5.825 ± 0.87 | 1.137 ± 0.30 | 1.191 ± 0.35[a] | 3.033 ± 1.126 | 2.665 ± 0.708 |
| $f_e$ (%) | 0.346 ± 0.09 | 0.611 ± 0.05[a] | 1.942 ± 1.603 | 4.55 ± 4.419[a] | 24.943 ± 10.341 | 0.157 ± 10.341[a] |
| λ ($h^{-1}$) serum | 4.582 ± 1.983 | 3.028 ± 1.151 | 0.12 ± 0.04 | 0.12 ± 0.04 | 3.434 ± 0.81 | 1.227 ± 0.51[a] |
| λ ($h^{-1}$) urine | 0.26 ± 0.02 | 0.17 ± 0.03[a] | 0.09 ± 0.05 | 0.07 ± 0.06 | 0.01 ± 0.27 | 0.03 ± 0.02 |
| $t_{1/2}$ (h) serum | 0.26 ± 0.07 | 0.26 ± 0.03 | 6.72 ± 2.189 | 6.77 ± 2.013 | 0.25 ± 2.846 | 0.43 ± 0.07 |
| $t_{1/2}$ (h) urine | 2.729 ± 0.17 | 4.479 ± 1.137[a] | 10.656 ± 4.044 | 26.364 ± 15.989 | 7.903 ± 1.571 | 6.107± 1.843[a] |
| MRT (h) | 0.33 ± 0.11 | 0.12 ± 0.09[a] | 8.856 ± 2.489 | 9.409 ± 3.163 | 0.08 ± 0.17 | 0.11 ± 0.01 |

The differences in certain pharmacokinetic parameters between pinocembrin, pinostrobin and liquiritigenin enantiomers demonstrate that these flavonoids may be stereospecifically metabolized. For instance, the significantly higher distribution space (Vss) of R-liquiritigenin than S-liquiritigenin resulting in a correspondingly higher serum elimination half-life. The dramatically increased $f_e$ of S-liquiritigenin (24.943 ± 10.341%) compared to that of R-liquiritigenin (0.157 ± 10.341%) denotes that a much larger percentage of S-liquiritigenin is excreted unchanged in the urine compared to its counterpart. These differences may be partially attributed to the observed deeper penetration into tissues (higher $V_{ss}$) of S-liquiritigenin, which are potentially attributable to stereospecific differences in tissue binding affinity. Additionally, the existence of stereopspecific renal transport is a potential explanation for this finding, though no studies currently exist. For pinocembrin and pinostrobin, the R-enantiomers showed significantly higher $f_e$ values, but only insignificant slightly higher clearances, and volumes of distribution ($V_{ss}$). Further, S-pinocembrin showed significantly greater urine $\lambda$ 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 non-renal clearance for all three 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.[68] 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). For our compounds of interest, it was observed that the volume of distribution ($V_{ss}$) of R-pinostrobin and S-pinostrobin was 16.591 ± 7.066 L/kg and 17.782 ± 7.322 L/kg (Table 6.1). 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 and liquiritigenin enantiomers since they also have significantly large $V_{ss}$ values. R-pinocembrin has an observed $V_{ss}$ of 1.872 ± 0.312 L/kg while S-pinostrobin a $V_{ss}$ of 1.831 ± 0.092 L/kg. Similarly R-liquiritigenin with a $V_{ss}$ of 6.43 ± 0.009 L/kg and S-liquiritigenin a $V_{ss}$ of 4.67 ± 7.816 L/kg. These similarly large volumes of distribution ($V_{ss}$) values correlate with the lipophilic nature of these compounds (XLogP values of 2.7, 3.1, and 2.2 for pinocembrin, pinostrobin, and liquiritigenin, respectively), which might indicate their preferential binding to tissues and preference to reside in the body.

It was also observed that the three flavonoids are predominately cleared via non-renal elimination (fraction excreted in urine, $f_e$ of 0.3-0.6%, 2-4.5%, and 0.2% for both pinocembrin, pinostrobin and R-liquiritigenin enantiomers, respectively) with the one exception of S-liquiritigenin, which showed an $f_e$ of 24.943 ± 10.341 %(Table 6.1).

The glucuronidated metabolites of pinocembrin, pinostrobin, and liquiritigenin previously identified in serum were also detected in the urine samples (Figure 6.4, 6.5, 6.6).

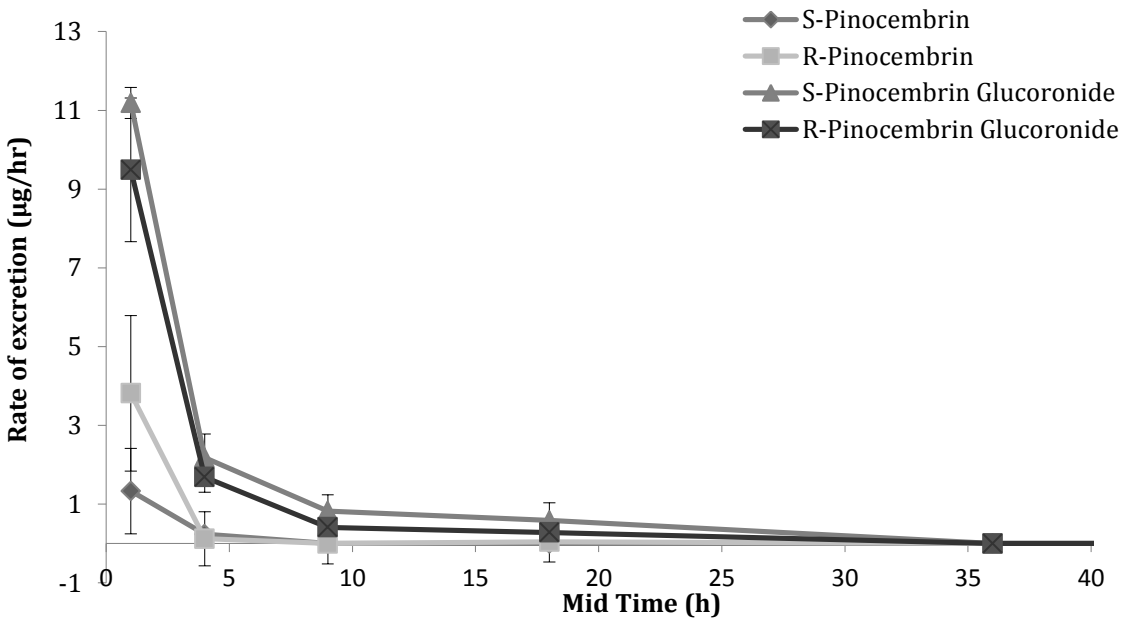


**Figure 6.4.** Rate of urinary excretion of free and glucuronidated pinocembrin enantiomers (µg) excreted in urine following intravenous administration of racemic pinocembrin (20 mg/kg) in rats (n=4, mean ± SEM).

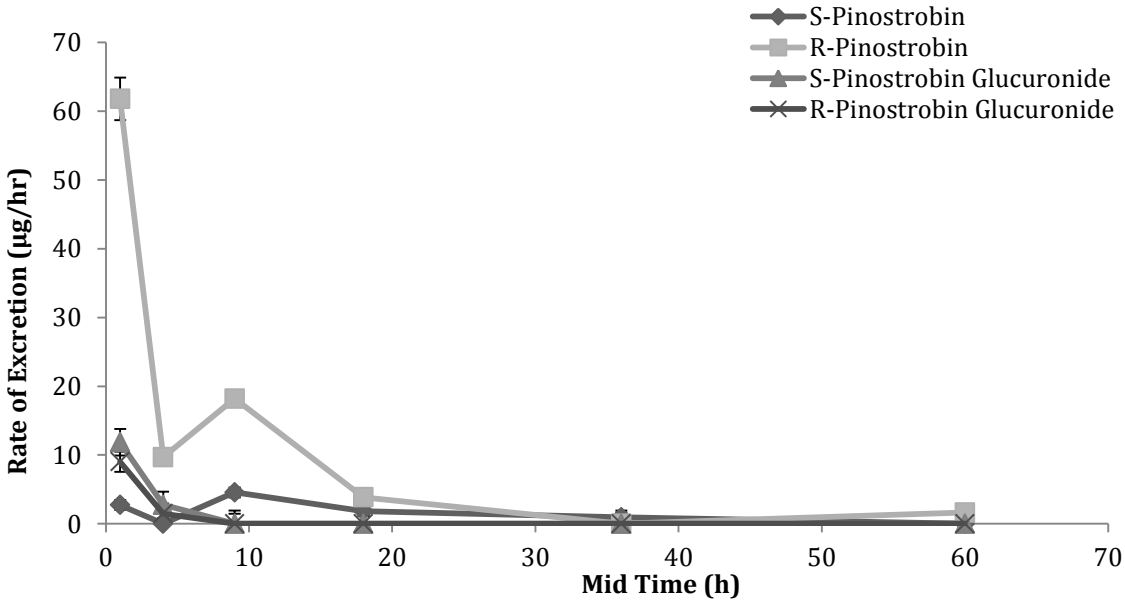


**Figure 6.5.** Rate of urinary excretion of free and glucuronidated pinostrobin enantiomers (µg) excreted in urine following intravenous administration of racemic pinostrobin (20 mg/kg) in rats (n=4, mean ± SEM).

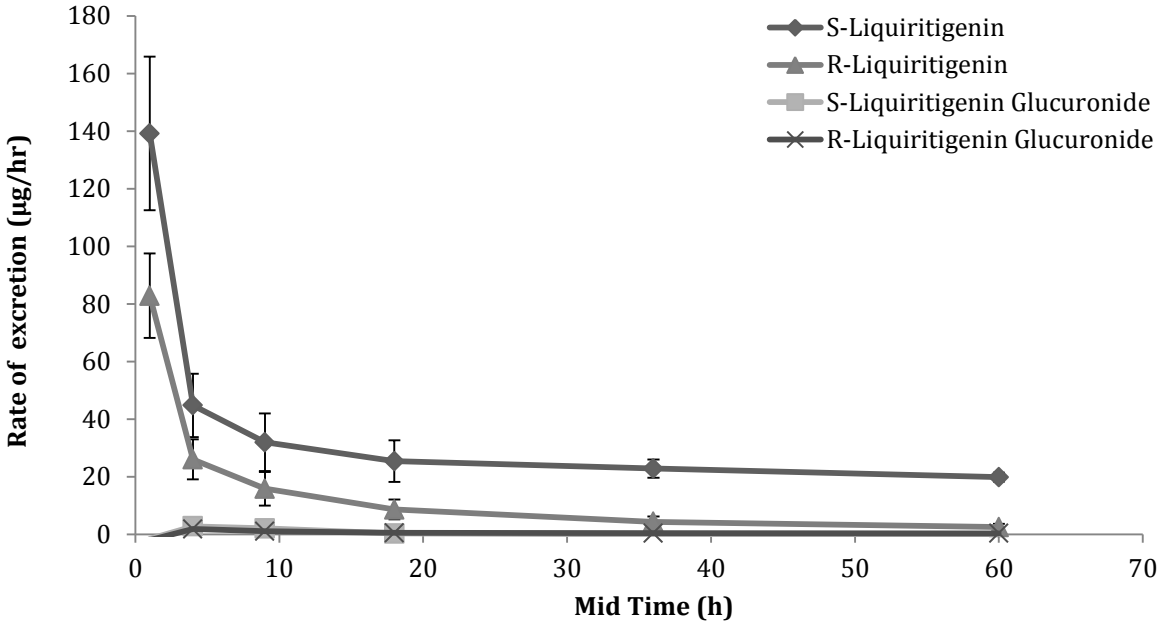


**Figure 6.6**. Rate of urinary excretion of free and glucuronidated liquiritigenin enantiomers (µg) excreted in urine following intravenous administration of racemic liquiritigenin (20 mg/kg) in rats (n=4, mean ± SEM).

In the case of the three 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 (3-80 hours among the three flavonoids) was significantly higher than the plasma half-life (0.25-7 hours among the three flavonoids). For instance, pinocembrin exhibited serum half-lives of 0.262 ± 0.07 h and 0.263 ± 0.03 h for S- and R-pinocembrin, respectively, while the urinary half-lives for S- and R-pinocembrin were 2.729 ± 0.17 h and 4.479 ± 1.137 h, respectively. In the case of pinostrobin, serum half-lives of 6.72 ± 2.189 h and 6.77 ± 2.013 h were observed for S- and R-pinostrobin, respectively, while the urinary half-lives for S- and R-pinostrobin were 10.656 ± 4.044 h and 26.364 ± 15.989 h, respectively. Similarly, the observed half-lives for S- and R-liquiritigenin were 0.53 ± 2.846 h and 1.548 ± 0.07 h, respectively, while the urinary

half-lives were 79.903 ± 1.571 h and 61.078 ± 1.843 h for S- and R-liquiritigenin, respectively (Table 6.1).

These discrepancies suggest that the serum half-life is likely significantly underestimating the overall half-life of pinocembrin, pinostrobin, and liquiritigenin 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).[64,65] Furthermore, it needs to be mentioned that the compounds of interest (pinocembrin, pinostrobin, and liquiritigenin) 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 6.1). It can be observed that employing serum significantly underestimates $AUC_{inf}$, and half-life, while λ was overestimated. Thus, urine may provide more significant pharmacokinetic parameters indicating that these chiral flavonoids have long half-lives up to 80 h) and high volumes of distribution suggesting they considerably distribute out of the vasculature.

The total cumulative urinary excretion plots (Figure 6.7, 6.8, 6.9) indicate that these three flavonoids undergo considerable phase II metabolism due to the significantly higher excretion of glucuronidated metabolites compared to the parent compounds. However, based on the low fraction excreted in urine ($f_e$ values of 0.3-0.6%, 2-4.5%, and

0.2% for both pinocembrin, pinostrobin and R-liquiritigenin enantiomers, respectively), it can be presumed that the majority of these compounds will exit the blood to be distributed into tissues ($V_{ss}$ values of 4-9 L/kg) and eventually be eliminated via non-renal routes, with the exception of S-liquiritigenin ($f_e$ 24.943 ± 10.341) (Figure 6.7, 6.8, 6.9).

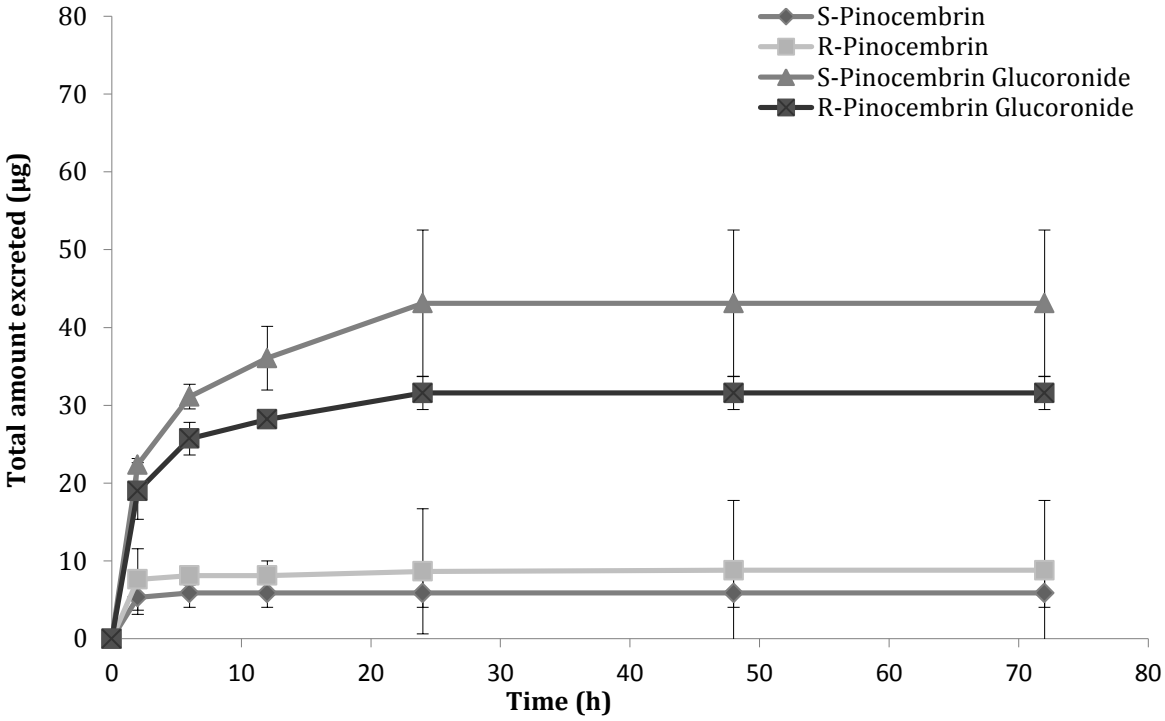


**Figure 6.7.** Cumulative free and glucuronidated pinocembrin enantiomers (µg) excreted in urine following intravenous administration of racemic pinocembrin (20 mg/kg) in rats (n=4, mean ± SEM).

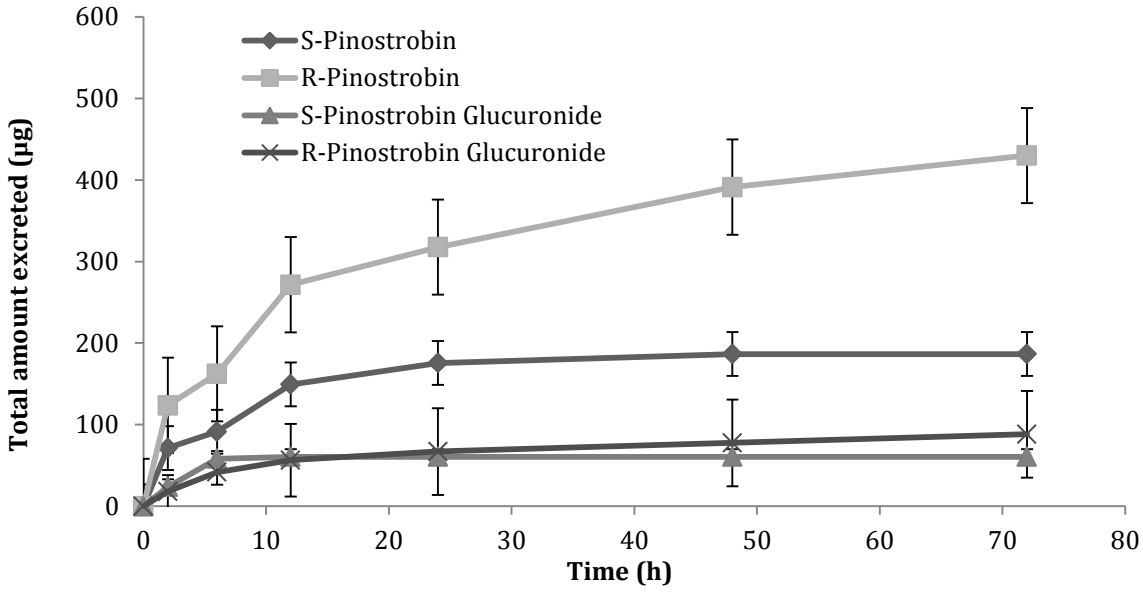


**Figure 6.8.** Cumulative free and glucuronidated pinostrobin enantiomers (µg) excreted in urine following intravenous administration of racemic pinostrobin (20 mg/kg) in rats (n=4, mean ± SEM).

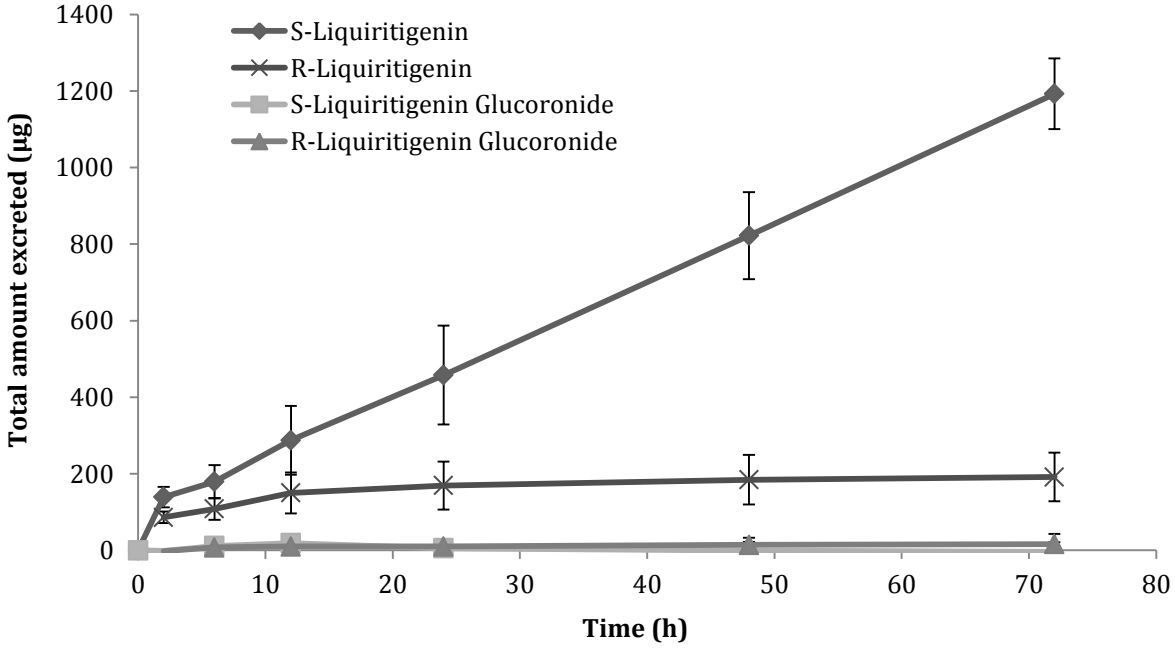


**Figure 6.9.** Cumulative free and glucuronidated liquiritigenin enantiomers (µg) excreted in urine following intravenous administration of racemic liquiritigenin (20 mg/kg) in rats (n=4, mean ± SEM).

In the rat pinocembrin, pinostrobin, and liquiritigenin are rapidly being metabolized to different glucuronides. Despite the differences in solubility and lipophilicity nature of these compounds (parent and glucuronidates compounds) it can be observed that they have similar rates of excretion (Figure 6.4, 6.5, 6.6). 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.

### *6.3.2 Stereospecific Pharmacokinetics of Oral Pinocembrin, Pinostrobin, and Liquiritigenin*

Following oral administration of pinocembrin (100 mg/kg) and liquiritigenin (50 mg/kg), the serum concentration vs. time curves indicated low absorption of free flavonoid (Figure 6.10 and 6.12). 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 (Table 6.2).

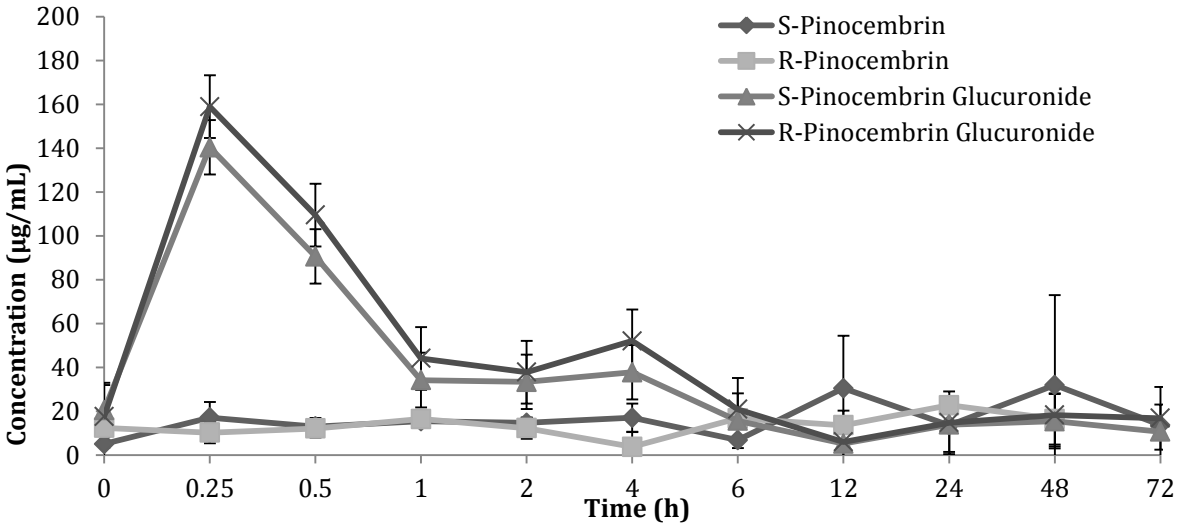


**Figure 6.10.** Concentration-time profile in serum of pinocembrin enantiomers following oral administration of racemic pinocembrin (100 mg/kg) in rats (n=4, mean ± SEM).

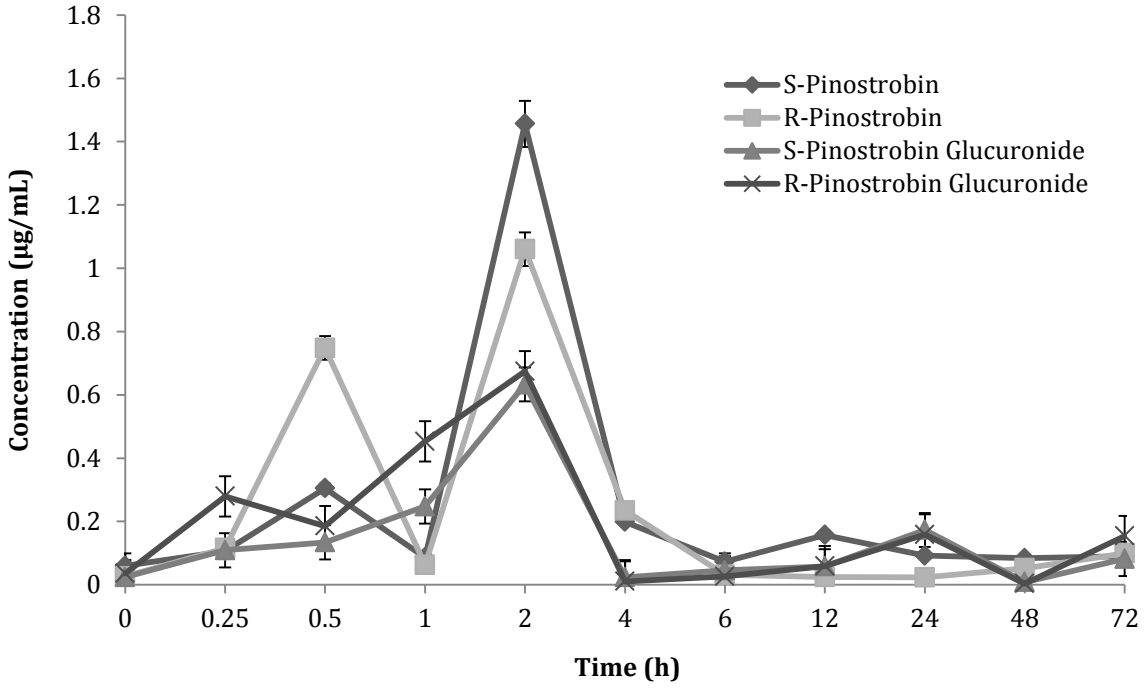


**Figure 6.11.** Concentration-time profile in serum of pinostrobin enantiomers following oral administration of racemic pinostrobin (100 mg/kg) in rats (n=4, mean ± SEM).

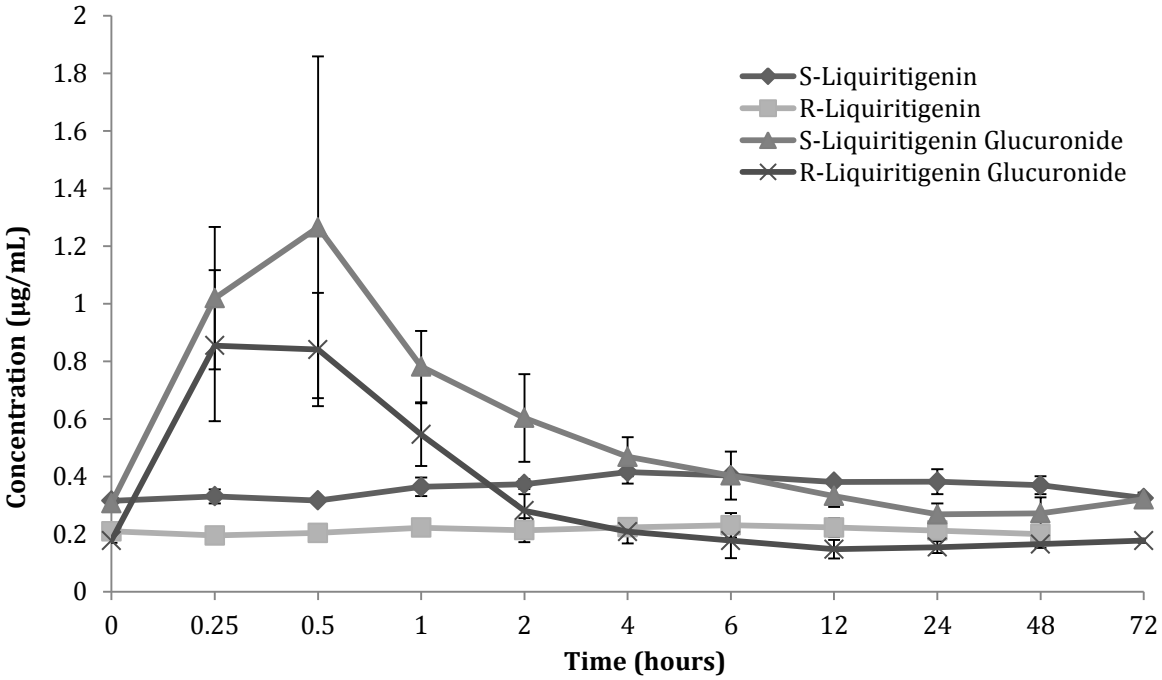


**Figure 6.12.** Concentration-time profile in serum of liquiritigenin enantiomers following oral administration of racemic liquiritigenin (50 mg/kg) in rats (n=4, mean ± SEM).

Pinocembrin, pinostrobin, and liquiritigenin enantiomers were all glucuronidated but to variable degrees. Pinocembrin and liquiritigenin were 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 and liquiritigenin 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 6.2). 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 (430.82 ± 21.354) in comparison to S-pinostrobin (33.83 ± 18.996). Bioavailability (F) was calculated by dividing the AUC of oral administration by the AUC of IV administration with dose adjustment. Significant differences in bioavailability were noted between pinostobin and liquiritigenin enantiomers. The calculated bioavailability of S-pinostrobin was approximately 2% whereas R-pinostrobin showed a calculated bioavailability of approximately 14% (Table 6.2). R-liquiritigenin had a 3 fold greater calculated bioavailabilty compared to S-liquiritigenin (42% vs 14% respectively). 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 and R-liquiritigenin glucuronides seen following enzyme incubation.

The values for $Vd_{ss}$ and CL were also calculated for each flavonoid following oral administration. These parameters vary from those calculated from IV studies. This is expected as 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, pinostrobin, and liquiritigenin remained low at <0.15% indicating very little of the administered doses are excreted in the urine. However, the observed stereospecific difference in $F_e$ of liquiritigenin enantiomers after intravenous administration also occurs with oral administration (11.73 ± 5.938 for S-liquiritigenin 6.81 ± 4.046 for R-liquiritigenin). The half-lives of pinocembrin, pinostrobin, and liquiritigenin enantiomers did not differ significantly and ranged from 4 to 44 hours (Table 6.2). These half-lives following oral administration were longer than those seen after IV administration (0.25-7 h).

**Table 6.2.** Stereospecific pharmacokinetics of pinocembrin, pinostrobin, and liquiritigenin in serum after PO administration in rats (100 mg/kg, 50 mg/kg for liquiritigenin) (mean ± SEM, n=4). [a]Denotes statistical significant difference ($P<0.05$) between enantiomers.

| Pharmacokinetic Parameter | S-pinocembrin | R-pinocembrin | S-pinostrobin | R-pinostrobin | S-liquiritigenin | R-liquiritigenin |
|---|---|---|---|---|---|---|
| $AUC_{inf}$ (μg*h/mL) | 570.27 ± 21.709 | 530.82 ± 82.061 | 33.83 ± 18.996 | 430.82 ± 21.354[a] | 128.36 ± 10.538 | 452.631 ± 36.157[a] |
| $Vd_{ss}/F$ (L/kg) | 3.80 ± 1.344 | 5.14 ± 1.805 | 67.13 ± 26.114 | 156.50 ± 30.423[a] | 2.53 ± 2.128 | 1.30 ± 1.003 |
| $CL_{total}/F$ (L/h/kg) | 2.82 ± 0.0842 | 2.83 ± 0.844 | 0.62 ± 0.19 | 6.27 ± 4.256[a] | 0.7 ± 0.85 | 1.45 ± 0.0845[a] |
| $F_e$ (%) | 2.2 ± 0.394 | 2.33 ± 0.565 | 0.75 ± 0.25 | 0.88 ± 0.38 | 11.73 ± 5.938 | 6.81 ± 4.046 |
| F (%) | 43.237 ± 1.22 | 57.8 ± 14.41 | 1.835 ± 1.427 | 13.835 ± 3.417[a] | 14.202 ± 7.314 | 41.763 ± 5.167[a] |
| $t_{1/2}$ (h) serum | 20.31 ± 8.408 | 27.07 ± 18.834 | 31.90 ± 5.624 | 44.22 ± 8.235 | 4.183 ± 14.537 | 6.257 ± 20.427 |

With respect to pinocembrin, this may in part be due to the higher dose 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-life of pinocembrin. Another, more likely, 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, pinostrobin, and liquiritigenin are demonstrating solubility-limited absorption where the rate of absorption is slowed due to poor solubility in the gastrointestinal tract.

Urine sample analysis following oral administration showed the presence of both the parent and glucuronide conjugate forms. The total cumulative urinary excretion plots following oral administration are reported in Figures 6.13, 6.14, and 6.15 for pinocembrin, pinostrobin, and liquiritigenin espectively. All three compounds are excreted predominantly in the glucuronidated form which parallels the results seen after IV administration of these compounds.

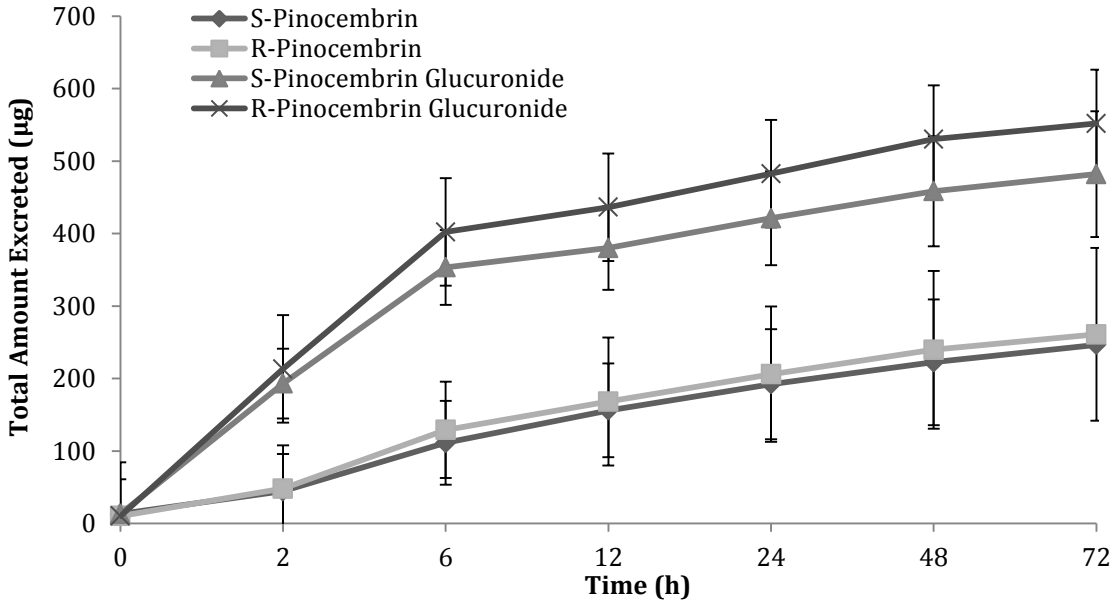


**Figure 6.13.** Cumulative free and glucuronidated pinocembrin enantiomers (µg) excreted in urine following oral administration of racemic pinocembrin (100 mg/kg) in rats (n=4, mean ± SEM).

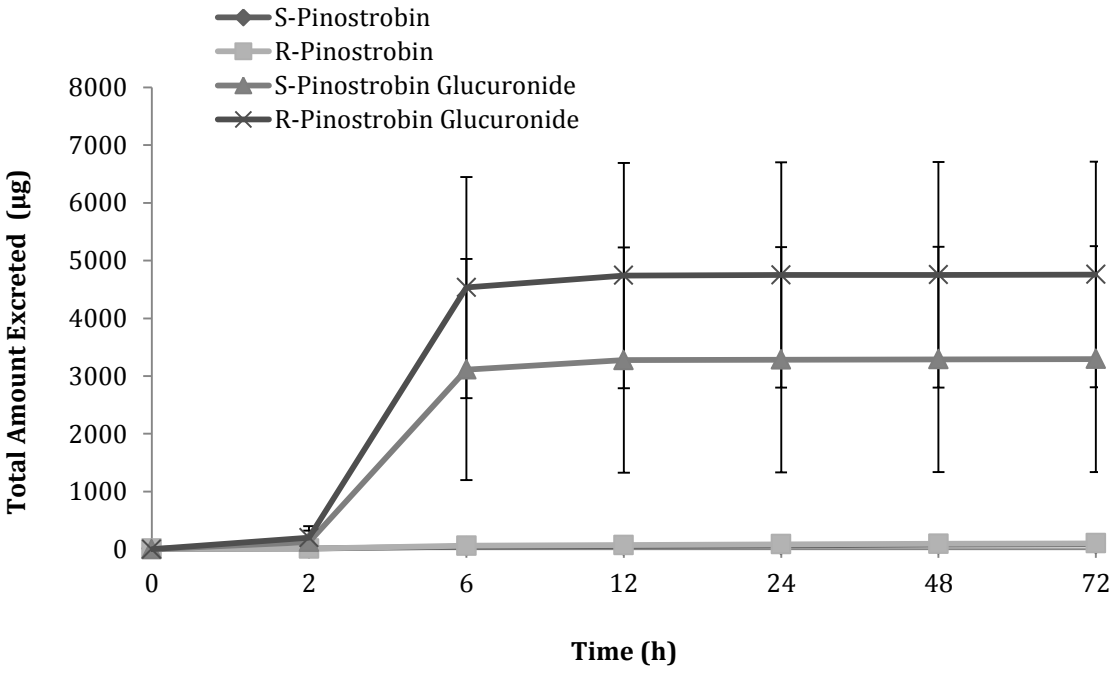


**Figure 6.14.** Cumulative free and glucuronidated pinostrobin enantiomers (µg) excreted in urine following oral administration of racemic pinostrobin (100 mg/kg) in rats (n=4, mean ± SEM).

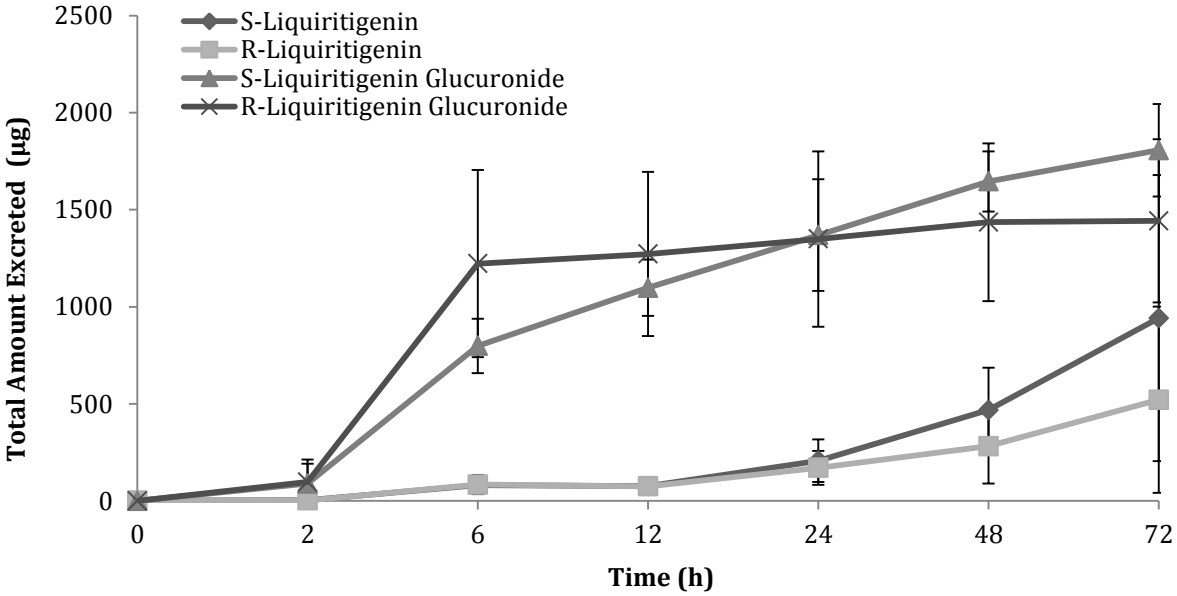


**Figure 6.15.** Cumulative free and glucuronidated liquiritigenin enantiomers (µg) excreted in urine following oral administration of racemic liquiritigenin (50 mg/kg) in rats (n=4, mean ± SEM).

The rate of urinary excretion plots for pinocembrin (Figure 6.16), pinostrobin (Figure 6.17), and liquiritigenin (Figure 6.18) 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.

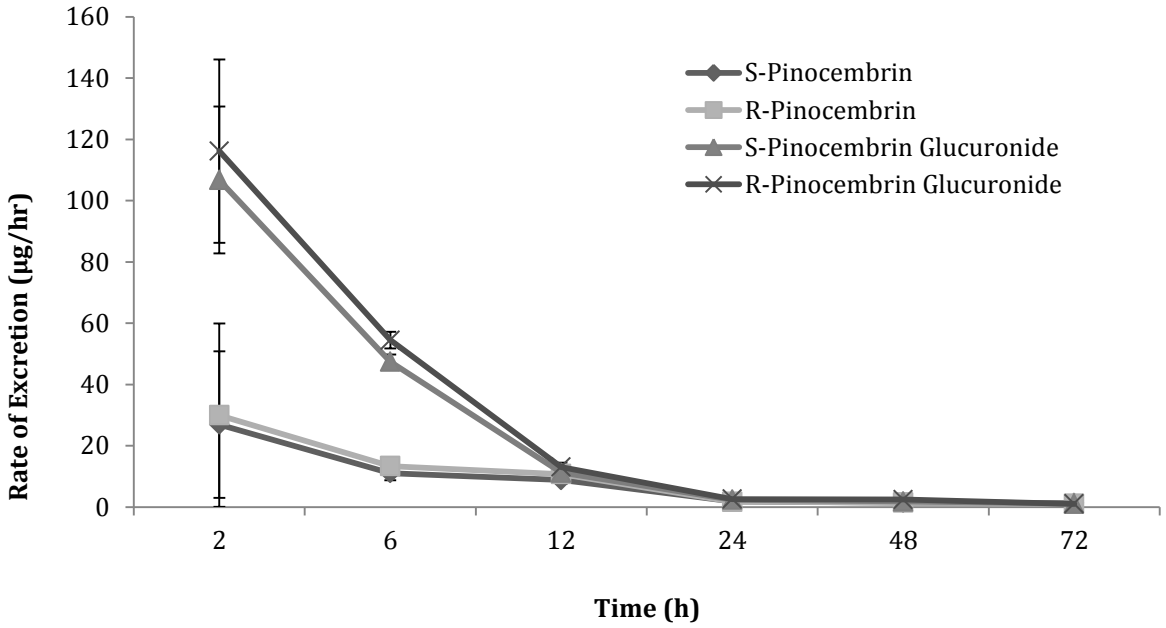


**Figure 6.16.** Rate of urinary excretion of free and glucuronidated pinocembrin enantiomers (µg) excreted in urine following oral administration of racemic pinocembrin (100 mg/kg) in rats (n=4, mean ± SEM).

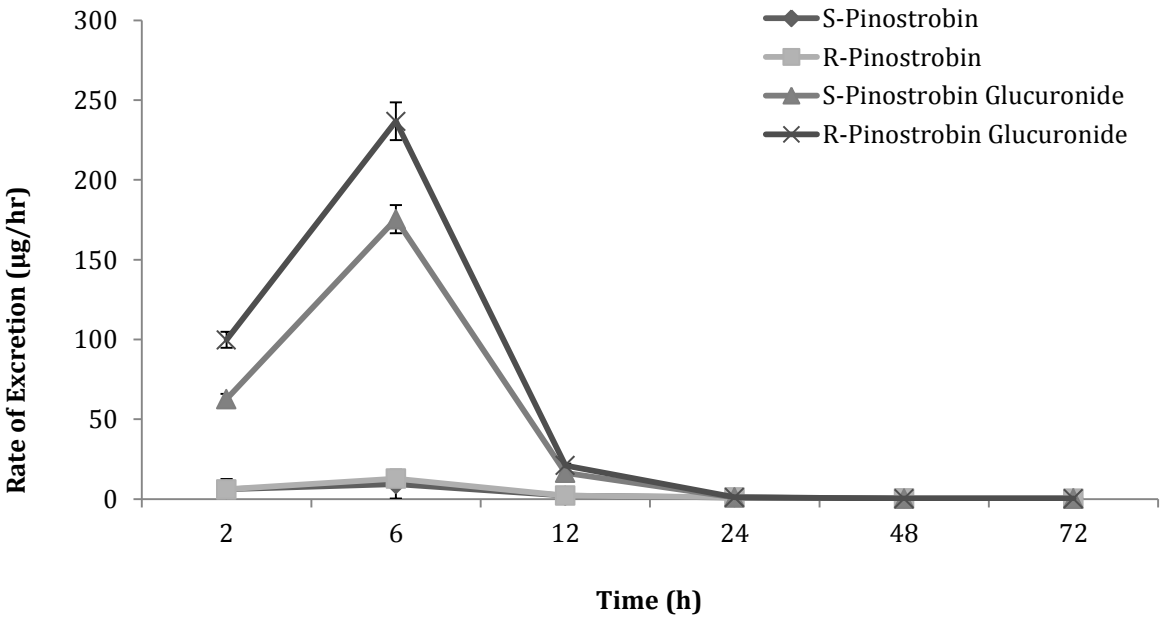


**Figure 6.17.** Rate of urinary excretion of free and glucuronidated pinostobin enantiomers (µg) excreted in urine following oral administration of racemic pinostrobin (100 mg/kg) in rats (n=4, mean ± SEM).

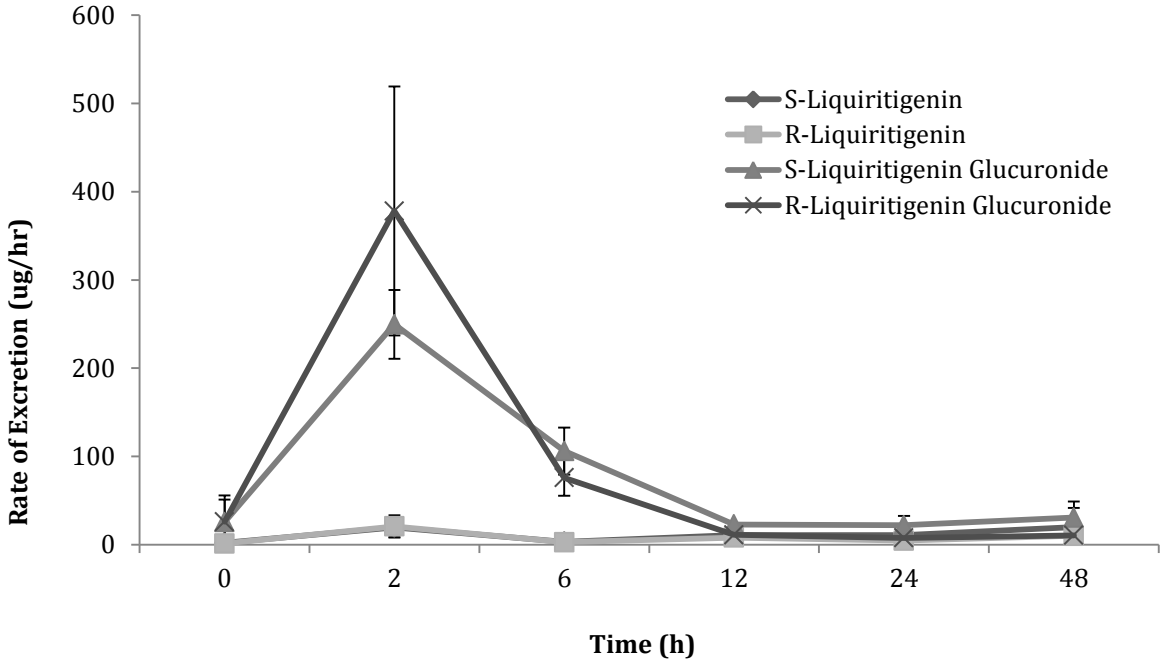


**Figure 6.18.** Rate of urinary excretion of free and glucuronidated liquiritigenin enantiomers (µg) excreted in urine following oral administration of racemic liquiritigenin (50 mg/kg) in rats (n=4, mean ± SEM).

## 6.4 Discussion

As has been stated, pharmacokinetic characterization and analysis of pinocembrin, pinostrobin, and liquiritigenin 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.[44] 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 6.1). Additionally, Yan *et al.* have recently assessed the achiral pharmacokinetics of pinocembrin after a 20 mg IV infusion over 30 minutes in humans.[43] The authors report a half-life of 47.4 ± 14.0 minutes, a $V_d$ (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 = 5.52 L/kg/h) would equal 6.9 L/min or 5.4 – 5.8 L/kg/h which parallels the results of the human study. 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.[69] In this study, the reported half life was 4.34 ± 0.24 minutes, the AUC $_{0\text{-}t}$ was 3817.80 ± 352.89 ng min/mL, 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 ± 5.624 h, R-pinostrobin 54.22 ± 8.235 h). Additionally, female Kunming rats were used rather than male Sprague-Dawley which were employed in our studies. Achiral liquiritigenin pharmacokinetics have been fairly well characterized. Many of the studies monitor the pharmacokinetics of liquiritigenin and other components of complex botanical mixtures or single plant extracts, making it difficult to know the true dose of pure liquiritigenin administered. Kang *et al.* utilizing intravenous dosing of liquiritigenin 20 mg/kg reported approximate plasma half-lives of 5-8 minutes, clearances of 50-70 mL/min/kg, $V_{ss}$ 240-190 mL/kg and AUC of 290-410 ug min/mL.[53,54] Data from the present study parallel these results with respect to AUC (280-385 ug min/mL) and clearance (50-30 mL/min/kg) but also demonstrate a comparitively greater half-life

(approximately 15 – 30 min). Pharmacokinetic parameters of orally administered liquiritigenin given alone or in a mixture (exact dose unknown) are highly variable between existing studies with approximate plasma half-lives of 3-11 h, and AUC of 19-6594 ug h/L. [46,47,49,53-55] The bioavailability reported by Kang *et al.* was 4-6% which is lower than the bioavailability reported herein.[54,70] This discrepancy in bioavailability likely results from differences in formulation. For IV and oral formulations, Kang *et al.* dissolved liquiritigenin in a 40:60 ratio of PEG 400:distilled water. These formulations presumably vary greatly from the PEG 600 and DMSO used in these studies which may alter the aqueous solubility and dissolution of liquiritigenin. Li *et al.* conducted the only study to date with respect to stereospecific pharmacokinetics of liquiritigenin in urine in humans after oral administration of a botanical mixture.[59] The authors reported fraction excreted unchanged in the urine ($f_e$ %) of 36.2 ± 19.0 S-liquiritigenin compared to 50.9 ± 21.6 R-liquiritigenin. The data presented here show significant stereoselectivity with an $f_e$ (%) 11.73 ± 5.938 S-liquiritigenin compared to 6.81 ± 4.046 after oral administration and 24.943 ± 10.341 S-liquiritigenin compared to 0.157 ± 10.341 after intravenous administration. It is possible that species dependant pharmacokinetics and stereoselective urinary excretion are responsible for these differences. All three flavanones examined pinocembrin, pinostrobin, and liquiritigenin each undergoes glucuronidation upon intravenous administration, as determined by serum and urine concentrations and later verified by treating plasma and urine samples with β-glucuronidase. In the case of liquiritigenin these data parallel findings previously mentioned.[53,54]

To our knowledge, this is the first literature report that has assessed the stereospecific pharmacokinetics of pinocembrin, pinostrobin, and liquiritigenin after

intravenous and oral administration of the pure racemates. Previous studies save one[59] have focused only on the racemic mixtures and utilized achiral analysis after oral ingestion. Our findings indicate that these three 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.[64,65] 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 through potential abberant calculations of $t_{1/2}$. This may occur because IV dosing concentrations are very close to the limit of detection, which may lead to the observation of only the distribution phase. Underestimation of plasma half-life due to assay sensitivity limits has been reported before in the case of procainamide.[66] 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 may beunderestimating the overall half-life of pinocembrin, pinostrobin, and liquiritigenin enantiomers due to assay sensitivity limits in serum. However, there are other statistical methods, such as the Fisher information, to weigh the data and account for poor method sensitivity. 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, pinostrobin, and liquiritigenin 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, 3.1, and 2.2 for pinocembrin, pinostrobin, and liquiritigenin, 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 three compounds are mainly excreted via non-renal routes, which is also verified by their generally low fraction excreted in urine ($f_e$) values of 3-7% (Table 6.1), except for S-liquiritigenin (24%). Based on these pharmacokinetic data, pinocembrin, pinostrobin, and liquiritigenin appear to be exiting the vasculature and distributing to the different tissues in the body.

## 6.5 Conclusions

The stereospecific pharmacokinetics of intravenously and orally administered pinocembrin, pinostrobin, and liquiritigenin in rats are reported for the first time within this chapter. These studies indicate that pinocembrin, pinostrobin, and liquiritigenin 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.

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# Chapter VII: In vitro Pharmacodynamic Characterization of Selected Chiral Flavonoids: Pinocembrin, Pinostrobin, and Liquiritigenin

## 7 Abstract

Pinocembrin, pinostrobin, and liquiritigenin belong to a subclass of flavonoids called flavanones – suspected to have particular involvement in the prevention of heart disease, colon cancer, and diabetes. Several potential mechanisms for the role of flavonoids and their subclasses in chronic disease prevention have been reported. To elucidate the potential role of these flavanones in heart disease, diabetes, and colon cancer, the bioactivity of racemic pinocembrin, pinostrobin, and liquiritigenin was measured in several *in vitro* assays with pathophysiological and pathological roles in heart disease, colon cancer, and diabetes etiology and pathophysiology. Pure enantiomeric forms of each flavonoid are also tested, where possible, to identify the stereospecific contribution to bioactivity.

## 7.1 Introduction

The epidemiological evidence for association of fruit and vegetable intake with decreased incidence and risk of chronic disease has been the major motivator for the search for active ingredients in the hopes of providing a reliable way to reproduce the observed benefits in future populations.[1] Over time, much of the literature has gone from speaking broadly in terms of fruits and vegetables, then to flavonoids, then to flavonoid subclasses, and finally individual flavonoids with respect to identifying the active ingredient.[1] Unfortunately, the broad and inclusive categories of causative agent has made it more difficult to take advantage of any observed benefits. In fact, investigating a potential therapeutic, the active ingredient of which is one or more compounds in a class of at least 4,000[1] with six different subclasses, not to mention chiral forms and metabolites, effectively creates a barrier to further development of these compounds. Previous studies have suggested some pharmacological activity for pinocembrin, pinostrobin, and liquiritigenin including anti-cancer, anti-inflammatory, anti-diabetic, and anti-oxidant activities (Chapter I).

When compounds with similar actions are present together, the combined effect may be predicted by additivity if the individual drugs are equally active. However, the effect of a combination of some drugs such as enantiomers can be exaggerated or attenuated. The exaggerated effect is termed synergistic, whereas the blunted effect is termed sub-additive or antagonistic.[2] In each of these cases, the individual compounds may contribute differentially to the effect. Assessment of activity that departs from additivity suggests that some kind of interaction is occurring when both compounds are present together.

Therefore, understanding the role of each flavonoid enantiomer in the pharmacological activity described for the racemic mixtures as well as the differences between the activity of aglycones and glycosides is important. One of the inherit difficulties of assessing the individual activity of enantiomers can be their lack of commercial availability. The degree of purity of the enantiomer is also critically important. The methods of stereoseparation usually require high resolution of the assay to obtain stereochemically pure enantiomers.

This chapter describes and compares the anti-diabetic, anti-oxidant, cardioprotective COX inhibitory and anti-cancer activities of pinocembrin, pinostrobin, and liquiritigenin and their enantiomers where possible.

## 7.2 Methods

### *7.2.1 Separation and Collection of Pure Enantiomers*

The limited commercial availability of pure enantiomers of pinocembrin, pinostrobin and liquiritigenin made necessary the manual separation and collection of pure enantiomers using the analytical methods described earlier in Chapters II, III, and IV. The collection of the pure enantiomers occurred following chiral chromatographic separation of multiple injections of racemic pinostrobin and liquiritigenin. 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 pinostrobin and liquiritigenin enantiomers

to verify their identity also limited the number of stereospecific pharmacological studies completed. CD spectra obtained for pinostrobin and liquiritigenin enantiomers were consistent with previously published data and are shown in the apendix.[3] Eventually as the project progressed, pure S-pinostrobin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and pure R-pinocembrin was procured via a custom synthesis by Toronto Research Chemicals (Toronto, ON, Canada).

### *7.2.2 Anti-diabetic activity*

#### 7.2.2.1 Chemicals and Reagents

Pinocembrin and liquiritigenin racemic mixtures were purchased from Extrasynthese (Genay Cedex, France). Racemic pinostrobin was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). Dimethyl sulfoxide (DMSO), poly(ethylene glycol) (PEG) 400, phosphate buffered saline (PBS), α-amylase from porcine pancreas type VI-B, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), and 4-nitrophenyl α-D-glucopyranoside were purchased from Sigma-Aldrich (St. Louis, MO, USA). Amylase HR reagent was purchased from Megazyme International Ireland (Wicklow, Ireland).

#### 7.2.2.2 α – Amylase inhibition assay preparation

Methanol was used to dissolve racemic pinocembrin, pinostrobin, and liquiritigenin as well as S-pinocembrin and R-pinocembrin at concentrations between 0 and 200 μg/mL. Subsequent experiments with pinostrobin and liquiritigenin used methanolic dilutions of the racemates and enantiomers at concentrations from 0 to 1 μg/mL. Amylase HR Reagent (100 μL) was prepared following directions accompanying the reagent,

Megazyme Amylase HR Reagent cat. No. R-AMHR4. This was added with 40 µL of flavonoid in methanol to a 96-well plate (40 µL of blank methanol in control wells). After incubation for 5 minutes at 37ºC, 60 µL of 0.1 mg/mL α-amylase in 0.1 M HEPES buffer (pH 6.9) was added to the plate wells. Additional incubation at 37ºC for 10 min followed, whereupon 20 µL of 3.25 M sodium hydroxide was added to each well to stop the reaction. The assay was performed in sextuplicate.

#### 7.2.2.3 α – Amylase inhibition assay description

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.[4] 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.

#### 7.2.2.4 α– Glucosidase inhibition assay preparation

Pinocembrin, pinostrobin, and liquiritigenin 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), 25µL of 20 mM p-nitrophenyl-α-D-glucopyranoside (PNPG) in phosphate

buffer, and 10µL of the flavonoids in DMSO were added to a 96-well plate (10µL 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.

#### 7.2.2.5 α– Glucosidase inhibition assay description

α-Glucosidase inhibition was determined through the colorimetric assay mentioned above.[115] 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.

#### 7.2.2.6 Statistical Analysis

Data were presented as mean and standard error of the mean (mean ± S.E.M.). Student's t-test was used to compare unpaired samples using Excel software. A p-value < 0.05 was considered statistically significant.

### *7.2.3 In vitro Cyclooxygenase-1 and -2 (COX) Inhibitory Activity*

#### 7.2.3.1 Chemicals and Reagents

Pinostrobin was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). S-pinocembrin was purchased from Sigma-Aldrich (St. Louis, MO, USA. Pinocembrin and liquiritigenin were purchased from Extrasynthese (Genay, France). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA). The COX Inhibitor Screening Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA, catalog No. 560131).

#### 7.2.3.2 Pre-Assay Preparations

On the day of the experiment racemic flavonoids and S-pinocembrin were dissolved in DMSO to yield concentrations of 1, 10, and 250 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 assess 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 Synergy HT 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 ($\%B/B_0$).

#### 7.2.3.3 Description of the COX Inhibitor Screening Assay

The COX Inhibitor Screening Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA, catalog No. 560131) and it 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.

#### 7.2.3.4 Statistical Analysis

Data were presented as mean and standard error of the mean (mean ± S.E.M.). Student's t-test was used to compare unpaired samples using Excel software. A p-value $< 0.05$ was considered statistically significant.

### *7.2.4 In vitro Anti-oxidant activity*

#### 7.2.4.1 Chemicals and Reagents

Pinostrobin was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). S-pinocembrin was purchased from Sigma-Aldrich (St. Louis, MO, USA. Pinocembrin and liquiritigenin were purchased from Extrasynthese (Genay, France). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA). The Anti-oxidant Assay Kit was purchased from Cayman Chemical Company (Ann Arbor, MI, USA, catalog No. 709001).

#### 7.2.4.2 Pre-Assay Preparations

On the day of the experiment racemic flavonoids 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 Synergy HT 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®.

#### 1.1.1.1 Description of the Anti-oxidant Assay

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, pinostrobin, and liquiritigenin as well as S-pinocembrin was measured.

#### 7.2.4.3 Statistical Analysis

Data were presented as mean and standard error of the mean (mean ± S.E.M.). Student's t-test was used to compare unpaired samples using Excel software. A p-value $< 0.05$ was considered statistically significant.

### *7.2.5 Cell size assay*

#### 7.2.5.1 Chemicals and Reagents

Racemic pinocembrin was purchased from Extrasynthese (Genay Cedex, France). 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 (Carslbad, 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.

#### 7.2.5.2 Cell size assay preparations

Cell culture methodology for ventricular cardiomyocyte plating was derived from previous work described by Wu *et al.*[5] Isolation of cardiomyocytes from ventricles of 1-day-old neonatal Sprague-Dawley rates was accomplished by 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.[6] Cells were cultured for 24 hours before treatment.

#### 7.2.5.3 Cell size assay description

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 permeablized 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 $4^0$C 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.[6,7]

### *7.2.6 In vitro Anti-cancer activity*

#### 7.2.6.1 Chemicals and Reagents

Pinostrobin was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). S-pinocembrin was purchased from Sigma-Aldrich (St. Louis, MO, USA. Pinocembrin and liquiritigenin were purchased from Extrasynthese (Genay, France). 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).

#### 7.2.6.2 Cell Culture

The cell lines used in these series of experiments were HT-29 (human colorectal carcinoma). Cells were obtained from the American Type Culture Association (ATCC, Manassas, VA, USA). All media preparation and other cell culture work were performed

in a laminar flow hood. The blower and UV light in the cell culture hood were turned on 15 - 20 minutes before each use. The working surface was sterilized with 75% ethanol before and after each use. HT-29 cell lines were supplemented with 10% heat-inactivated FBS and were treated with penicillin-streptomycin (10.0 mg/l). Cells were stored in 75 $cm^2$ tissue cell culture flasks (15 cm × 8.5 cm × 3.5 cm, TPP, Switzerland), and incubated at 37°C in a 5% $CO_2$ atmosphere using a Forma Scientific $CO_2$ water jacketed incubator from Thermo Scientific (Waltham, MA, USA).

#### 7.2.6.3 Cell Subculture and Cell Number

Thirty minutes prior to subculturing cell lines, media, PBS, and a trypsin-EDTA solution were placed in a 37°C Precision Scientific Inc. reciprocal shaking water bath (Artisan Scientific Corporation, Champaign, IL, USA). The trypsin-EDTA solution was comprised of 0.5% trypsin and 0.2% EDTA/0.9% NaCl diluted in PBS to prepare a 10% working solution. Next, the cell flask was removed from the 5% $CO_2$ incubator and cells were observed under the light microscope to determine the percent confluency and their general health. A percentage of confluency of 50-75% was desired; after confluency was determined, media were aspirated and the cells were washed with 5.0 ml PBS. After a gentle wash, PBS was aspirated and 1.0 ml of the trypsin-EDTA working solution was added; then the flask was placed in the 5% $CO_2$ incubator for 2 – 4 minutes depending on the cell line. The flask was then removed from the incubator and when cell detachment was confirmed with the light microscope, trypsin-EDTA containing detached cells was transferred to a 15 ml conical tube containing 9.0 ml PBS. The conical tube was centrifuged at 700 r.p.m. for 5 minutes. Following centrifugation, the conical tube was removed and the supernatant was aspirated, leaving the cell pellet undisturbed.

Resuspension of cells was attained by adding 5.0 ml of fresh media followed by careful pipetting for 3 minutes; 10.0 µl of resuspended cells was removed and diluted 4 times in trypan blue. The trypan blue solution was added to a Hausser BRIGHT-LINE counting chamber (1.0 mm deep) from Optic Planet, Inc. (Northbrook, IL, USA) where the number of live cells was verified, and the number of dead cells was recorded. If it was determined that the number of dead cells surpassed 10% of the total population of healthy cells, the cells were excluded from future experiments and a new generation of the cell line was thawed.

The total number of cells in the flask was determined using the following equation:

$$Cells/ml = (\#\ cells/4) \times (dilution) \times (1 \times 10^4)$$

Media containing cells and fresh media were added in determined volumes to a fresh 75 $cm^2$ flask depending on the observed cell number attained with the previous equation, and the desired cell seeding number. The flask was then placed into the 5% $CO_2$ incubator at 37°C. Cell subculture was performed 2-3 times per week depending on the growth rate of each particular cell line and the observed confluency.

The optimal cell seeding numbers for each cell line was determined by preliminary cell seeding number experiments. Cells were seeded in number $1 \times 10^4$, $2 \times 10^4$, $3 \times 10^4$ and so on until a final seeding number of $5 \times 10^4$ per well in a 96-well plate (Costar 3595, Costar Corp., Cambridge, MA, USA) was attained. The 96-well plates were incubated in a 5% $CO_2$ atmosphere for 72 h. After incubation, medium was aspirated; 20.0 µl of 10% Alamar blue (resazurin) fluourescent dye solution was added to the cells. The 96-well plates were incubated at 37°C in 5% $CO_2$ atmosphere for 3 hours,

then removed from the incubator and placed at room temperature in a drawer to protect them from light for 30 minutes. Next, the 96-well plates were placed into a Synergy HT multi-well plate reader (Biotek® Instruments Inc., Winnoski, VT, USA) using Gen 5 software from Biotek®. Fluorescence was read at an excitation of 530 nm and an emission of 590 nm. Standard curves of cell seeding number and fluorescence were generated. The optimal cell seeding number for each cell line to be used in this series of experiments was chosen form the linear portion of the generated curve. All cell lines were seeded at a density of 5,000 cells per well.

#### 7.2.6.4 HT-29 Anti-proliferation Model

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, pinostrobin, liquiritigenin, 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, pinostrobin, and liquiritigenin 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.

#### 7.2.6.5 Description of the Alamar Blue Assay

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, pinostrobin, and liquiritigenin.

#### 7.2.6.6 Data Analysis

Data were analyzed as mean percent of viable cells ± standard deviation in Microsoft Excel®

### *7.2.7 CYP 2D6 Inhibition*

#### 7.2.7.1 Chemicals and Reagents

CYP 2D6 inhibitory activity of racemic pinocembrin, pinostrobin, and liquiritigenin 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.

#### 7.2.7.2 CYP 2D6 Inhibition assay preparations

Concentrations of 0.01, 0.1, 1, 10, 50, and 100 μM of racemic pinocembrin, pinostrobin, and liquiritigenin 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.

#### 7.2.7.3 CYP 2D6 Inhibition assay description

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%.

#### 7.2.7.4 Statistical Analysis

Data were presented as mean and standard error of the mean (mean ± S.E.M.). Student's t-test was used to compare unpaired samples using Excel software. A p-value < 0.05 was considered statistically significant.

## 7.3 Results and Discussion

*In vitro* studies of the pharmacological activity of the stereoisomers of pinocembrin, pinostrobin, and liquiritigenin 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.

*In vitro* studies of each stereoisomer of pinocembrin, pinostrobin, and liquiritigenin are needed to fully characterize their therapeutic potential. Although the isolation of stereoisomers is possible with the validated HPLC methods described in Chapters II – IV, 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 chapter, 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, pinostrobin, and liquiritigenin, and explore the contribution of the flavonoid enantiomers to any observed pharmacological activity. Flavonoid racemates were tested first in each assay to screen for positive dose response curves.

### *7.3.1 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.[8] Flavonoids from several subclasses have been shown to inhibit various subtypes of CYP 450 drug metabolizing enzymes.[9] Pinocembrin, pinostrobin, and liquiritigenin racemates were assessed for their inhibitory activity of CYP 2D6.

#### 7.3.1.1 Pinocembrin

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 (Figure 7.1).

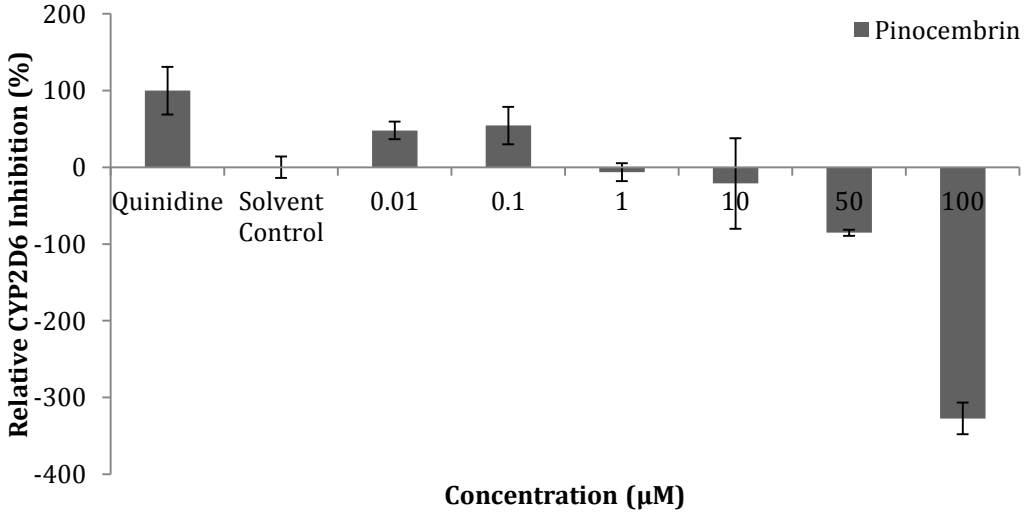


**Figure 7.1.** CYP 2D6 Inhibition (%) of pinocembrin racemate, quinidine (positive control), and blank methanol (solvent control) from 0.01 to 100 μM (n=3, mean ± SEM).

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.[10] Honey was found to have no significant effect on CYP 2D6 activity in this study.[10]

#### 7.3.1.2 Pinostrobin

Similar to pinocembrin, the CYP 2D6 inhibition of racemic pinostrobin was weak and only appeared at low concentrations (Figure 7.2). 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.

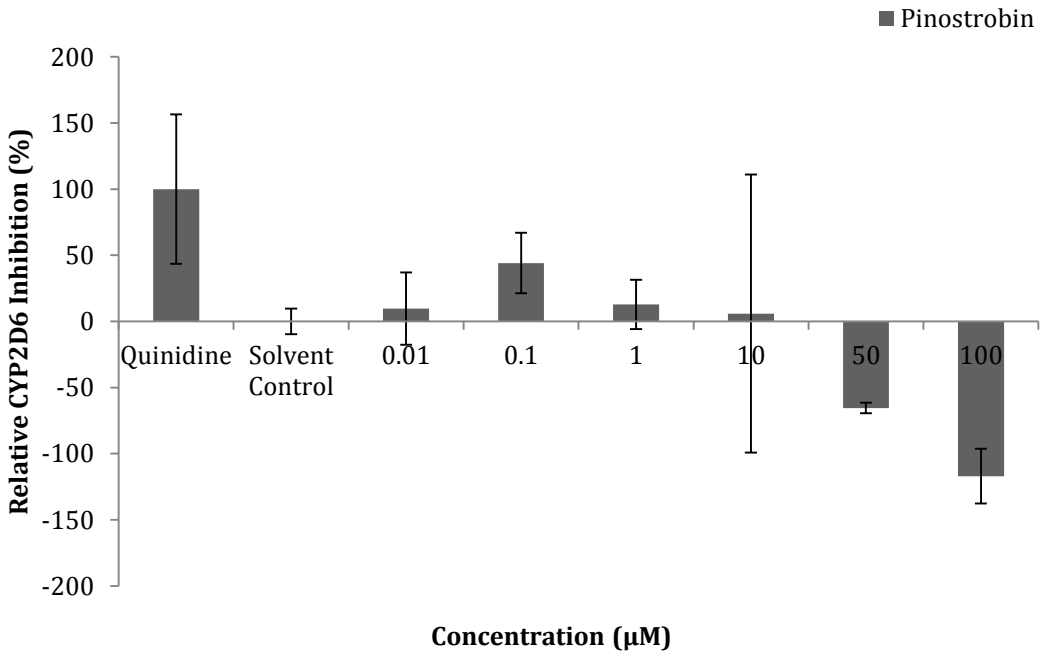


**Figure 7.2.** CYP 2D6 Inhibition (%) of pinostrobin racemate, quinidine (positive control), and blank methanol (solvent control) from 0.01 to 100 μM (n=3, mean ± SEM).

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.[121]

#### 7.3.1.3 Liquiritigenin

The CYP 2D6 inhibitory activity of the liquiritigenin racemate was assessed (Figure 7.3). No significant inhibition was observed at any concentration. Further, neither inhibition nor induction appeared in a dose dependent manner, leaving weak activity and high variability the hallmarks of the assay.

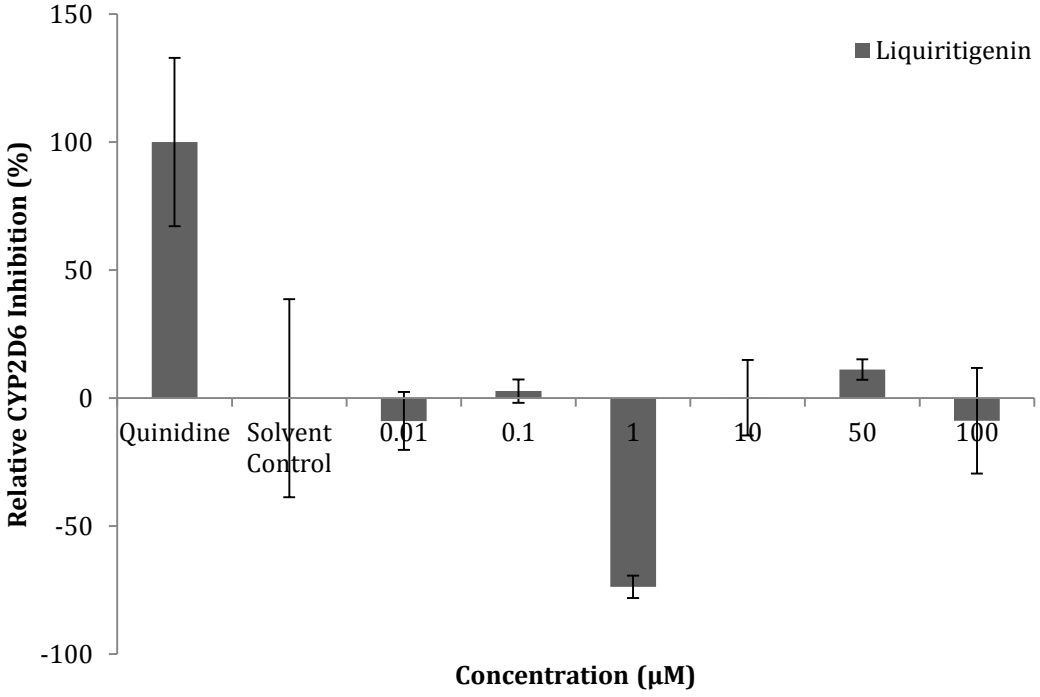


**Figure 7.3.** CYP 2D6 Inhibition (%) of liquiritigenin racemate, quinidine (positive control), and blank methanol (solvent control) from 0.01 to 100 μM (n=3, mean ± SEM).

A previous study reported liquiritigenin to possess overall moderate CYP 2D6 inhibition.[11] However, at 10 μM liquiritigenin showed around 60% activation of CYP 2D6, similar to the effect seen at 1 μM in these results.

After screening racemic pinocembrin, pinostrobin, and liquiritigenin for CYP 2D6 inhibitory activity, the results suggest that pinocembrin and pinostrobin may be weak inhibitors at low concentrations and liquiritigenin is potentially a non-inhibitor. 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.[12] 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 and the high variability seen in the liquiritigenin screen, it was decided that CYP 2D6 inhibition would not be a suitable screen to pursue for investigating individual flavonoid enantiomer activities. However, it should be noted that although CYP 2D6 is considered non-inducible by pharmaceuticals, little is known about the effect of flavonoids on CYP 2D6 non-inducibility, warranting further study.

### *7.3.2 In vitro Anti-Cancer Activity*

Anti-cancer activity has been observed in all three of the compounds mentioned. (Chapters II-IV) Screening for anti-proliferative activity using the Alamar Blue (resazurin) fluorescent cell viability measurement is relatively simple and accurate. The non-fluorescent dye resazurin can be metabolized by viable cells, whereas non-viable cells cannot. Resorufin is the fluorescent metabolite of resazurin, and can be detected

using a plate reader. Relative number of viable cells can be determined using this measurement.

The anti-proliferative activity of racemic pinocembrin, pinostrobin, and liquiritigenin on HT-29 (human colorectal adenocarcinoma) cancer cell line are shown in Figure 7.4**.** 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 (Figure 7.4 A). Racemic pinostrobin showed a slight decrease in cell viability at the low concentrations, however, cell viability increased as concentrations increased (Figure 7.4 B). Racemic liquiritigenin showed moderate anti-proliferative activity that was dose dependent (Figure 7.4 C).

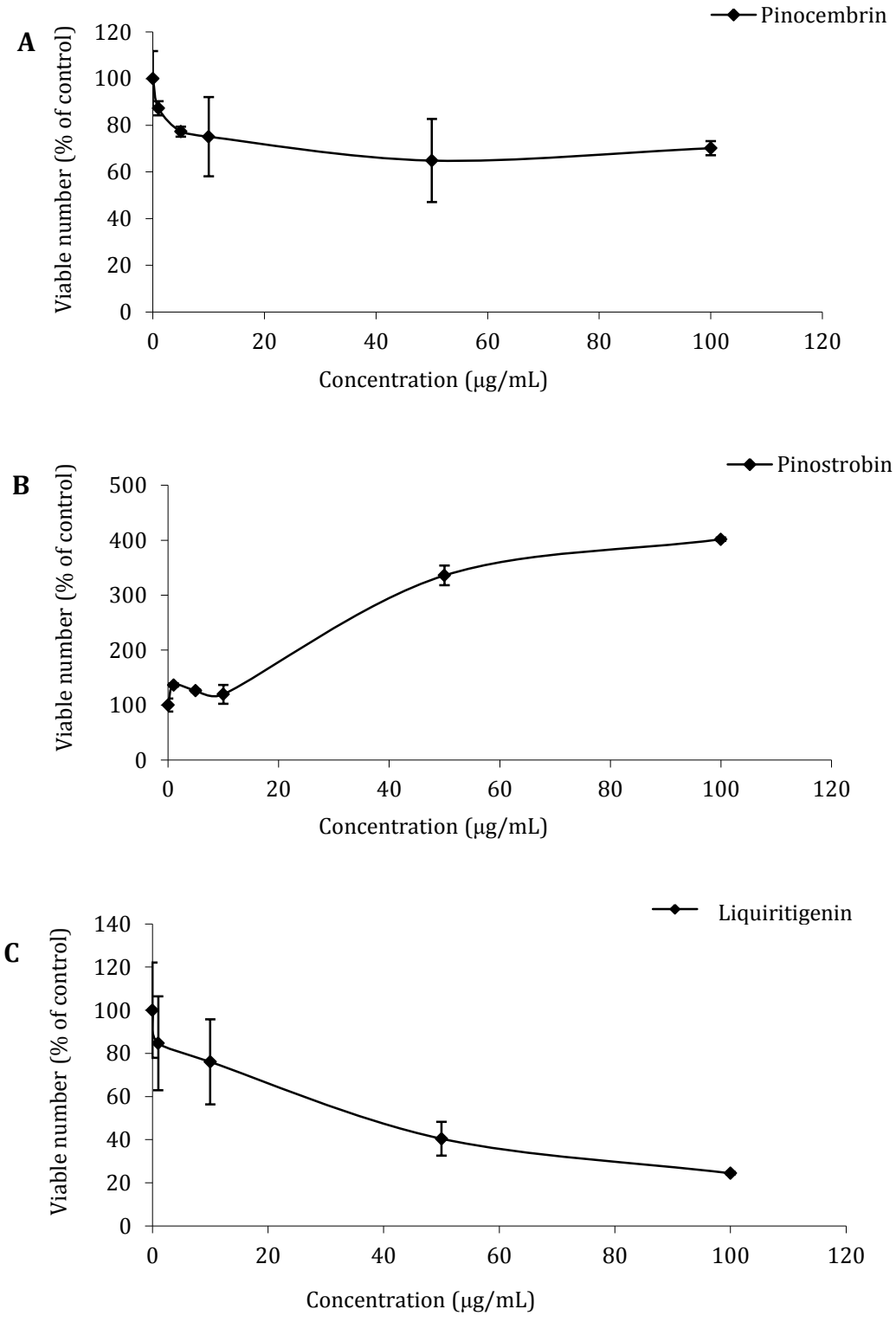


**Figure 7.4.** HT-29 Cell viability after administration of racemic pinocembrin (A), pinostrobin (B), and liquiritigenin (C).

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, pinostrobin, and liquiritigenin. 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 three flavonoids. As observed in other pharmacologic assays using these compounds, unexpected results occurred at high concentrations with respect to pinostrobin. Poor solubility at high concentrations may explain these data.

### *7.3.3 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.[13,14] 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.[15] 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. The anti-oxidant activity assay reported here measures direct anti-oxidant activity via the formation of the radical cation $ABTS^{\bullet+}$ from the oxidation of ABTS by the radical ferryl myoglobin. Inhibitory activity is assessed by the test compounds ability to prevent the oxidation of ABTS to $ABTS^{\bullet+}$ compared to the positive control Trolox® (a water soluble tocopherol analogue). $ABTS^{\bullet+}$ is quantified spectrophotometrically with UV detection.

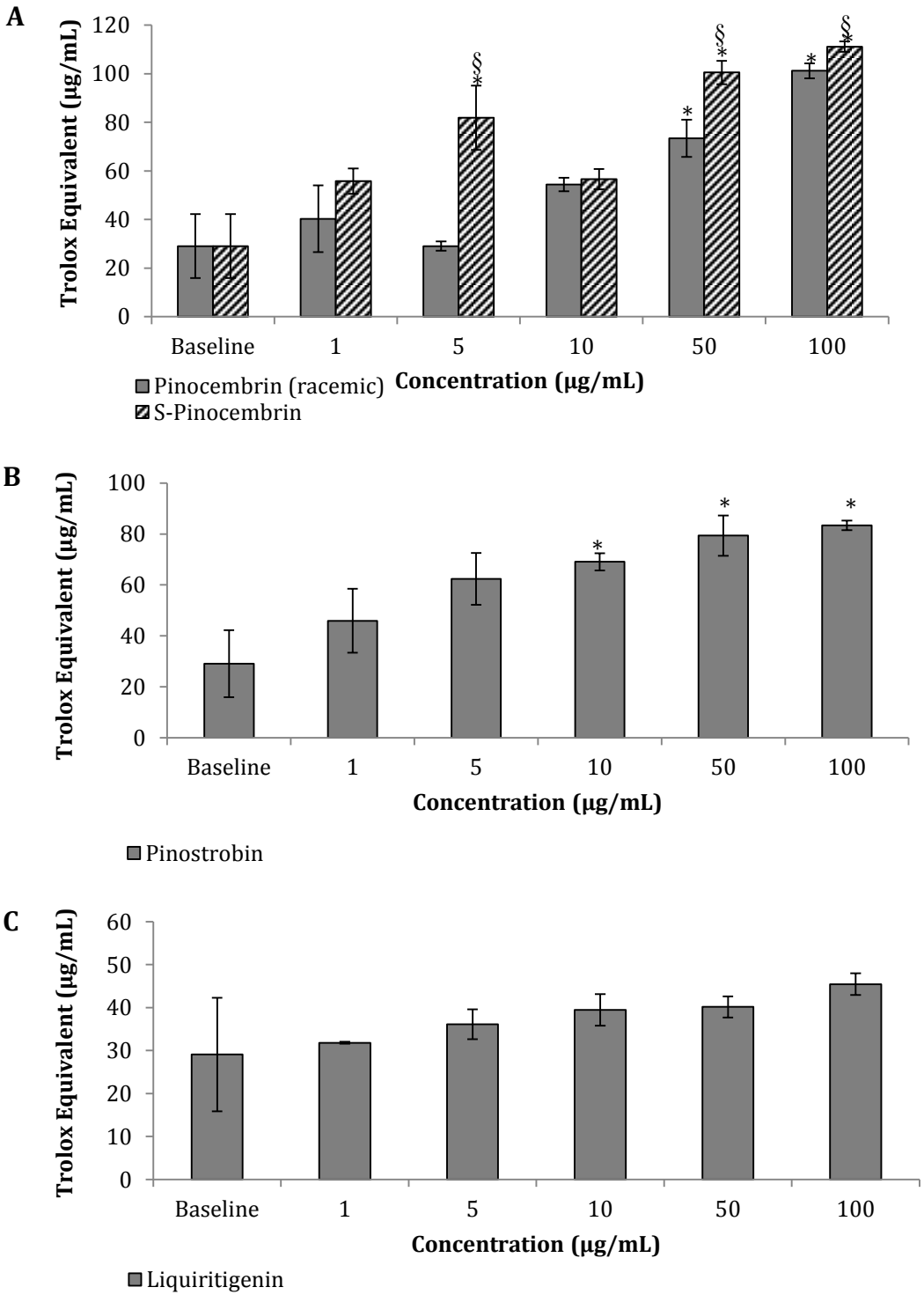


**Figure 7.5.** Anti-oxidant capacity (Trolox® equivalent) of racemic pinocembrin and S-pinocembrin (A), racemic pinostrobin (B), and racemic liquiritigenin(C) (n = 4, mean ± SEM) compared to DMSO alone (baseline). * Represents a significant difference between racemate and baseline antioxidant activity in DMSO alone ($P < 0.05$). § Represents a significant difference between S-pinocembrin and racemic pinocembrin ($P < 0.05$).

Racemic pinocembrin showed anti-oxidant activity greater than baseline at 10, 50, and 100 µg/mL in a dose dependent manner (Figure 7.5 A). S-Pinocembrin showed greater anti-oxidant activity than its racemate at concentrations of 5, 50, and 100 µg/mL (Figure 7.5 A). 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 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 or differences in physical chemical properties such as solubility, which can differ between racemates and pure enantiomers.[16,17] The anti-oxidant activity of the pinostrobin racemate was significantly greater than baseline at the three highest concentrations tested (Figure 7.5 B). Racemic liquiritigenin did not show anti-oxidant activity greater than baseline over the concentration range tested (1 – 100 µg/mL) as is shown in Figure 7.5 C. 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.

### *7.3.4 Anti-diabetic Activity*

α-Amylase and α-glucosidase are carbohydrate hydrolyzing enzymes located in the gastrointestinal tract. Before glucose can be readily absorbed into the blood stream, it must first be liberated from structural forms of higher complexity such as starches and

carbohydrates. This is a primary role of α-amylase and α-glucosidase. 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.

#### 7.3.4.1 α-Glucosidase inhibition

Inhibition of α-glucosidase by racemic pinocembrin, pinostrobin, and liquiritigenin 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.[18] Results are reported in Figure 7.6.

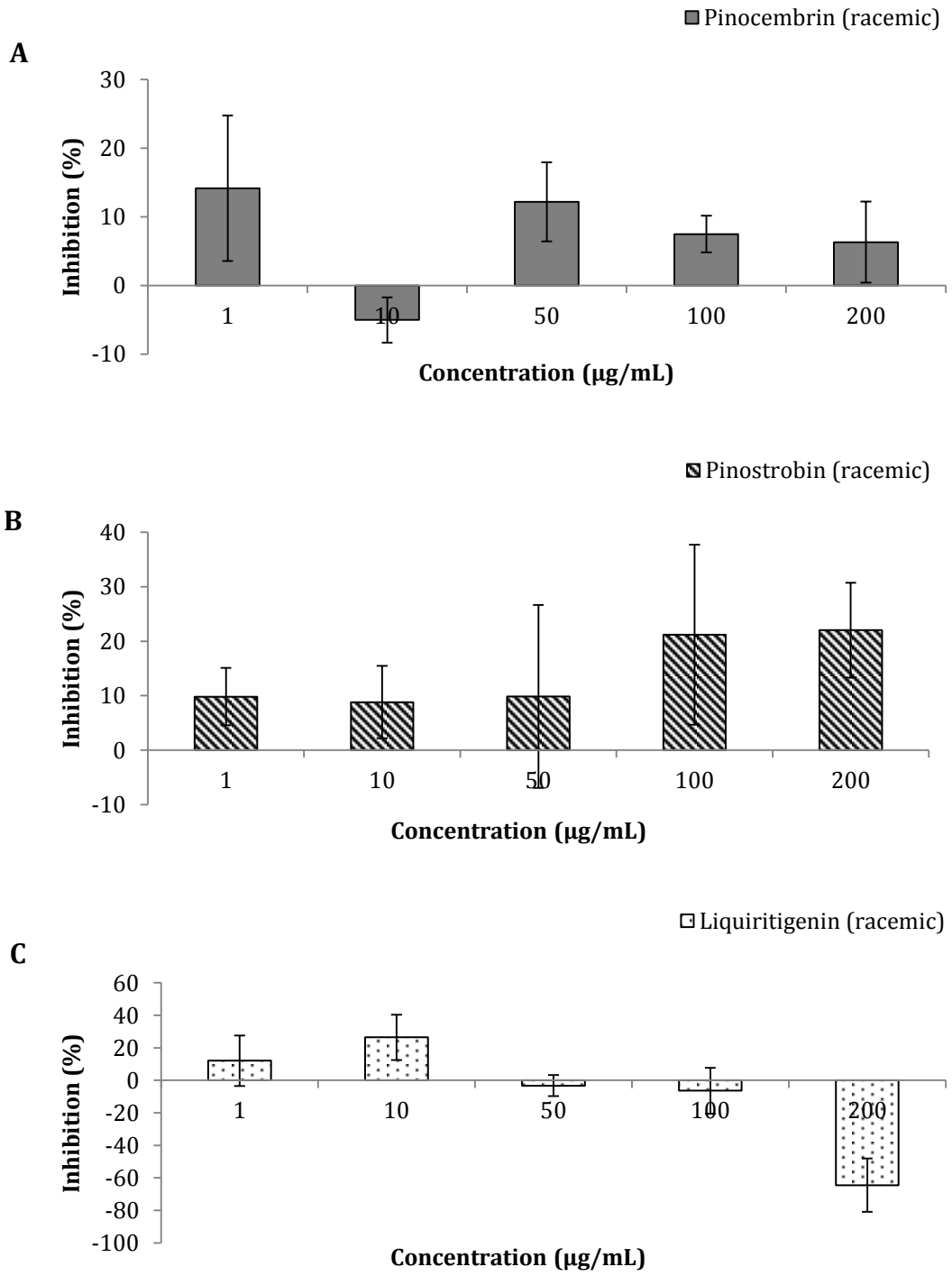


**Figure 7.6.** Alpha-glucosidase percent inhibition of racemic pinocembrin (A), pinostrobin (B), and liquiritigenin (C) ($n$ = 6 ± SEM).

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 (Figure 7.6 A). At 10 µg/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, disqualified it for further enantiospecific screening (Figure 7.6 B). Racemic liquiritigenin also showed weak and variable α-glucosidase inhibitory activity, which apparent induction at the higher concentrations (Figure 7.6 C). The lack of reproducible concentration dependent α-glucosidase inhibitory activity by any of the tested racemic flavonoids made it an unattractive assay for further enantiospecific investigation.

#### 7.3.4.2 α-Amylase Inhibition

##### 7.3.4.2.1 *Pinocembrin*

The pinocembrin racemate was screened for α-amylase inhibition with the results presented in Figure 7.7. 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, pinostrobin, and liquiritigenin to α-amylase inhibition. As previously mentioned, pure pinocembrin enantiomers were separated by HPLC, however eventually S-pinocembrin was available for purchase from Sigma-Aldrich (St. Louis, MO, USA), while pure R-pinocembrin was eventually 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 7.7.

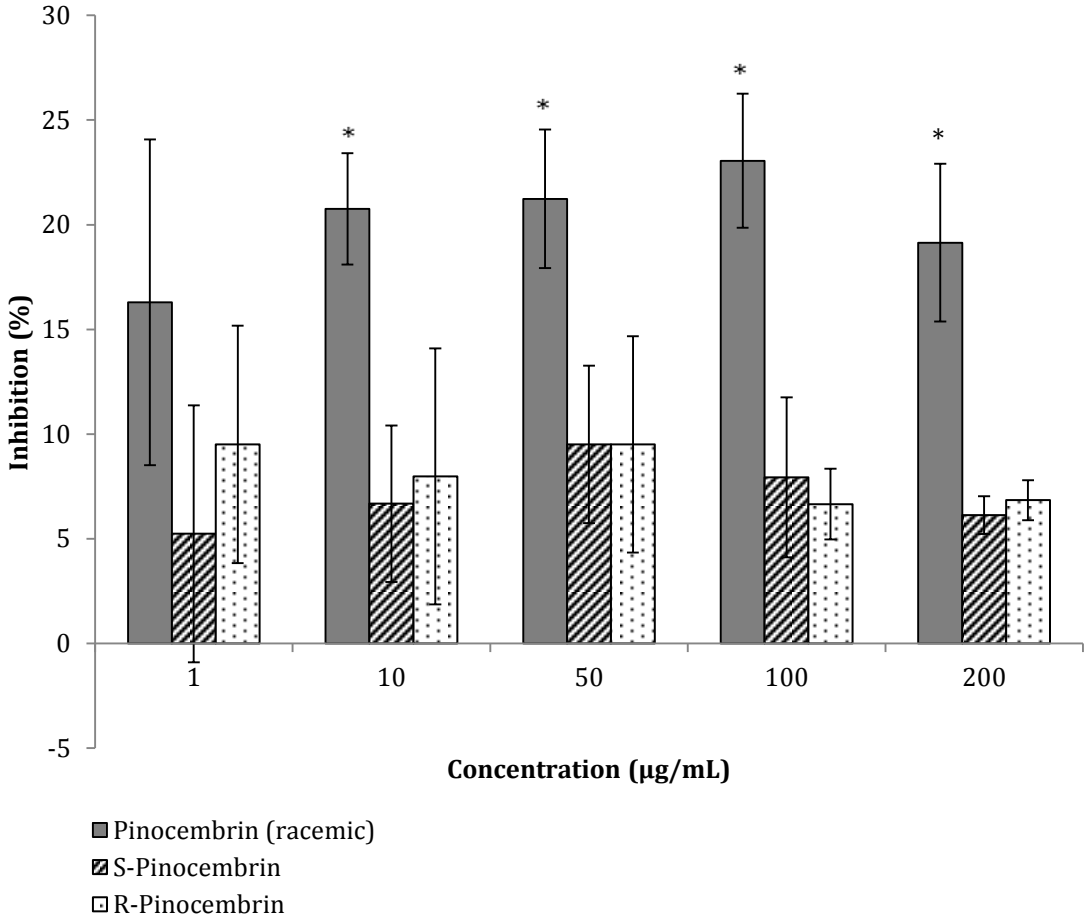


**Figure 7.7.** Alpha-amylase percent inhibition of racemic pinocembrin and its pure enantiomers ($n$ = 6 ± SEM). * Represents a significant difference between racemic pinocembrin and its enantiomers ($P < 0.05$).

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.

7.3.4.2.2 *Pinostrobin*

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 7.8). 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.

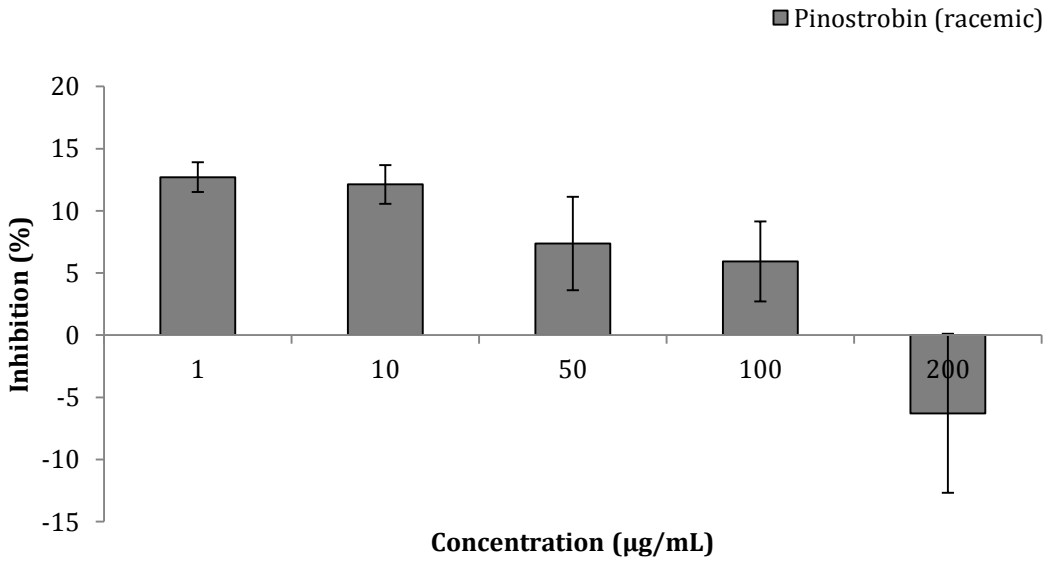


**Figure 7.8.** Alpha-amylase percent inhibition of racemic pinostrobin ($n$ = 6 ± SEM). *

Figure 7.9 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.

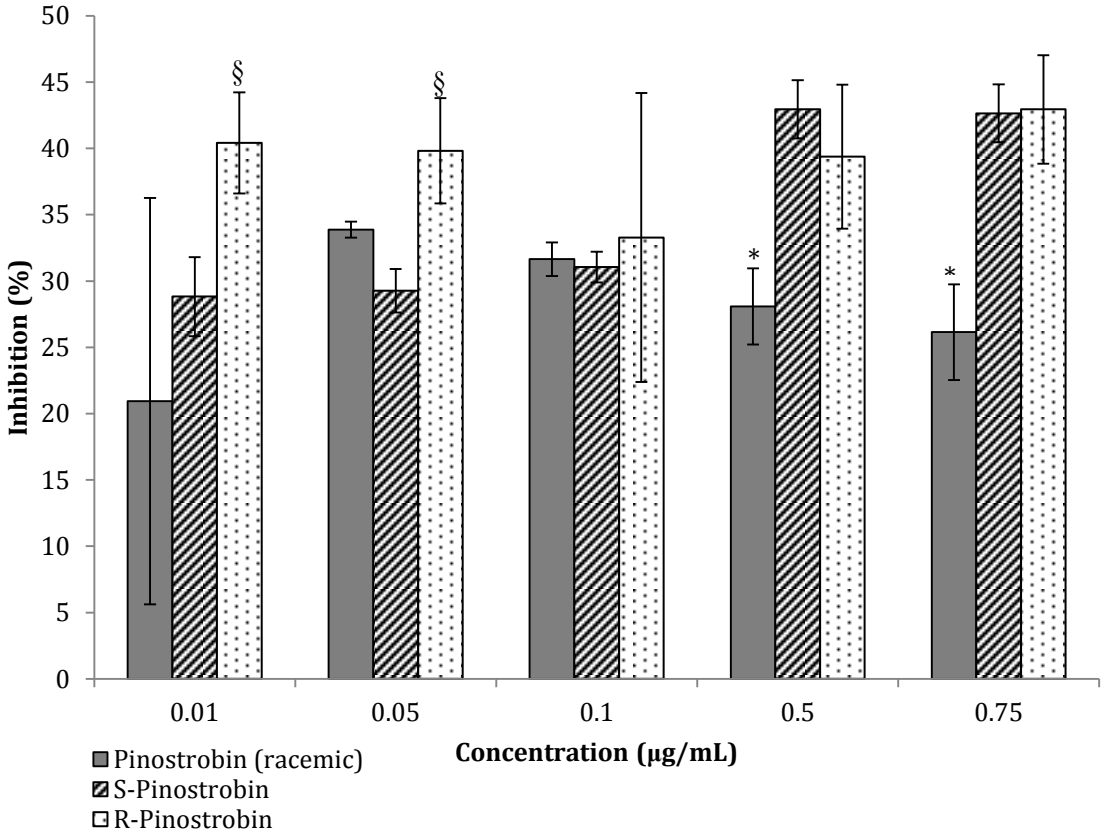


**Figure 7.9.** Alpha-amylase percent inhibition of racemic pinostrobin and its pure enantiomers ($n$ = 6 ± SEM). § Represents a significant difference between S-pinostrobin and R-pinostrobin ($P < 0.05$). * Represents a significant difference between racemic pinostrobin and its enantiomers ($P < 0.05$).

7.3.4.2.3 *Liquiritigenin*

The liquiritigenin racemate was also initially assessed for α-amylase inhibitory activity at the concentration range of 1 – 200 µg/mL (Figure 7.10). Weak inhibition was observed at 1 µg/mL. Subsequent ascending concentration point showed induced activity of the enzyme. Similar to pinostrobin any positive dose response curve demonstrated by racemic liquiritigenin would be occurring at least at concentrations smaller than 1 µg/mL. It was again decided to repeat the α-amylase inhibition assay again at the lower concentration range of 0.01 – 0.75 µg/mL.

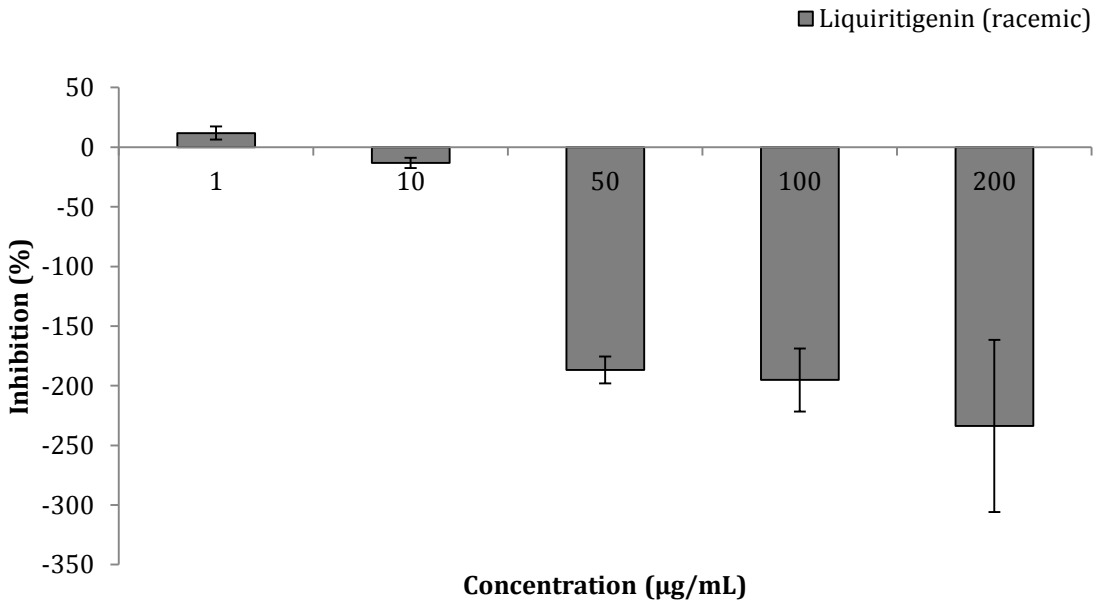


**Figure 7.10.** Alpha-amylase percent inhibition of racemic liquiritigenin and its pure enantiomers ($n$ = 6 ± SEM). * Represents a significant difference between racemic liquiritigenin and its enantiomers ($P < 0.05$).

α-Amylase inhibition activity of racemic liquiritigenin and its enantiomers was measured over the concentration range of 0.01 – 0.75 µg/mL (Figure 7.11). The dose dependent decrease of enzyme inhibition demonstrated by the liquiritigenin racemate is apparent. Greater percent inhibition at the two lowest concentration points (15% at 0.01 µg/mL and 12% at 0.05 µg/mL) was noted when compared to the lowest concentration point (11% at 1 µg/mL) of the higher concentration range (1 – 200 µg/mL) assay. This observation is not noted in the pure enantiomers, which demonstrated significantly higher α-amylase inhibition at several concentrations than racemic liquiritigenin.

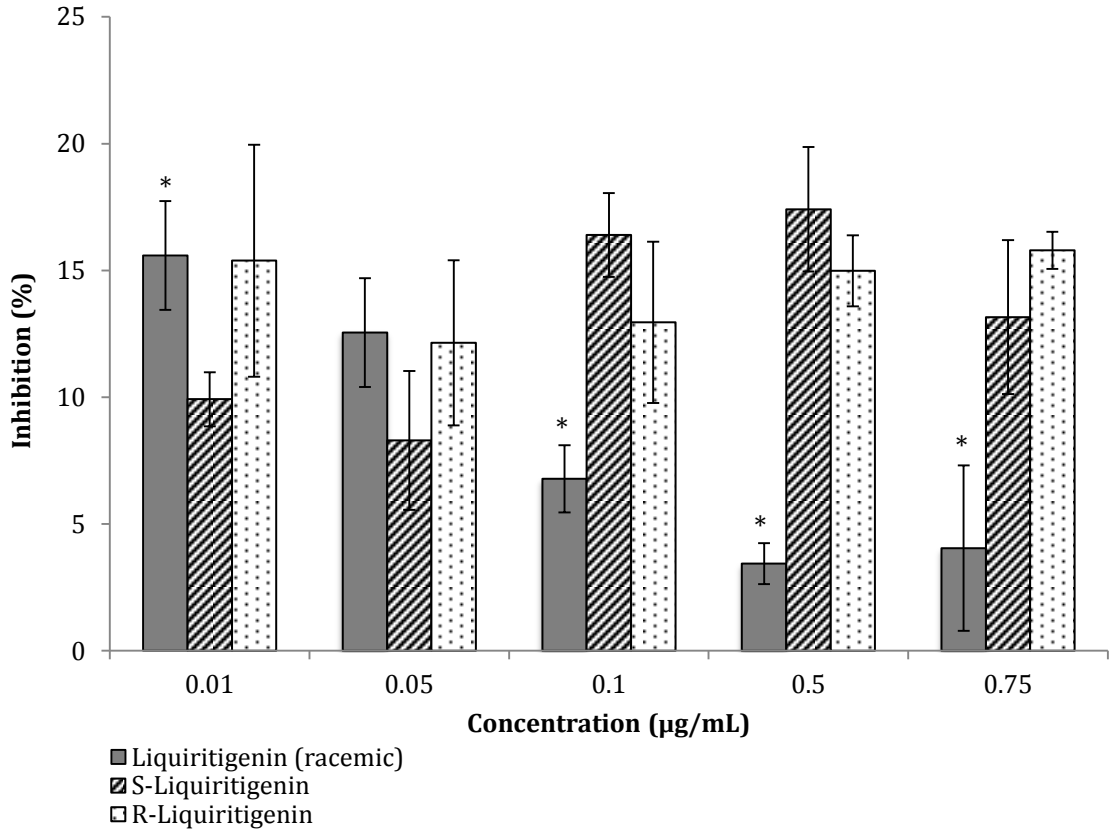


**Figure 7.11.** Alpha-amylase percent inhibition of racemic liquiritigenin and its pure enantiomers ($n$ = 6 ± SEM). * Represents a significant difference between racemic liquiritigenin and its enantiomers ($P < 0.05$).

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.[19] The combined effect of weight loss and postprandial glucose lowering may contribute to the association of flavonoid intake and decreased risk of diabetes.[20,21] α-Amylase inhibition has been demonstrated in products known to contain pinocembrin and pinostrobin, such as honey.[22] Since performing these experiments, α-glucosidase inhibition was observed by pure S-pinocembrin and pure S-liquiritigenin extracted from a Chinese medicinal plant in one study.[23] 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 three compounds. Assessment of α-amylase inhibition of racemic pinocembrin, pinostrobin, and liquiritigenin as well as their pure enantiomers is reported for the first time. α-amylase inhibition was seen by all three 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.

### *7.3.5 In vitro Cyclooxygenase Activity*

Cyclooxygenases (COXs) contain cyclooxygenase and peroxidase activities. Prostaglandins can be synthesized from several essential fatty acid precursors including arachidonic acid. COX activity will result in the production of $PGG_2$ and $PGH_2$ which are

unstable endoperoxide intermediates. COX catalyzes the conversion of arachidonic acid to $PGH_2$, the first step in the biosynthesis of prostaglandins, thromboxanes, and prostacyclins. 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. Cyclooxygenases have been involved in gastric and colorectal carcinogenesis; and in particular, COX-2 polymorphisms were demonstrated to be present in patients with gastric or colorectal lesion. The assay measures COX inhibition 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.

#### 7.3.5.1 Pinocembrin

Racemic pinocembrin, S-pinocembrin, and R-pinocembrin were assessed for their COX-1 and COX-2 inhibitory activity using a commercially available ELISA assay. The results are presented in Figure 7.12. Racemic pinocembrin had COX-1inhibitory activity roughly equivalent to ibuprofen at 1 µg/mL with decreasing inhibition at 10 and 250 µg/mL. Conversely, the enantiomers 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, with R-pinocembrin also showing greater COX-1 inhibition.

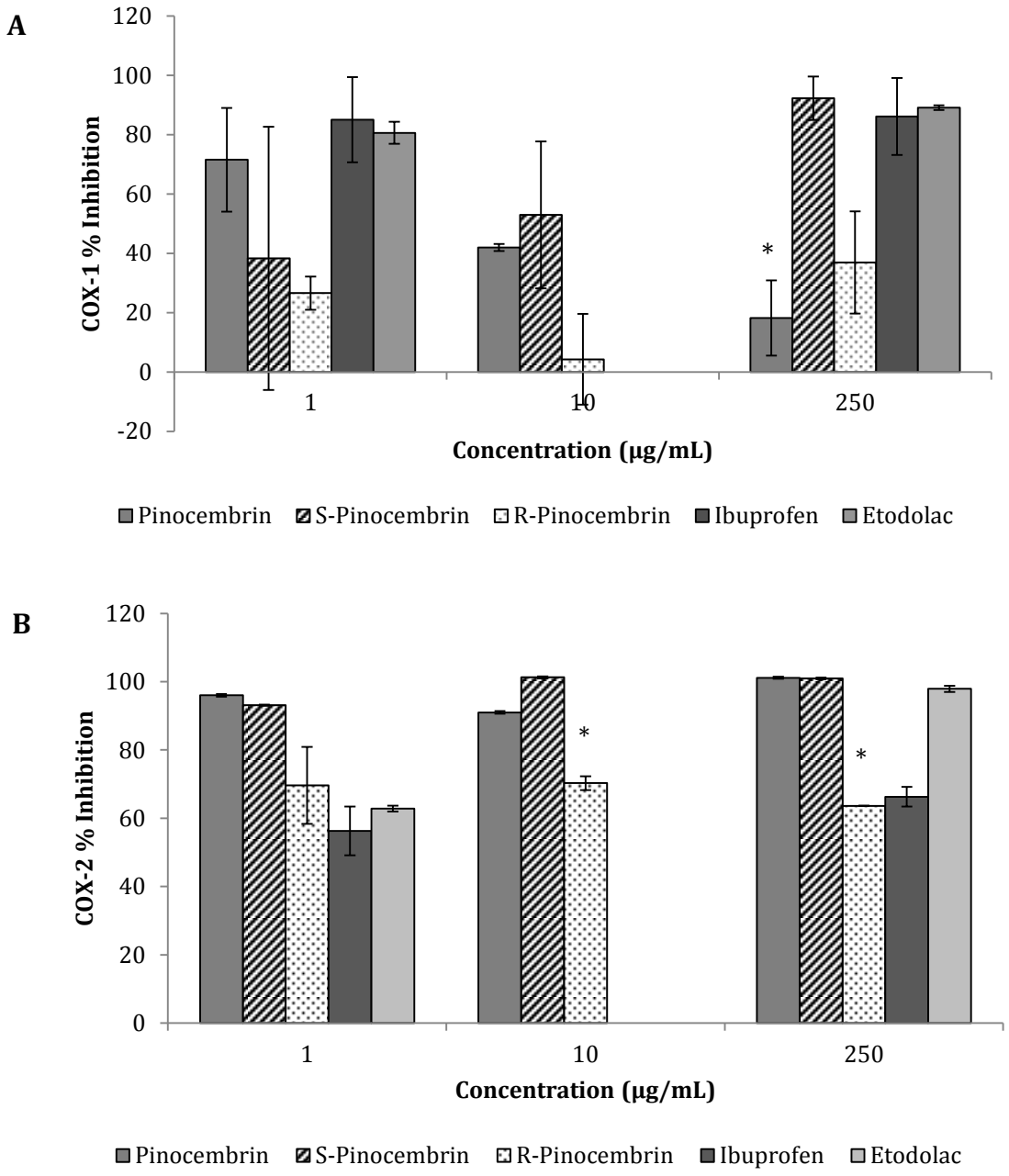


**Figure 7.12.** Cyclooxygenase-1 (COX-1) (A) and cyclooxygenase-2 (COX-2) (B) activity after addition of racemic pinocembrin, S-pinocembrin , R-pinocembrin and ibuprofen and etodolac (positive controls) at concentrations 1.0 – 250.0 µg/ml (n = 3, mean ± SEM).* Represents a significant difference between racemic pinocembrin and its enantiomers ($P < 0.05$).

Both racemic pinocembrin and S-pinocembrin demonstrated high COX-2 inhibition at all concentrations tested, exceeding that of the positive control etodolac even at the lowest concentration (1µg/mL) suggesting high potency. Interestingly, the COX-2 inhibition demonstrated 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 (Figure 7.12 B). R-Pinocembrin showed significantly less activity than S-pinocembrin or the racemate at 10 and 250 µg/mL. R-pinocembrin was unique in that it was a much less potent COX-2 inhibitor than S-pinocembrin or the racemate.

#### 7.3.5.2 Pinostrobin

Racemic pinostrobin was screened for its COX-1 and COX-2 inhibitory activity using a commercially available ELISA assay. The results are presented in Figure 7.13. The pinostrobin racemate showed variable and weak COX-1 inhibitory activity at all concentrations tested. 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.

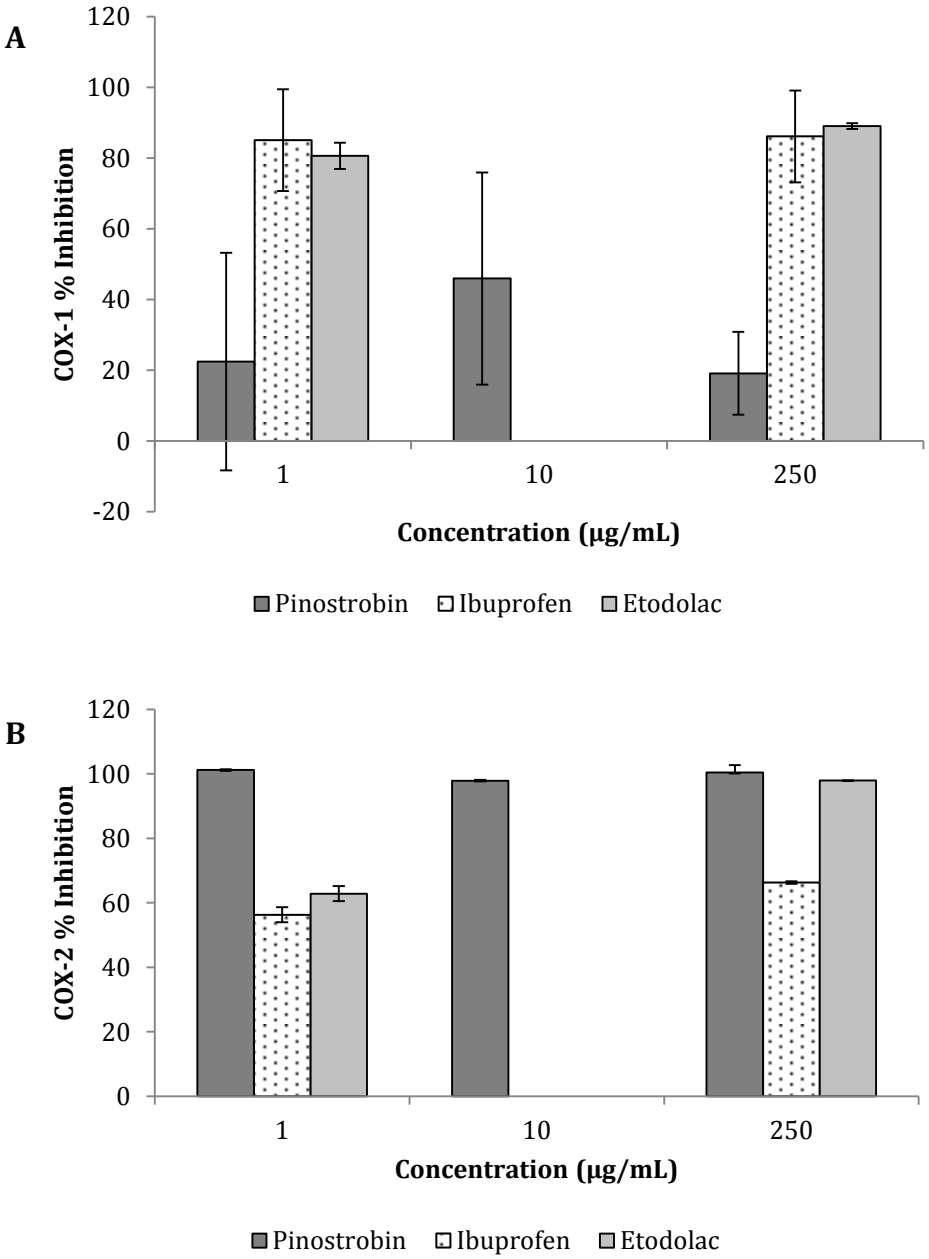


**Figure 7.13.** Cyclooxygenase-1 (COX-1)(A) and cyclooxygenase-2 (COX-2)(B) activity after addition of racemic pinostrobin and ibuprofen and etodolac (positive controls) at concentrations 1.0 – 250.0 µg/ml (n = 3, mean ± SEM).

#### 7.3.5.3 Liquiritigenin

Racemic liquiritigenin was assessed for its COX-1 and COX-2 inhibitory activity using a commercially available ELISA assay. The results are presented in Figure 7.14. The liquiritigenin racemate showed COX-1 inhibitory activity approximately equivalent to ibuprofen (positive control) at 1 µg/mL, with decreasing inhibition at the remaining consecutive concentrations. At 250 µg/mL racemic liquiritigenin demonstrated poor COX-1 inhibition compared to the positive controls (ibuprofen and etodolac). The results of the COX-2 inhibition assay were similar to both pinocembrin and pinostrobin racemates. Potent COX-2 inhibition was apparent, with percent inhibition approaching 100% at the lowest concentration tested (1 µg/mL) as well as both subsequent concentrations (10 and 250 µg/mL).

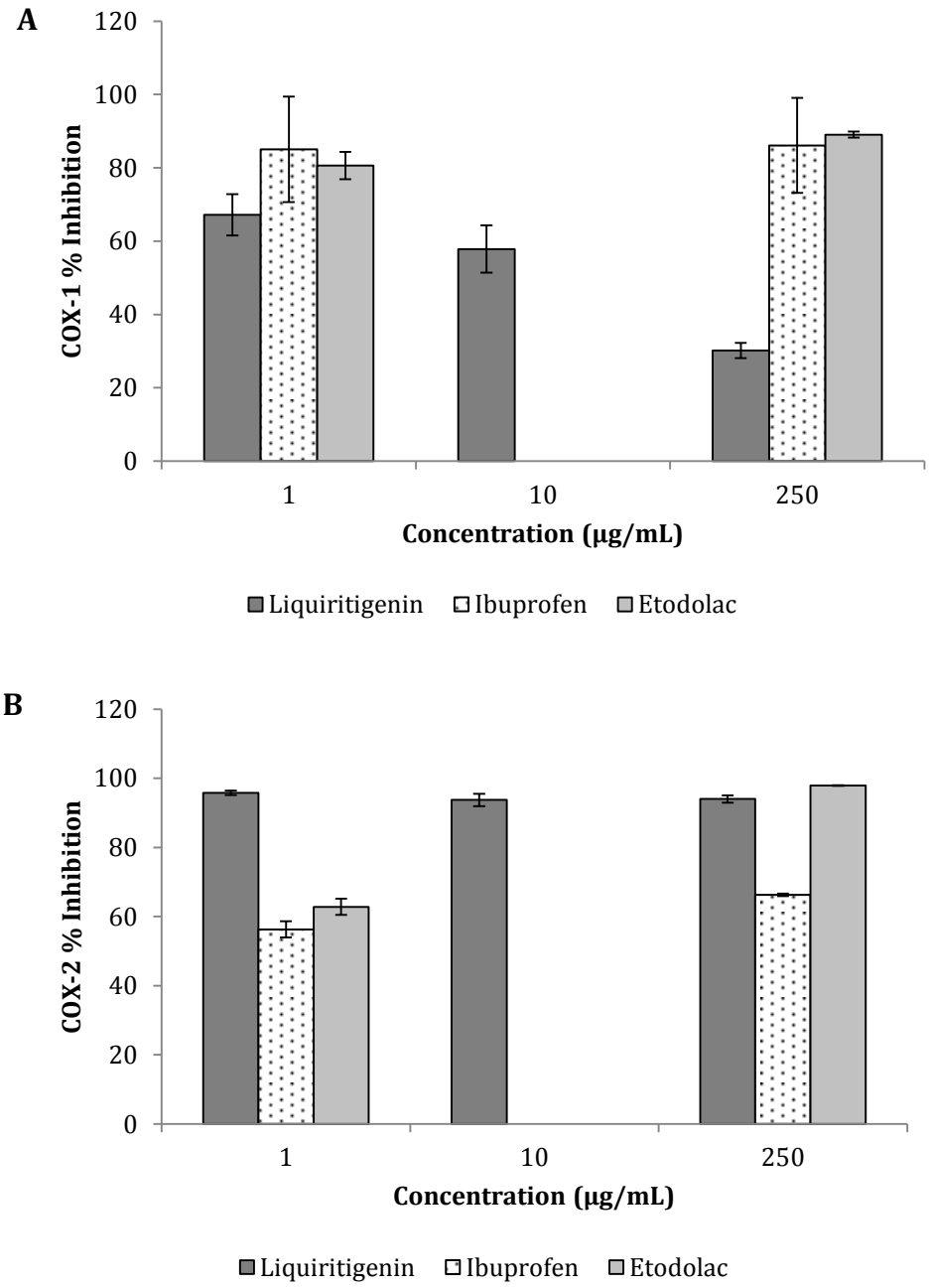


**Figure 7.14**. Cyclooxygenase-1 (COX-1)(A) and cyclooxygenase-2 (COX-2)(B) activity after addition of racemic liquiritigenin and ibuprofen and etodolac (positive controls) at concentrations 1.0 – 250.0 µg/ml (n = 3, mean ± SEM). Values are expressed as percentage (%).

In summary, racemic pinocembrin, pinostrobin, liquiritigenin 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-Pinocembrin showed increasing COX-1 inhibition with increasing concentration, with R-pinocembrin showing weak and variable COX-1 inhibition 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. R-pinocembrin was the exception, showing COX-2 inhibition similar to positive controls, but weaker than racemic and S-pinocembrin. Given the limitations with the procurement and stereoselective separation and collection of pure enantiomers of pinostrobin and liquiritigenin, as well as the anti-oxidant and COX inhibition results demonstrated by pinocembrin and its readily available enantiomer S-pinocembrin, pinocembrin was chosen as a focus for further enantiospecific characterization of potential activity with relevance to heart disease.

### *7.3.6 Cell 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.

#### 7.3.6.1 Pinocembrin

Racemic pinocembrin, S-pinocembrin, and R-pinocembrin were assessed for their effect on their prevention of cardiac myocyte hypertrophy. In Figure 7.15 A, 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.

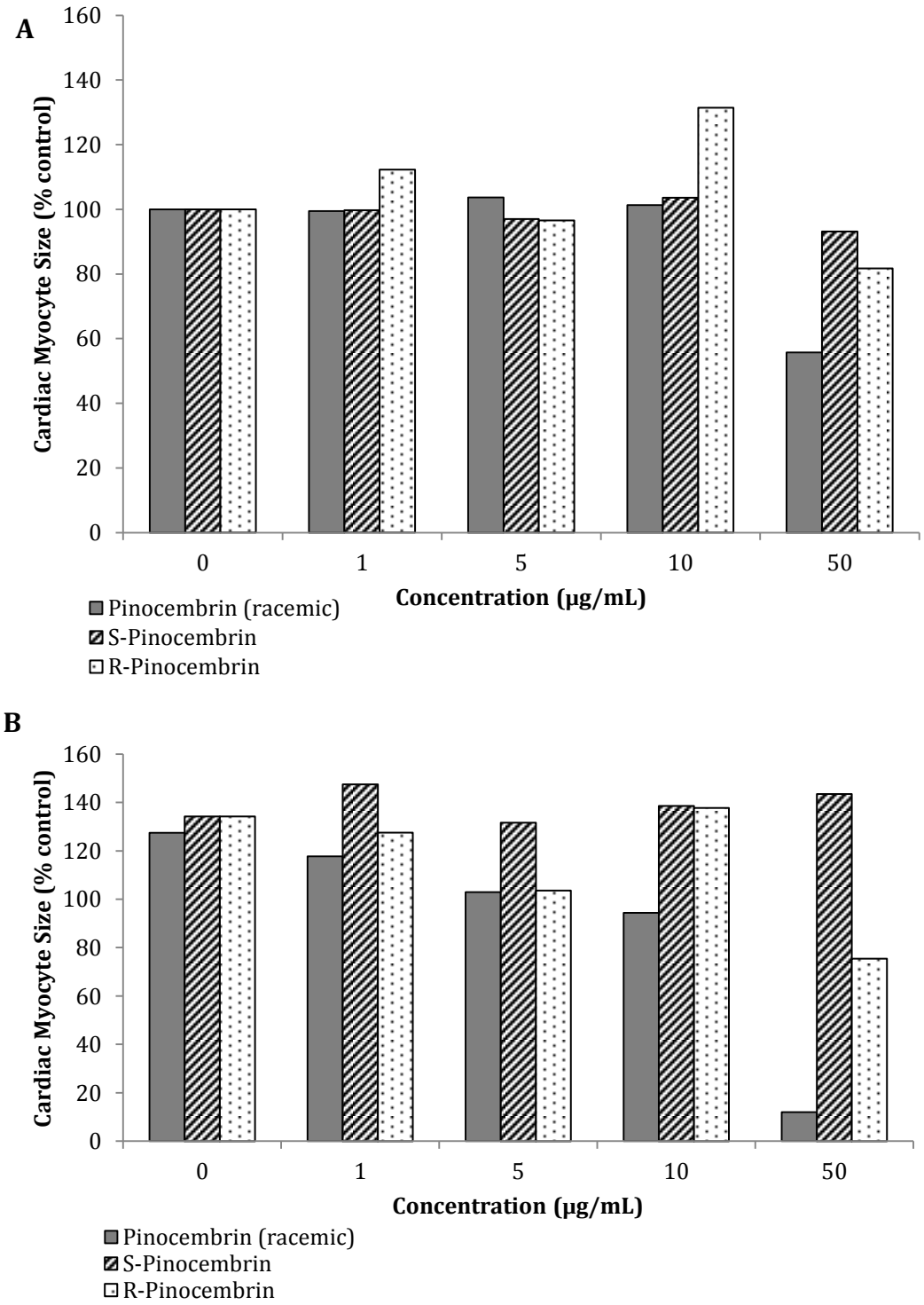


**Figure 7.15.** Effect of racemic pinocembrin, S-pinocembrin, and R-pinocembrin on cardiac myocyte size (percent of control) before (A) and after (B) addition of endothelin-1 to induce hypertrophy.

Figure 7.15 B 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.

## 7.4 Conclusions

Overall, these studies have demonstrated that pinocembrin, pinostrobin, and liquiritigenin 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, pinostrobin, and liquiritigenin 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 antioxidant 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 and liquiritigenin to elucidate their potential differences in anti-inflammatory and antioxidant activity.

These studies demonstrated the utility and necessity of developing stereoselective HPLC methods for chiral flavonoids. The validated HPLC methods described in Chapters II, III, and IV were successfully used to isolate the stereoisomers of pinocembrin, pinostrobin, and liquiritigenin that were not initially or currently 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.

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# Chapter VIII: Summary and Conclusions

The direct chromatographic approach for chiral flavonoid analysis has dominated this field of investigation over the last thirty years, despite the development of multiple methods and techniques. By direct chromatography, resolution is generally achieved through chiral polymer phases of oligosaccharides and their derivatives. Capillary electrophoresis has been subject to grater use in conjunction with chiral mobile phase additives. Indirect derivatization methods have been very limited and mostly observational. There has been an undeniable increase in awareness and interest in developing stereospecifc techniques to analyze chiral flavonoids. However, in part due to the large number of compounds in this class, there remains a lack of stereospecific assays published in the literature for many chiral flavanones. Even fewer of these studies are developed and validated in biological matrices according to industry best practice. Research of this quality is needed in these areas and is necessary to verify and optimize any beneficial effects found in *in vitro* bioactivity assays as well as identifying the active ingredient in epidemiological studies. Finally, it is necessary to identify and prevent any toxicity in the growing population of nutraceutical product consumers.

To further these goals, methods were developed using direct enantioselective HPLC for pinocembrin, pinostrobin, and liquiritigenin racemates. The developed methods are rapid, sensitive, reproducible, accurate, and stereospecific. The methods were validated in biological matrices using FDA and ICH guidelines. Application of the methods in pharmacokinetic sample analysis, as well as quantititative content analysis of several natural health products was performed.

The analysis of flavonoid containing botanical supplements was performed using three LC/MS methods for stereospecific detection of liquiritigenin, pinocembrin, and pinostrobin. Stereospecific quantification of the flavonoid enantiomers was performed. In 14 of the 19 products analyzed, expected or advertised flavonoids were detected, including one product that met the label claims of racemic flavonoid content within 100-120%. However, variability between products containing the same plant extract was observed. This was true even in products with the same indication for use. Variability in the quantity and recommended dose of a purported active ingredient between manufacturers and formulations could prevent reliable replication of therapeutic results obtained from clinical trials, or even anecdotal reports. This observation is significant in that occurred between products that were licenced by Health Canada – potentially giving consumers the impression that they are similar or identical products with similar or identical ingredients. This variability may result in large part from the unavailability of detection methods that can allow reasonable analysis and quantification. The methods used in this study allow for the assessment of natural health products and dietary supplements to ensure quality and content. This is especially relevant as more is discovered about the pharmacological effects and potential health benefits of flavonoids from basic and epidemiologic research. Manufacturers of natural health products that incorporate flavonoids into their formulations should be particularly careful to accurately label their products and adhere to regulatory requirements. Additionally, uniformity between manufactured batches is another source of potential variability in flavonoid content that manufacturers are recommended to monitor. A further understanding of

flavonoid enantiomeric and glycosidic forms would also provide more comprehensive scientific regulation of products containing them.

The stereospecific pharmacokinetics of intravenously and orally administered pinocembrin, pinostrobin, and liquiritigenin in rats were reported for the first time. These studies indicate that pinocembrin, pinostrobin, and liquiritigenin are highly distributed and rapidly glucuronidated. Elimination via non-renal routes is likely. This may indicate that high concentrations in the liver and gastrointestinal tract are possible. Low bioavailability and short half-lives are characteristic of all three compounds. Assessment of the distribution of the flavonoids revealed differences with respect to chirality that translated to the overall pharmacokinetic profile.

Pharmacological activity of racemic pinocembrin, pinostrobin, and liquiritigenin was established in a variety of exploratory and well established *in vitro* assays. Obtaining and collecting pure enantiomers for testing in the assays proved successful, yet time consuming and expensive. However, in those assays where pure enantiomers were tested against racemates for pharmacologic activity, pharmacodynamic differences were apparent. For example, pinocembrin, pinostrobin, and liquiritigenin enantiomers demonstrated concentration-dependent alpha amylase inhibitory activity characteristically different than the racemates. This observation held in multiple activity screens. Pinocembrin was singled out as having promise in screens relevant to heart disease, as it demonstrated both antioxidant 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 and liquiritigenin to elucidate their potential differences in anti-inflammatory and antioxidant 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 quantities 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. However, the objectives of the study, which were to: develop stereospecific HPLC or LC-MS detection methods for flavonoids of interest, assess stereospecific content of flavonoids of interest in selected natural health products, perform stereospecific pharmacokinetic studies of intravenously administered flavonoids in the rat, and evaluate stereospecific pharmacodynamics of flavonoids of interest *in vitro,* were successfully carried out. The data supported the hypothesis by indicating that stereospecific differences in formulation content, pharmacokinetic disposition, and pharmacodynamic behaviour do exist. Of particular note was the dramatic predominance of renal S-liquiritigenin excretion compared to R-liquiritigenin. Further study investigating the potential involvment of a stereospecific renal transporter may be indicated. Additionally, the observation of CYP 2D6 induction by pinocembrin and pinostrobin was remarkable, as CYP 2D6 is generally thought to be non-inducible by most pharmaceuticals.

Finally, further clinical investigations and studies of chiral flavonoids including toxicokinetics in humans are needed to form a complete clinical picture in light of these findings.

# Appendix

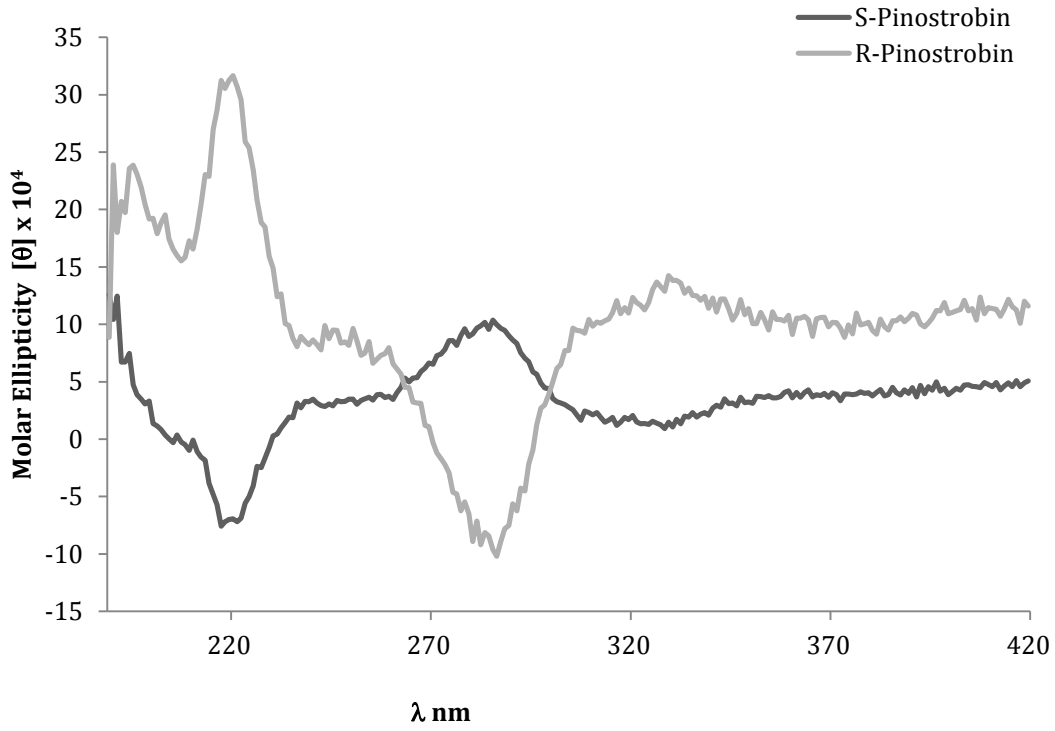


**Figure 1.** Circular dichroism (CD) spectrum of S-pinostrobin and R-pinostrobin.

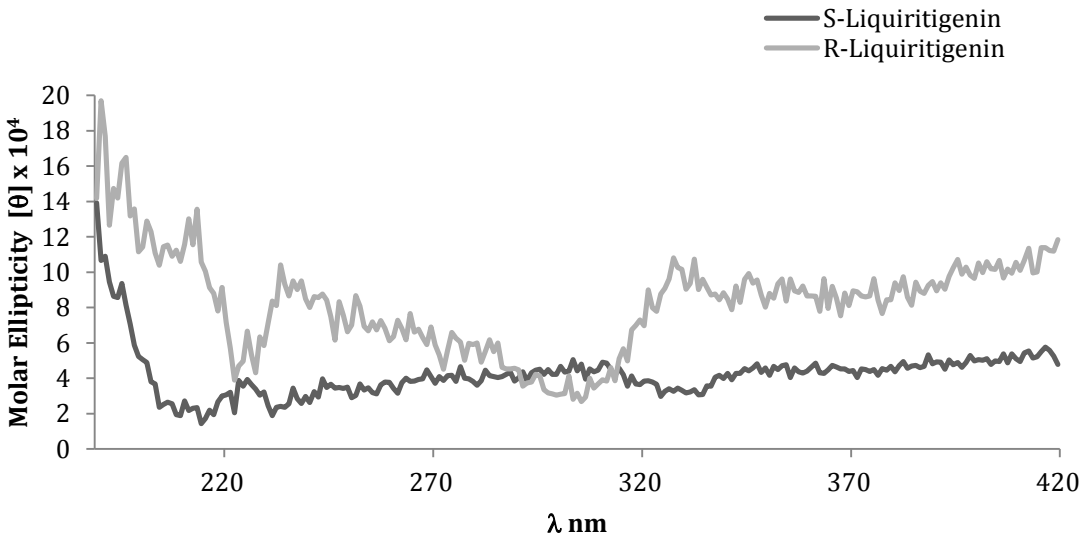


**Figure 2.** Circular dichroism (CD) spectrum of S-liquiritigenin and R-liquiritigenin.