

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

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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.

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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 (1,2,3). 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 1) has recently been shown to be a highly selective estrogen receptor β 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 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 3) has exhibited multiple 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.

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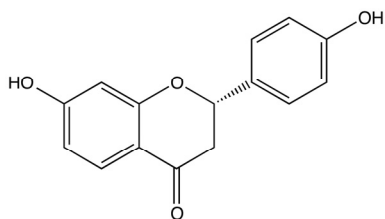


Figure 1. Structure of S(-)-liquiritigenin

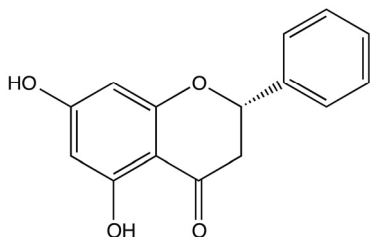


Figure 2. Structure of S(+)-pinocembrin

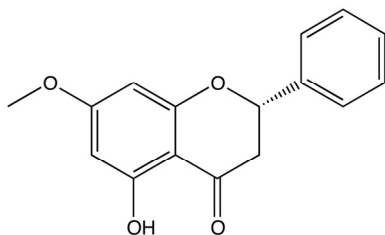


Figure 3. Structure of S(+)-pinostrobin

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 liquiritigenin, 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.

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.

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.

Table 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.

Samples were centrifuged at 2,000 rpm for 5 min. The supernatant was collected and then divided into two groups with 100 μ l 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 μ l aliquot was dried to completion under a stream of nitrogen gas and reconstituted in 200 μ l phosphate buffer saline (PBS). To this, 20 μ l of β -glucosidase from almonds was added and then incubated at 37°C for 48 h in a hot water bath. Internal standard (25 μ l) 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 μ l mobile phase and 10 μ l 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 μ l aliquots, 25 μ l of internal standard was added. Samples were dried under a stream of nitrogen gas, residues reconstituted in 200 μ l mobile phase, and 10 μ l was injected into the LC/MS system. By subtracting of the concentration of the non-enzymatic incubated sample from the incubated sample, the amount of glycoside can be determined.

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 200°C and a block temperature of 200°C. 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 μ g/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 $\times$ 4.6mm i.d., 5- μ m 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. ($\pm$)-Pinocembrin was used as an internal standard. S(-)liquiritigenin, R(+)-liquiritigenin, and ($\pm$)-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 $\times$ 4.6mm i.d., 5 μ m 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(+)-pinocembrin 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 $\times$ 4.6mm i.d., 5- μ m 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.

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 2, 3, and 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.

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.

Table 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(-)liquiritigenin			R(+)liquiritigenin		
	Claimed	Aglycone	Glycoside	Claimed	Aglycone	Glycoside
1	†	0.08	0.007	†	0.099	0
2	†	0.11	0.04	†	0.170	0
3	†	0.36	0	†	0.447	0
4	†	4.12	0	†	5.94	0
5	†	1.24	0.72	†	1.87	0.76
6	†	10.8	8.3	†	13.2	9.16
7	†	0.43	0	†	0.93	0
8	†	4.15	0	†	4.84	0
9	†	4.03	0	†	4.52	0
10	†	53.2	0	†	56.7	0

Table 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(+)pinocembrin			R(-)pinocembrin		
	Claimed	Aglycone	Glycoside	Claimed	Aglycone	Glycoside
1	†	1.62	0	†	0.887	0
2	†	1046	124	†	274	62.9
3	†	0	0	†	0	0
4	†	7.85	13.1	†	5.21	7.07
5	†	1.14	10.1	†	0.953	0
6	†	1.17	2.2	†	0.705	1.02
7	†	0	0	†	0	0
8	6000	649	8028	6000	471	5510
9	2.19%	531	5370	2.19%	424	3431

Table 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.

Number	S(+)-pinostrobin			R(-)-pinostrobin		
	Claimed	Aglycone	Glycoside	Claimed	Aglycone	Glycoside
1	†	15.1	0	†	12.9	0
2		0	0		0	0
3	†	42.6	33.7	†	54.7	0
4		0	0		0	0
5	†	0	0	†	0	0
6		61.8	0		77.4	0
7		0	0		0	0
8	†	0	0	†	0	0
9	†	28.9	0	†	35.2	0

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.

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.

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