Natural Health Research Institute: Scientific Symposium

The Effectiveness of Natural Products for Women’s Health

8th Annual NHRI Scientific Symposium

Presented by: UIC College of Pharmacy
Safety and Efficacy of Botanical Dietary Supplements As Alternatives to Hormone Replacement Therapy

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University of Illinois College of Pharmacy
UIC/NIH Botanical Center for Dietary Supplements Research

- 1998 – US Congress appropriated funds for the NIH Office of Dietary Supplements to establish botanical research centers
- 1999 – UIC Botanical Center founded
- First Director, Norman R. Farnsworth
UIC Botanical Dietary Supplement Research Philosophy

Any botanical dietary supplement to be studied in humans must be:

- Botanically authenticated
- Biologically standardized
- Chemically standardized (based on active constituents)
- Manufactured under GMP
- Have a [proposed] mechanism of action
- Safe
Steps in Developing a Botanical Dietary Supplement for Clinical Evaluation

1. Search scientific and ethno-medical botanical literature [NAPRALERT, Pubmed, etc.]
2. Acquire plant material according to Good Agricultural & Collection Practices
3. Determine bioassays to assess MOA & Safety
4. Perform isolation of the active compounds
5. Chemically characterize active compounds to be used as biomarkers for standardization
6. Standardize extract for (pre-)clinical studies
7. Develop chemically & biologically standardized formulation under GMP guidelines
8. Conduct Phase I and Phase II clinical trials
UIC Botanicals for Women’s Health

- **Angelica sinensis**
  - Estrogenic, serotonergic, QR Induction, antioxidant, anti-inflammatory
- **Cimicifuga (Actea) racemosa** (black cohosh)
  - Serotonergic, antioxidant, anti-inflammatory
- **Vitex agnus-castus**
  - Serotonergic, anti-inflammatory, QR induction, antioxidant
- **Valeriana officinalis**
  - Serotonergic, anti-inflammatory
- **Trifolium pratense** (red clover)
  - Estrogenic, progestogenic, anti-inflammatory, QR induction, antioxidant
- **Humulus lupulus** (hops)
  - Estrogenic, anti-inflammatory, QR induction, antioxidant
- **Viburnum prunifolium**
  - Serotonergic, anti-inflammatory, QR induction
- **Glycyrrhiza glabra** (licorice)
  - Estrogenic, anti-inflammatory, QR induction
- **Glycyrrhiza uralensis**
  - Estrogenic, anti-inflammatory
Project 1: Metabolomics: Characterization of Botanical Chemistry and Synergy

Guido Pauli, Shao-Nong Chen

- **Aim 1**: Chemodiversity Profiling of Bioactive Metabolites
- **Aim 2**: Metabolomic Standardization Methods
- **Aim 3**: Study of Botanical Synergy

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**Herbal Metabolome**

<table>
<thead>
<tr>
<th>1° Metabolites</th>
<th>2° Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquitous/Unspecific Metabolites</td>
<td>Bioactive 2° Metabolites</td>
</tr>
<tr>
<td>Rare: Bioactive 1° Metabolites</td>
<td>Inactive 1°/2° Metabolites</td>
</tr>
</tbody>
</table>
Project 2: Botanical Modulation of Estrogen Carcinogenesis
Judy Bolton, Birgit Dietz

AIM 1
1) Aromatase
2) P4501B1
3) P4501A1

AIM 2
DNA OXIDATION

AIM 3
GENOTOXICITY
BOTANICAL

4) COMT
5) NQO1

6) GST

7) ROS

GENOTOXICITY
BOTANICAL

N^7G-4-OHE_2
N^3A-4-OHE_2

Testosterone/Androstendione

4-OHE_2
4-MeOE_2
4-OHE_2-SQ
4-OHE_2-Q

DNA Conjugates

DEPURINATING ADDUCTS
Aim 1: Metabolism & bioavailability of active compounds

- In vitro and in vivo metabolism
- Identification of enzymes responsible for metabolism
- Investigation of enzyme inhibition by botanicals
- Bioavailability in rats

Aim 2: Investigation of botanical-drug interactions

- Inhibition of phase I and phase II metabolism
- Induction of phase I and phase II enzymes
- Induction/inhibition of drug transporters
- Identification of compounds responsible for enzyme inhibition/induction and transporter inhibition/induction
Core B: Botanical Integrity
GF Pauli, SN Chen, DD Soejarto

Core C: Bioassay
Judy Bolton, Birgit Dietz

Core D: Analytical
Richard van Breemen, Dejan Nikolic, David Lankin

- Botanical authentication and extraction
- Chemical and biological standardization
- Determination of mechanisms of action
- Identification of active constituents
Clinical Trials of Botanical Dietary Supplements at the UIC Botanical Center

- Phase I investigation of safety and pharmacokinetics of hops. In progress.
Alternatives to Hormone Replacement Therapy: Screening Botanicals for Estrogens

- Hormone replacement therapy (HRT) in women might increase risks of cardiovascular disease, breast cancer and dementia.
- Therefore, women are seeking safer alternatives to HRT.
- Many women are using botanical dietary supplements such as black cohosh, red clover or hops for the management of menopausal symptoms.
- The mission of the UIC/NIH Botanical Center is to investigate the safety and efficacy of botanical alternatives to HRT.
- Finding alternatives to HRT that prevent instead of causing cancer is a goal of the Center.
Hops: Promising Botanical for Postmenopausal Women’s Health

- Hops (Humulus lupulus L.) used during the brewing of beer as a preservative and flavoring agent.
- Under investigation for managing hot flashes in menopausal women.
Identification of Active Compounds in Botanicals
PUF-LC-MS vs Bioassay Guided Fractionation
Pulsed Ultrafiltration-Mass Spectrometric Screening for Ligands of ER-α and ER-β

1. Binding

Preincubate Extract and Estrogen Receptor

2. Ultrafiltration Separation

2a. Wash Unbound to Waste

2b. Elute Bound Ligands into Trapping Column

3. LC-MS Identification

Elute desalted ligands into MS

HPLC Pump

Injector

Ligand-ER Complexes

Unbound Compounds

Ultrafiltration LC-MS Assay of Hop Extract for Ligands to Estrogen Receptors

Retention time (min)

LC-MS response

Xanthohumol
[M-H]⁻ m/z 353
No binding

8-Prenylnaringenin
[M-H]⁻ m/z 339

6-Prenylnaringenin
[M-H]⁻ m/z 339

Isoxanthohumol
[M-H]⁻ m/z 353

ER-β Binding Assay
ER-α Binding Assay
Control
Metabolism of Xanthohumol Determined Using Human Liver Microsomes and Hepatocytes

Identification of CYP450 Enzymes that O-demethylate Isoxanthohumol to Form 8-PrenylNaringenin

- mAb inhibitors of CYP450 enzymes were incubated with human liver microsomes, isoxanthohumol and NADPH.
- 8-PrenylNaringenin was measured using LC-MS.
- CYP1A2 O-demethylates isoxanthohumol to form 8-prenylNaringenin.

Conversion of Xanthohumol to Isoxanthohumol and 8-Prenynaringenin

Although estrogenic 8-prenynaringenin is only a minor constituent of hop products, it can be formed in vivo by hepatic metabolism of isoxanthohumol.

LC-MS-MS Analysis for Standardization of Hop Extract for Phase I Clinical Investigation

Retention time (min)

Thermo TSQ Quantum; YMC AQ 2 x 100 mm, 3 μm. Total analysis time 15 min.
Active Compounds Identified and Measured in Standardized Ethanol Extract of Hops

- **8-PrenylNaringenin**: 0.35% (wt) estrogenic
- **6-PrenylNaringenin**: 1.77% (wt) weak estrogen
- **Isoxanthohumol**: 1.07% (wt) weak estrogen
- **Xanthohumol**: 33.84% (wt) cancer prevention agent (non-estrogen)
Investigation of Possible Induction of Human Cytochrome P450 Enzymes by Hops

- Although hops (Humulus lupulus L.) are used to brew beer, little is known about possible interactions of hop extracts or their constituents with drug metabolizing enzymes.
- An *in vitro* assay utilizing human hepatocytes in cell culture was used to evaluate the potential for cytochrome P450 induction by hops.
- Cells were incubated with hop extracts or with vehicle only (control) to induce or inhibit drug metabolizing enzymes.
- Cytochrome P450 enzyme assays were carried out using standard substrates for the isoforms CYP3A4 and CYP1A2.
- UHPLC-MS-MS and SRM were used with the Nexera-8030 system to enhance the throughput, precision and accuracy of the enzyme activity assay.
CYP1A2 and CYP3A4 Activities of Human Hepatocytes after Treatment with Hops

Conclusion: Hops do not induce drug metabolizing enzymes.
Inhibition of Human CYP450s by Hops and Prenylated Flavonoids Isolated from Hops (Humulus lupulus)

- In addition to induction of drug metabolizing enzymes, inhibition of these enzymes is a common mechanism for drug-botanical interactions.
- The standardized hop ethanolic extract and active prenylated phenols were assayed for cytochrome P450 (CYP450) inhibition.
- When inhibition of CYP450 enzymes was observed, IC$_{50}$ values (concentrations required to inhibit 50% enzyme activity) were determined for inhibitors of specific enzymes.
Screening of Prenylated Phenols from Hops and a Hop Extract for Inhibition of Cytochrome P450 Enzymes

<table>
<thead>
<tr>
<th>Compound</th>
<th>CYP1A2</th>
<th>2B6</th>
<th>2C8</th>
<th>2C9</th>
<th>2C19</th>
<th>2D6</th>
<th>2E1</th>
<th>3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% Inhibition (1 µM) ± S.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-PN</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
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<tr>
<td></td>
<td>55.8 ±</td>
<td>32.2 ±</td>
<td>43.8 ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-PN</td>
<td>0.7</td>
<td>&lt; 10</td>
<td>1.2</td>
<td>ND</td>
<td>0.2</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>87.7 ±</td>
<td>51.0 ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>1.1</td>
<td>31.6 ± 8.0</td>
<td>8.6</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>47.8 ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XN</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>5.1</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>26.7 ±</td>
<td>36.4 ±</td>
<td>92.7 ±</td>
<td>69.8 ± 10.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.5 ±</td>
<td>13.7 ±</td>
<td>19.2 ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hop extract (5 µg/mL)</td>
<td>0.8</td>
<td>1.9</td>
<td>1.2</td>
<td>63.8 ± 2.0</td>
<td>0.1</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
<td>&lt; 10</td>
</tr>
<tr>
<td></td>
<td>88.4 ±</td>
<td>34.7 ±</td>
<td>97.6 ±</td>
<td>92.8 ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>48.2 ±</td>
<td>98.9 ±</td>
<td>96.3 ±</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 Mix¹</td>
<td>16.4 ±</td>
<td>14.9 ±</td>
<td>92.7 ±</td>
<td>65.5 ±</td>
<td>20.8 ±</td>
<td>29.2 ±</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>1.4</td>
<td>0.6</td>
<td>75.9 ± 2.1</td>
<td>6.8</td>
<td>4.9</td>
<td>&lt; 10</td>
<td>2.3</td>
</tr>
</tbody>
</table>

¹ Mixture of 6-PN, 8-PN, IX, and XN at concentrations identical to the 5 µg/mL hop extract
**IC$_{50}$ Values (µM) for the Inhibition of Cytochrome P450 Enzymes by a Hop Extract and Hop Prenylated Phenols**

<table>
<thead>
<tr>
<th>Compound</th>
<th>CYP1A2</th>
<th>CYP2C8</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hops</td>
<td>9.4 ± 0.6</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>3.3 ± 0.7</td>
</tr>
<tr>
<td>IX</td>
<td>ND²</td>
<td>0.2 ± 0.1</td>
<td>2.1 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>8-PN</td>
<td>1.1 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>XN</td>
<td>ND</td>
<td>1.1 ± 0.1</td>
<td>3.3 ± 0.4</td>
<td>ND</td>
</tr>
<tr>
<td>6-PN</td>
<td>ND</td>
<td>1.9 ± 0.1</td>
<td>5.9 ± 0.7</td>
<td>ND</td>
</tr>
</tbody>
</table>

1. Data expressed as mean ± SD
2. ND: Not determined since ≤ 50% inhibition at 10 µM
Phase I hops Clinical Study Design

- Healthy women
- Post-menopausal
- Not on hormone replacement therapy
- No beer for 1 month before or during study

5 women → Low dose 1 capsule\(^1\) daily, 5 days → Medium dose 2 capsules daily, 5 days → High dose 3 capsules daily, 5 days

- Urine collected during first 24 h.
- Blood drawn hourly for first 24 h then daily through day 5.
- Women monitored for another 7 days for adverse effects.
- Urine and blood analyzed for effects on blood chemistry, hormone levels, etc.
- Urine and blood analyzed using UHPLC-MS-MS for 8PN, 6PN, IX, XN, and their conjugates in support of pharmacokinetics study.
- Data will be used for design of Phase II study of safety and efficacy.

\(^1\)Each capsule was standardized to 0.25 mg 8PN, 1.30 mg 6PN, 0.80 mg IX, 21.3 mg XN
Concentrations of hop compounds increased after enzymatic deconjugation, indicating that most hop flavonoids in serum were conjugated.
Concentration-time Curves for Pharmacokinetics Evaluation of Hop Flavonoids in Human Serum

**Xanthohumol**
- 1.0 mg 8-PN
- 0.5 mg 8-PN
- 0.25 mg 8-PN

**Isoxanthohumol**

**8-Prenylnaringenin**

**6-Prenylnaringenin**
Assessment of Pharmacokinetics

- WinNonlin 6.2 (Pharsight; Sunnyvale, CA)
- AUC, $C_{\text{max}}$, $T_{\text{max}}$, $T_{1/2}$, $V_{d/F}$, $Cl/F$ were determined.
- Non-compartmental analysis (NCA) was used for pharmacokinetics modeling.
- Urinary excretion was $\leq 2\%$ of XN, IX, 6PN, and 8PN
- Challenges
  - Interconversion of compounds
  - Enterohepatic recirculation
## Pharmacokinetics of Hop Prenylated Flavonoids

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Low Dose</th>
<th>Med Dose</th>
<th>High Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>XN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUC(_0\text{-}\infty)</strong></td>
<td>h·ng/ml</td>
<td>73.2 ± 60.1</td>
<td>166.9 ± 62.1</td>
<td>322.6 ± 169.2</td>
</tr>
<tr>
<td><strong>(T_{1/2})</strong></td>
<td>h</td>
<td>18.3 ± 5.3</td>
<td>9.5 ± 2.2</td>
<td>20.7 ± 12.7</td>
</tr>
<tr>
<td><strong>Cmax</strong></td>
<td>ng/ml</td>
<td>4.4 ± 3.0</td>
<td>22.2 ± 12.1</td>
<td>27.6 ± 8.9</td>
</tr>
<tr>
<td><strong>IX</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUC(_0\text{-}\infty)</strong></td>
<td>h·ng/ml</td>
<td>149 ± 86</td>
<td>418 ± 446</td>
<td>546 ± 301</td>
</tr>
<tr>
<td><strong>(T_{1/2})</strong></td>
<td>h</td>
<td>27.5 ± 5.7</td>
<td>24.8 ± 24.3</td>
<td>19.9 ± 8.7</td>
</tr>
<tr>
<td><strong>Cmax</strong></td>
<td>ng/ml</td>
<td>5.7 ± 2.7</td>
<td>19.1 ± 15.5</td>
<td>37.6 ± 17.6</td>
</tr>
<tr>
<td><strong>8PN</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUC(_0\text{-}\infty)</strong></td>
<td>h·ng/ml</td>
<td>24.3 ± 8.9</td>
<td>69.2 ± 38.9</td>
<td>88.6 ± 52.6</td>
</tr>
<tr>
<td><strong>(T_{1/2})</strong></td>
<td>h</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>&gt;20</td>
</tr>
<tr>
<td><strong>Cmax</strong></td>
<td>ng/ml</td>
<td>1.4 ± 0.3</td>
<td>3.9 ± 2.6</td>
<td>6.7 ± 3.8</td>
</tr>
</tbody>
</table>
Enrollment and Randomization of Menopausal Women in a Phase II Trial of Safety and Efficacy of Black Cohosh and Red Clover vs. Prempro and

- Patients screened: n=1450
- Patients randomized: n=89
- Placebo: n=22
- Prempro® (0.625mg/2.5mg): n=23
- Black cohosh (128 mg): n=22
- Red clover (120 mg): n=22

Primary outcome to be measured: Reduction of hot flashes
Phase II Clinical Trial Primary Outcome: Reduction of Hot Flashes in Postmenopausal Women


Trifolium pratense

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Graph showing the reduction of vasomotor symptom intensity over time for different treatments:

- **1** Black Cohosh
- **2** Red Clover
- **3** Placebo
- **4** Prempro®

The graph indicates a significant reduction in vasomotor symptom intensity for the treatments compared to the placebo group. The asterisks (*) denote statistical significance.
Enzyme assays and mRNA measurements indicate that the hop clinical extract does not significantly induce CYP1A2 or CYP3A4 (according to FDA guidelines).

The hop extract inhibited CYP2C8, CYP2C9 and CYP 2C19 with IC$_{50}$ values of 0.8, 0.9, and 3.3 μg/mL, respectively.

Isoxanthohumol (IX) was the strongest inhibitor of CYP2C8 with an IC$_{50}$ of 0.2 μM.

8-Prenylnaringenin (8-PN) was the most effective inhibitor of CYP2C9 and CYP2C19 with IC$_{50}$ values of 1.1 and 0.4 μM, respectively. 8-PN inhibited CYP1A2 with an IC$_{50}$ of 1.1 μM.

After rapid absorption in the Phase I study, prenylated hop phenols were conjugated so that only low levels of free flavonoids were observed in human serum.

Enterohepatic recirculation and long half-lives were observed.

In vivo studies are needed to evaluate significance of these inhibition data for hops and to investigate efficacy of hop extracts for the management of menopausal symptoms.
Conclusions

- The safety and efficacy of botanical dietary supplements used by menopausal women are under investigation using botanically authenticated, chemically and biologically standardized extracts prepared using GMP.

- Mechanisms of action are being determined, and synergy of constituents are under investigation.

- Safety studies include Phase I maximum tolerated dose and pharmacokinetic studies as well as evaluations of drug-botanical interactions such as induction and inhibition of drug metabolizing enzymes.

- Efficacy studies have included Phase II randomized, placebo-controlled clinical trials.
Acknowledgements

- NIH grants P50 AT00155 and P50 AT00155S1 from the Office of Dietary Supplements and the National Center for Complementary and Alternative Medicine
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- Shimadzu Instruments, Naturex and Pharmavite
- Harald Schwarz and Hopsteiner
- All the faculty, students and postdoctoral fellows of the UIC/NIH Botanical Center for Dietary Supplements Research, and especially the founding director, Norman R. Farnsworth.