CITY UNIVERSITY OF NEW YORK GRADUATE SCHOOL
Ph.D. PROGRAM IN BIOLOGY – 2009 PLANT SCIENCES FIRST EXAMINATION

Reading List and Preparation Instructions

Reading List

Basic botany, systematics, plant morphology

Raven, P.H., R.F. Evert & S.E. Eichhorn. 2005. Biology of Plants, 7th ed. W.H. Freeman and Company, New York, NY.

Judd, W.S, C.S. Campbell, E.A. Kellogg, P.F. Stevens & M.J. Donoghue. 2008. Plant Systematics: A Phylogenetic Approach, 3rd ed. Sinauer Associates, Sunderland, MA. **Chapters 1–7.**

Biostatistics

Sokal, R.R. & F.J. Rohlf. 1995. Biometry, 3rd Ed. WH Freeman, NY.

Phytochemistry

Reference: Dewick, Paul M. 2002. Medicinal natural products: a biosynthetic approach. Second Ed., John Wiley and Sons, NY.

Papers from which essay questions will be asked (manuscripts will be provided at the exam):

1. B U Jaki, SG Franzblau, LR Chadwick, DC Lankin, F Zhang, Y Wang, GF Pauli (2008) Purity-activity relationships of natural products: The case of anti-TB active ursolic acid. *J. Nat. Prod.*, 71 (10): 1742-1748
2. K M. Newton, S D. Reed, A Z. LaCroix, L Grothaus, K Ehrlich, J Guiltinan, (2006) Treatment of vasomotor symptoms of menopause with Black Cohosh, multibotanicals, soy, hormone therapy, or placebo. *Ann Intern Med.* 145:869-879.

Plant Molecular Biology and Genetics

Reference: Lodish, Berk, Zipursky, Matsudaira, Baltimore, Darnell. 2004. Molecular Cell Biology, 6th ed. W.H. Freeman, NY. Chapters 3, 4, 5, 6, 7, 8, 13, 22.4)

Papers from which essay questions will be asked (manuscript will **NOT** be provided at the exam):

Li, F., Vallabhaneni, R., and Wurtzel, E.T. (2008) *PSY3*, a new member of the phytoene synthase gene family conserved in the Poaceae and a key regulator of abiotic-stress-induced root carotenogenesis. *Plant Physiology* 146 (3): 1333-1345 http://www.plantphysiol.org/cgi/reprint/146/3/1333 (plus link to supplemental data).

Preparation for the exam

The exam will be given on one day and consist of three sections (700 pts). Session I (100 pts) must be handed in before the start of the exam in both electronic and hard copy. Sessions II and III (each 300 pts) will draw from the reading list above. Students must pass with a minimum of 70% or 490 points.

SESSION I. ESSAY to submit before the morning session. (TOTAL 100 pts)

Instructions: Submit the Session I essay in hard copy and in electronic form as a PDF file. Do not put your name on either, use your student number for filename and for marking the hard copy. Make sure that in "properties" there is no notation of "author".

Present a subject, problem, hypothesis, theory, or controversy you consider important to plant sciences. **The essay must be hypothesis driven.** The essay should show relevance across the botanical subdisciplines. The essay should be both a review and a synthesis and demonstrate the level of scholarship, criticism, and independent thinking we require at the doctoral level. Your topic may be a large or a small one; broad or highly specialized; and you must communicate how the chosen topic is relevant to a major concept. We wish to measure the ability to understand and to synthesize information and ideas from more than one discipline of biology. The paper should have a title and begin with a **one paragraph abstract/summary which includes your hypothesis.** The paper should be paginated and written with 11 pt. Arial or equivalent font, one-inch margins, and a **maximum of five (5) pages of double spaced text** followed by a minimum of 15 (complete) literature citations provided in the format required by the journal, Plant Physiology (see: http://www.plantphysiol.org/misc/ifora.shtml). Following the literature citation section, provide the names (from faculty in the CUNY Biology PhD program) of two potential "reviewers" along with their areas of expertise which you feel make them appropriate to reviewers of your manuscript. *Note: an essay based largely on the published work or grant proposals of faculty staff members or scientists at other institutions is not acceptable. The essay must be your own idea and not the product of a collaborative effort. Faculty should not be consulted in development of your essay.*

SESSION II. MORNING (TOTAL 300 pts)

Instructions:

1-Answer definitions and short answer botany questions (100 pts)

2- Write an essay in each of the two subject areas (plant systematics, plant morphology) (100 pts each essay, for a total of 200 pts). For each topic areas, students must choose one of the two essay questions.

SESSION III. AFTERNOON (TOTAL 300 pts)

Instructions: Answer one essay in each of the three subject areas (biostatistics, molecular biology, phytochemistry) (100 pts each essay, for a total of 300 pts). For each topic area, students should choose one of the two essay questions.

Treatment of Vasomotor Symptoms of Menopause with Black Cohosh, Multibotanicals, Soy, Hormone Therapy, or Placebo

A Randomized Trial

Katherine M. Newton, PhD; Susan D. Reed, MD MPH; Andrea Z. LaCroix, PhD; Louis C. Grothaus, MS; Kelly Ehrlich, MS; and Jane Guiltinan, ND

Background: Herbal supplements are widely used for vasomotor symptoms.

Objective: To test the efficacy of 3 herbal regimens and hormone therapy for relief of vasomotor symptoms compared with placebo.

Design: 1-year randomized, double-blind, placebo-controlled trial conducted from May 2001 to September 2004.

Setting: Group Health, Washington State.

Participants: 351 women age 45 to 55 years with 2 or more vasomotor symptoms per day; 52% of the women were in menopausal transition and 48% were postmenopausal.

Measurements: Rate and intensity of vasomotor symptoms (1 = mild to 3 = severe), and Wiklund Vasomotor Symptom Subscale.

Interventions: 1) Black cohosh, 160 mg daily; 2) multibotanical with black cohosh, 200 mg daily, and 9 other ingredients; 3) multibotanical plus dietary soy counseling; 4) conjugated equine estrogen, 0.625 mg daily, with or without medroxyprogesterone acetate, 2.5 mg daily; or 5) placebo.

Results: Vasomotor symptoms per day, symptom intensity, Wiklund Vasomotor Symptom Subscale score did not differ between the herbal interventions and placebo at 3, 6, or 12 months or for the average over all the follow-up time points ($P > 0.05$ for all comparisons) with 1 exception: At 12 months, symptom intensity was significantly worse with the multibotanical plus soy intervention than with placebo ($P = 0.016$). The difference in vasomotor symptoms per day between placebo and any of the herbal treatments at any time point was less than 1 symptom per day; for the average over all the follow-up time points, the difference was less than 0.55 symptom per day. The difference for hormone therapy versus placebo was − 4.06 vasomotor symptoms per day for the average over all the follow-up time points (95% CI, −5.93 to − 2.19 symptoms per day; $P < 0.001$).

Limitations: The trial did not simulate the whole-person approach used by naturopathic physicians. Differences between treatment groups smaller than 1.5 Vasomotor symptoms per day cannot be ruled out.

Conclusion: Black cohosh used in isolation, or as part of a multibotanical regimen, shows little potential as an important therapy for relief of vasomotor symptoms.

Ann Intern Med. 2006;145:869-879. www.annals.org
For author affiliations, see end of text.
Clinical Trials Registration number: *NCT00169299*

Hormone therapy remains the recommended treatment for vasomotor symptoms, but trials have shown serious risks with even short-term use (1, 2). The use of herbs, particularly black cohosh, multibotanical supplements, and dietary soy for menopausal symptoms has grown dramatically (3–6). Few of these approaches have been scientifically evaluated. Women and providers are seeking safe, effective alternatives to hormone therapy. We designed the Herbal Alternatives for Menopause Trial (HALT) to provide rigorous evidence on the efficacy and short-term safety of commonly used naturopathic approaches for management of vasomotor symptoms.

Methods

Design Overview and Setting

HALT was a 1-year double-blind, randomized, controlled trial designed to investigate the effects of 3 naturopathic approaches for vasomotor symptom relief and hormone therapy compared with placebo. Study methods have been described elsewhere (7). The Group Health Institutional Review Board approved this study, and a data and safety monitoring committee monitored it. The study was conducted at Group Health, an integrated health plan in Washington State.

Participants

Eligibility criteria were as follows: age 45 to 55 years; late menopausal transition (≥1 skipped menses within the preceding 12 months) or postmenopausal (no bleeding within 12 months, or follicle-stimulating hormone level > 20 IU/mL if patient had undergone hysterectomy without bilateral oophorectomy); and 2 or more vasomotor symptoms per day over 2 weeks (≥6 moderate to severe symptoms). Women in menopausal transition were included because many are highly symptomatic and trial data are lacking for this group. Exclusion criteria were the following: contraindications to hormone therapy; use of hor-

See also:

Print

Editors' Notes 870
Editorial comment. 924
Summary for Patients. I-25

Web-Only

Appendix Table
CME quiz
Conversion of figures and tables into slides

© 2006 American College of Physicians

Context

Caution about taking estrogen for treating postmenopausal vasomotor symptoms has led to increasing substitution of herbal regimens despite few tests of their effectiveness.

Contribution

The authors randomly assigned 351 perimenopausal or postmenopausal women to herbal treatments (black cohosh, multibotanicals, or multibotanicals plus counseling about dietary soy), estrogen with or without progesterone, or placebo. At 3, 6, and 12 months, patients receiving the herbal interventions had the same change in vasomotor symptoms as those receiving placebo (except for more severe symptoms at 12 months for patients taking multibotanicals plus dietary soy). Estrogen substantially decreased vasomotor symptoms.

Cautions

Most participants were white and were well-educated.

Implications

Herbal regimens did not reduce postmenopausal vasomotor symptoms in this sample of women.

—The Editors

mone therapy or oral contraceptives within 3 months before the trial; use of herbal medicines for menopausal symptoms within 1 month before the trial; soy allergy; bilateral oophorectomy; history of breast cancer; and nonadherence during the run-in period (<80% of capsules taken).

From May 2001 through August 2003, women were recruited by using direct mail. Screening calls determined initial eligibility. Women attended an orientation visit at which eligibility was confirmed, physical measurements were collected, and placebo medication and questionnaires for the 2-week run-in period were provided.

Randomization and Interventions

Participants were randomly assigned by using SAS software (SAS Institute, Inc., Cary, North Carolina), stratified by previous hormone therapy and hysterectomy; block sizes within strata ranged from 5 to 25. Treatment assignments were sent to the University of Washington Research Pharmacy, where medications were bottled and labeled with a sequential identification number without treatment group indication. At the randomization visit, vasomotor symptom diaries and medication counts were examined to confirm eligibility and adherence. The study nurse determined the appropriate stratum, assigned the participant the next study number in that stratum without knowledge of group assignment, and distributed study medications.

The publication of results from the Women's Health Initiative (WHI) estrogen–progestin trial (2) raised new concerns about the safety of estrogen therapy. New study participants were given the choice of 5-arm (including hormone therapy) versus 4-arm (no hormone therapy) randomization. Current participants gave consent again, incorporating risk estimates from the WHI, and were given the option of finding out whether they had been assigned to hormone therapy; 16 were unblinded, 1 discontinued use of the study drug, and all remained enrolled. Following publication of the WHI Memory Study (8, 9), we informed participants of those findings and restricted randomization to herbs and placebo. No further women were unblinded or discontinued use of the study drug.

Naturopathic medicine provided the model for study interventions. The herbal products, doses, and soy diet were based on approaches used by naturopaths when the study was designed (7). The study groups were as follows: 1) black cohosh (*Actaea racemosa* or *Cimicifuga racemosa*, 160 mg daily; 2.5% triterpene glycosides; 70% ethanol extract); 2) multibotanical; 3) multibotanical plus soy diet counseling; 4) conjugated equine estrogen, 0.625 mg daily, with (for women with a uterus) or without (for women without a uterus) medroxyprogesterone acetate, 2.5 mg; and 5) placebo. The multibotanical delivered daily doses of the following: black cohosh, 200 mg; alfalfa (*Medicago sativa)*, 400 mg; boron, 4 mg; chaste tree (*Vitex agnus-castus*), 200 mg; dong quai (*Angelica sinensis*), 400 mg; false unicorn (*Chamaelirium luteum*), 200 mg; licorice (*Glycyrrhiza glabra)*, 200 mg; oats (*Avena sativa*), 400 mg; pomegranate (*Punica granatum*), 400 mg; Siberian ginseng (*Eleutherococcus senticosus,* standardized constituents 0.8% eleutherosides E and B), 400 mg.

Black cohosh was provided by Pure World, Inc., (Hackensack, New Jersey). The multibotanical, ProGyne, was purchased from Progena Professional Formulations (Albuquerque, New Mexico) and encapsulated to study specifications. Both companies follow current good manufacturing practices, and single batches were used. Consumer Lab.com (White Plains, New York) tested the products after the study commenced. Dong quai, false unicorn, and pomegranate were not detected, suggesting that they were of poor quality or did not contain the tested marker compounds. Other marker compounds were detected in the approximate doses as labeled (7). The **Appendix Table** (available at www.annals.org) describes the herbal products in detail.

To facilitate blinding, medications and lactose placebo were encapsulated to provide 2 white and 2 blue capsules to each woman, and medication boxes were labeled with a unique identification number that did not indicate study group.

The soy food intervention was modeled after a successful 5-a-day intervention (10). We chose soy foods because of uncertainty about the efficacy of isoflavone supplements and because naturopaths commonly recommend whole soy foods. Participants received 5 telephone calls from a clinical dietitian and a 34-page booklet recommending 2 soy

food servings per day (12 to 20 g of soy protein) (7). Other participants received 1 telephone call and a similar booklet reinforcing fruit and vegetable intake. Participants were instructed not to discuss dietitian calls with study nurses and were unaware that soy counseling was linked to the multibotanical.

Outcomes and Measurements

The primary outcomes were change from baseline (measured over 2-week run-in period) to 3, 6, and 12 months (each measured for 4 weeks) and change from baseline to the average for all follow-ups with regard to the mean frequency and intensity of vasomotor symptoms (daytime hot flashes plus night sweats) and the mean Wiklund Vasomotor Symptom Subscale score (11, 12). We also evaluated change from baseline to follow-up (months 3, 6, and 12 and average change) for daytime hot flash rate, night sweat rate, and the total Wiklund Menopause Symptom Scale score. Symptom diaries and global ratings of menopause symptoms are almost universally used in studies of vasomotor symptoms. Participants used a vasomotor symptom diary to record daytime hot flashes and night sweats, rating intensity as mild, moderate, or severe (scale, 1 to 3), as recommended by the U.S. Food and Drug Administration (13). Women completed the Wiklund Menopause Symptom Scale, rating the severity of 13 menopausal symptoms (sweats, hot flashes, sleep disturbance, fatigue, vaginal dryness, depression, headache, irritability, muscle/joint pain, breast tenderness, nervousness, palpitations, and dizziness/fainting on a scale of 0 (none) to 10 (severe) (11). Soy food intake was monitored by using a self-reported, validated soy food questionnaire (14).

Follow-up Procedures

Participants returned to the research clinic at 3, 6, and 12 months. Questionnaires were collected, and study medication was dispensed. Medication adherence counts were conducted by staff without participant contact and without knowledge of treatment assignment. Study nurses encouraged adherence and monitored adverse events during monthly telephone calls and at visits. Adverse events were identified through participants' responses to the question, "Have you had any medical problems or been hospitalized?" Responses were recorded and adverse events were followed until they were resolved. The study nurse determined intensity (mild, moderate, or severe) and whether the event was serious (yes or no). Events were reviewed by the study physician, who determined whether the event was study related, without knowledge of group assignment. Adverse events were coded by using the *Coding Symbols for Thesaurus of Adverse Reaction Terms* (15).

The Data and Safety Monitoring Committee reviewed study progress and unblinded outcome and safety data 5 times.

Statistical Analysis

The study was powered to detect an effect of herbs halfway between the expected effects of hormone therapy and placebo, an outcome that we hypothesized would be meaningful.

Treatment effects, the difference between each treatment group and the placebo group with regard to the mean change from baseline, and the associated 95% CIs and P values were estimated by using a multivariate mixed model (PROC MIXED in SAS). We used an unstructured covariance matrix for the repeated measures, with separate parameter estimates for women in the hormone therapy group, since this structure best fit the data. Mixed models increase statistical power because of their ability to use all follow-up data and to better handle missing data. Although retention rates were very high, mixed-model analysis allowed us to use a true intention-to-treat approach, including data from all 351 randomly assigned women ($n = 349$ in adjusted analyses because of missing covariate data). The two Wiklund measures were analyzed after square-root transformation to normalize their distributions.

Mixed models were evaluated with and without adjustment for covariates. All models included a term for randomization protocol (4-arm vs. 5-arm). The adjusted models also controlled for age, body mass index (BMI), hysterectomy, menopausal status (menopausal transition vs. postmenopausal), and previous hormone therapy. All covariates except for BMI were selected a priori because of their hypothesized correlations with study outcomes and exposures. Results from the adjusted and unadjusted models were identical; we present only the adjusted results.

We also tested whether treatment effects differed by 4-arm versus 5-arm randomization (arm by-treatment interaction). Since they did not differ, we present the results based on all randomly assigned women. An "as-treated" sensitivity analysis, restricted to women who took at least 80% of their study medications, was conducted, but results were similar and are not presented. Finally, we used mixed models to test whether the effect of each treatment varied in a statistically significant manner with BMI (nonobese [BMI < 30 kg/m^2] vs. obese), hysterectomy, menopausal status, previous use of hormone therapy, and baseline symptom rate ($<$7 symptoms per day vs. $\geq$7 symptoms per day).

Adverse events rates were compared between each group and placebo by using chi-square tests or Fisher exact test (if expected count was $<$5). The study biostatistician conducted all analyses.

Role of the Funding Sources

This study was funded by the National Institute on Aging and National Center for Complementary and Alternative Medicine. These agencies did not participate in the design, conduct, or analysis of the study or in decisions to submit the manuscript for publication. The National Institute on Aging did participate in decisions related to recruitment redesign in response to the release of the WHI findings and had a representative who attended all Data and Safety Monitoring Committee meetings.

RESULTS

Participants and Follow-up

We mailed 157 493 informational brochures and received 3443 responses (**Figure 1**). The baseline visit was attended by 509 women; of the 398 eligible women, 351 consented and were randomly assigned as follows: black cohosh ($n = 80$); multibotanical ($n = 76$); multibotanical plus soy counseling ($n = 79$); conjugated equine estrogen with medroxyprogesterone acetate ($n = 29$ women with a uterus) or without medroxyprogesterone acetate ($n = 3$ women without a uterus, all receiving unopposed estrogen); or placebo ($n = 84$). We enrolled 159 women under the 5-arm randomization scheme and 192 under the 4-arm randomization scheme; 147 of 183 women who were given a choice selected the 4-arm protocol. Ninety-two percent of women completed the study (327 of 351), and 87% (306 of 351) were taking study medication at 12 months.

Baseline characteristics were similar across treatment groups, with the exception of BMI (**Table 1**). On average, BMI was lower in the black cohosh group than in the placebo group and was higher in the hormone therapy group than in the placebo group. Average age was 52.2 years; 93% of participants were white, and all had at least a high school education. Women averaged 6.5 vasomotor symptoms per day (SD, 3.7; range, 1.4 to 24), and 34% averaged at least 7 symptoms per day at baseline. Average symptom intensity was 1.8 (on a scale of 1 to 3); 29% of participants reported symptom intensity averaging at least 2.0. The average Wiklund Menopause Symptom Scale score was 2.3 (SD, 1.2; range, 0.2 to 6.5) and the average Wiklund Vasomotor Symptom Subscale score was 4.5 (SD, 2.0; range, 0.8 to 10). Of 183 women (52%) who were in menopausal transition at baseline, 79 (46.6%) achieved 12 months of amenorrhea during the study.

Among women assigned to the soy food intervention, 77% completed 3 or more telephone calls (mean, 3.6). At baseline, women reported an average of 0.6 serving of soy per day. On average, women in the multibotanical plus soy intervention increased dietary soy by 1.1 servings per day between baseline and 3 months, compared with 0.1 serving per day in the other 4 groups.

Primary Outcomes

The average adjusted number of vasomotor symptoms per day (**Figure 2**) and the Wiklund Vasomotor Symptom Subscale score (**Figure 3**) decreased between baseline and 3 months in all groups. There were no statistically significant differences in the average adjusted change in vasomotor symptoms per day or in vasomotor symptom intensity between the herbal interventions and placebo at 3, 6, or 12 months, or for the average over all the follow-up time points, with 1 exception: At 12 months, the multibotanical plus soy intervention had higher (worse) symptom intensity relative to placebo ($P = 0.016$) (**Table 2**). The average difference in vasomotor symptoms per day between the placebo and herbal treatments groups was less than 1 symptom per day at 3 months and less than 0.6 symptom per day for the average over all the follow-up time points. The average adjusted difference for hormone therapy compared with placebo was −4.55 (95% CI, −6.51 to −2.59) vasomotor symptoms per day at 3 months ($P < 0.001$) and −4.06 (CI, −5.93 to −2.19) vasomotor symptoms per day for the average over all the follow-up time points ($P < 0.001$) (**Table 2**).

There were no statistically significant differences in the Wiklund Vasomotor Symptom Subscale score between any of the herbal interventions and placebo at 3, 6, or 12 months or for the average over all the follow-up time points (**Table 2**). The Wiklund Vasomotor Symptom Subscale score was statistically significantly lower with hormone therapy than with placebo at all follow-up time points.

Additional Analyses

There were no statistically significant differences in hot flashes per day or night sweats per day between any of the herbal interventions and placebo at 3, 6, or 12 months or for the average over all the time points, with 1 exception: At 3 months, the black cohosh group had 0.38 less night sweat per day than the placebo group (CI, −0.72 to −0.04; $P = 0.030$) (**Table 3**). The difference between the herbal treatments and placebo was less than 0.6 hot flash per day and less than 0.4 night sweat per day at any time point; the differences in the average over all the time points were even smaller. The differences in hot flashes per day and night sweats per day between hormone therapy and placebo were statistically significant at all times points; over all follow-up time points, the average difference was nearly −3 hot flashes per day and nearly −1 night sweat per day (**Table 3**).

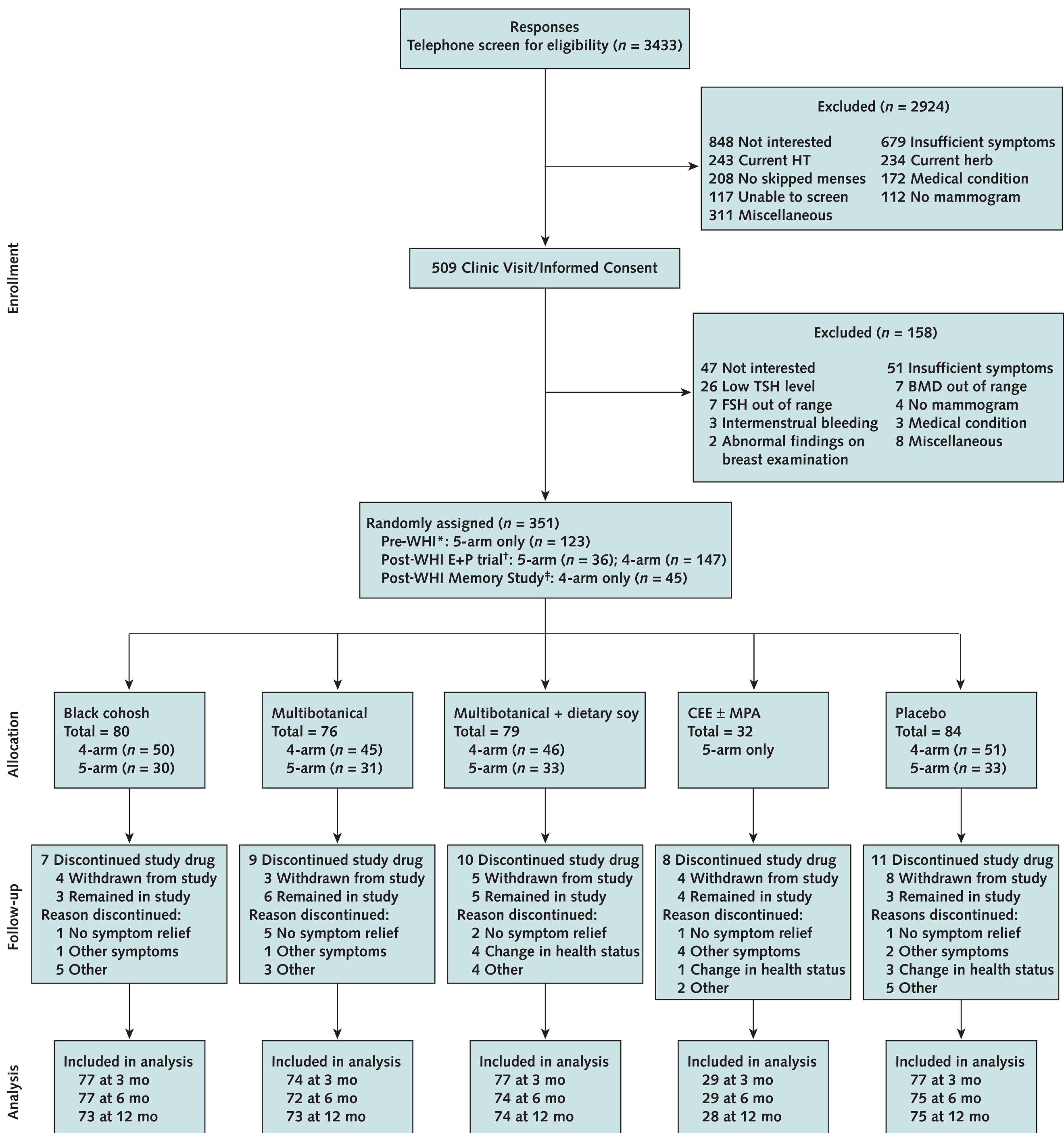
There were no statistically significant differences in the Wiklund Menopause Symptom Scale score between any of the herbal interventions and placebo at 3, 6, or 12 months or for the average over all the time points (**Table 3**). All differences between hormone therapy and placebo were statistically significant at all times (**Table 3**).

As-treated analyses, limited to women with at least 80% adherence, and separate analyses for women in the 4-arm and 5-arm randomization schemes, yielded similar results (data not shown). Results did not vary (no statistically significant treatment by subgroup interaction) when results were examined by baseline number of vasomotor symptoms (<7 vs. ≥7 per day), 4-arm versus 5-arm randomization scheme, baseline menopausal status, previous use of hormone therapy, hysterectomy (yes or no), or BMI (not obese [<30 kg/m^2] vs. obese) (data not shown).

Adverse Events and Adherence

Women assigned to hormone therapy reported more breast pain ($P < 0.001$) and menstrual disorders ($P = 0.04$) compared with placebo (**Table 4**). There were no statistically significant differences between any of the 4 groups and placebo in the proportion of women with up-

Figure 1. **Participant recruitment and retention, Herbal Alternatives for Menopause Trial (HALT).**



Reasons for discontinuing therapy are not mutually exclusive. All participants received allocated intervention. *In the original enrolled plan, all participants were enrolled in 1 of 5 groups. †After publication of the Women's Health Initiative (*WHI*) estrogen–progestin (E + P) trial (2), women were given the option of 4-arm (without conjugated equine estrogen [*CEE*]) or 5-arm randomization. ‡After publication of the WHI Memory Study (8, 9), randomization to CEE was stopped and only 4-arm randomization (that is, random assignment to herb or placebo, excluding hormone therapy) was used. BMD = bone mineral density; FSH = follicle-stimulating hormone; HT = estrogen with or without progestin; MPA = medroxyprogesterone acetate, 2.5 mg (women without a uterus were randomly assigned to CEE only); soy = counseling to increase dietary soy; TSH = thyroid-stimulating hormone.

Table 1. **Baseline Clinical and Demographic Characteristics by Randomization Group**

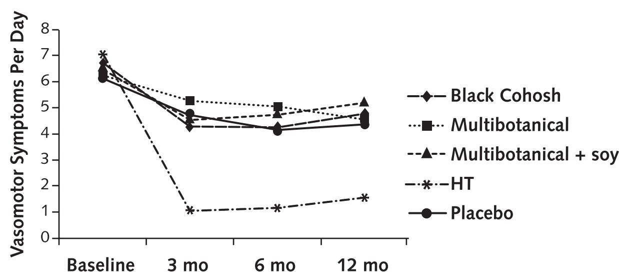
Characteristic	All Participants (*n* = 351)	Black Cohosh Group (*n* = 80)	Multibotanical Group (*n* = 76)	Multibotanical plus Soy Counseling Group (*n* = 79)	Conjugated Equine Estrogen with or without Medroxyprogesterone Acetate Group (*n* = 32)	Placebo Group (*n* = 84)
Mean age (SD), *y*	52.2 (2.4)	52.0 (2.2)	52.2 (2.5)	52.5 (2.5)	52.3 (2.6)	52.0 (2.5)
Mean body mass index (SD), *kg/m²*	28.6 (6.2)	27.3 (5.0)	28.4 (6.3)	28.4 (5.7)	31.5 (7.9)	29.2 (6.4)
Race/ethnicity, *n (%)*						
White	323 (93)	73 (91)	72 (99)	74 (95)	30 (94)	74 (88)
African-American	9 (3)	3 (4)	1 (1)	3 (4)	0 (0)	2 (2)
Other	15 (4)	4 (5)	0 (0)	1 (1)	2(6)	8 (10)
Greater than high school education, *n (%)*	331 (95)	77 (96)	72 (97)	75 (95)	28 (88)	79 (94)
Menopausal transition (vs. postmenopausal), *n (%)*	183 (52)	39 (49)	39 (52)	43 (54)	20 (63)	42 (50)
Hysterectomy*, *n (%)*	38 (11)	9 (11)	7 (9)	8 (10)	3 (9)	11(13)
Previous hormone therapy, *n (%)*	140 (40)	32 (40)	31 (41)	32 (41)	10 (31)	35 (42)
Mean vasomotor symptoms per day (SD), *n*	6.5 (3.7)	6.7 (3.0)	6.2 (3.6)	6.5 (3.9)	6.8 (4.9)	6.2 (3.7)
Hot flashes	4.6 (3.1)	4.7 (2.5)	4.4 (3.0)	4.6 (3.2)	5.0 (4.4)	4.3 (3.0)
Night sweats	1.9 (1.2)	2.0 (1.2)	1.8 (1.1)	1.9 (1.2)	1.8 (1.0)	1.9 (1.2)
Mean vasomotor symptom intensity (SD)†	1.80 (0.39)	1.78 (0.39)	1.78 (0.39)	1.77 (0.35)	1.82 (0.40)	1.85 (0.41)
Average symptoms moderate to severe (vs. mild), *n (%)*	101 (29)	21 (26)	20 (28)	22 (26)	12 (38)	26 (31)
Mean Wiklund Menopause Symptom score (SD)	2.3 (1.2)	2.2 (1.2)	2.2 (1.1)	2.2 (1.2)	2.1 (1.0)	2.5 (1.2)
Mean Wiklund Vasomotor Symptom Subscale score (SD)	4.5 (2.0)	4.4 (1.9)	4.3 (1.9)	4.3 (2.1)	4.5 (2.0)	4.9 (2.0)
Average ≥7 vasomotor symptoms per day, *n (%)*	120 (34)	33 (41)	23 (30)	28 (35)	12 (38)	24 (29)

* Women with hysterectomy had at least 1 ovary.
† 1 = mild; 2 = moderate; 3 = severe.

per or lower gastrointestinal symptoms; nausea and vomiting; fatigue, asthenia, or malaise; or headaches or migraine. Too few severe adverse events occurred to make meaningful group comparisons (1 case of endometrial cancer in the multibotanical plus soy group 2.8 months after randomization; 1 case of breast cancer in the multibotanical group 4.7 months after randomization).

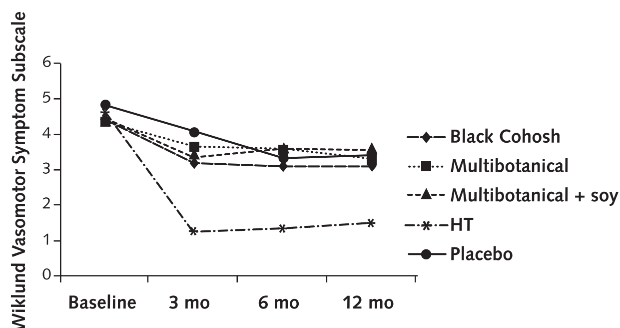
Over the 12-month follow-up period, the overall study sample on average took 86% of their pills; among individual study groups, adherence was 88% for black cohosh, 80% for multibotanical, 87% for multibotanical plus soy, 87% for hormone therapy, and 82% for placebo. These adherence figures include all 351 women; adherence was set to 0 for those who dropped out or stopped using study medication (**Table 4**). The primary reasons for discontinuation of study medication use or study withdrawal were no symptom relief (*n* = 10), other symptoms (*n* = 8), or change in health status (*n* = 8) (**Figure 1**).

Figure 2. **Adjusted mean number of vasomotor symptoms per day, by study group.**



Adjusted for age (continuous), body mass index (kg/m², continuous), hysterectomy (yes or no), previous use of hormone therapy (*HT*) (yes or no), menopausal status (menopausal transition vs. postmenopausal), and randomization arm (4-arm without hormone therapy vs. 5-arm with hormone therapy).

Figure 3. **Adjusted mean Wiklund Vasomotor Symptom Subscale scores, by study group.**



Adjusted for age (continuous), body mass index (kg/m², continuous), hysterectomy (yes or no), previous use of hormone therapy (*HT*) (yes or no), menopausal status (menopausal transition vs. postmenopausal), and randomization arm (4-arm without hormone therapy vs. 5-arm with hormone therapy).

Table 2. **Difference in Adjusted Mean Change in Vasomotor Symptom Frequency and Intensity and Wiklund Vasomotor Symptom Subscale Score between Intervention and Placebo Groups***

Variable	Black Cohosh Group (n = 80)		Multibotanical Group (n = 76)		Multibotanical plus Soy Counseling Group (n = 79)		Conjugated Equine Estrogen with or without Medroxyprogesterone Acetate Group (n = 32)	
	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo
Vasomotor symptoms per day (hot flashes plus night sweats)								
3 mo	–0.96 (–2.03 to 0.11)	0.079	0.41 (–0.67 to 1.50)	0.45	–0.53 (–1.60 to 0.54)	0.33	–4.55 (–6.51 to –2.59)	<0.001
6 mo	–0.48 (–1.63 to 0.66)	0.41	0.80 (–0.36 to 1.96)	0.178	0.32 (–0.83 to 1.47)	0.59	–3.86 (–5.73 to –2.00)	<0.001
12 mo	–0.18 (–1.30 to 0.93)	0.74	0.09 (–1.03 to 1.20)	0.88	0.49 (–0.62 to 1.60)	0.39	–3.76 (–5.76 to –1.76)	<0.001
Treatment effect over all follow-up time points	–0.54 (–1.47 to 0.38)	0.25	0.43 (–0.50 to 1.37)	0.36	0.09 (–0.83 to 1.02)	0.84	–4.06 (–5.93 to –2.19)	<0.001
Vasomotor symptom intensity (1 = mild, 2 = moderate, 3 = severe)								
3 mo	0.03 (–0.08 to 0.14)	0.59	0.08 (–0.03 to 0.19)	0.174	0.03 (–0.08 to 0.14)	0.60	0.07 (–0.17 to 0.31)	0.57
6 mo	0.01 (–0.11 to 0.12)	0.91	0.09 (–0.03 to 0.21)	0.126	0.06 (–0.06 to 0.17)	0.35	–0.13 (–0.34 to 0.08)	0.23
12 mo	0.05 (–0.07 to 0.17)	0.46	0.11 (–0.02 to 0.23)	0.089	0.15 (0.03 to 0.27)	0.016	0.05 (–0.15 to 0.26)	0.63
Treatment effect over all follow-up time points	0.03 (–0.07 to 0.12)	0.57	0.09 (0.00 to 0.19)	0.063	0.08 (–0.02 to 0.17)	0.108	0.00 (–0.19 to 0.18)	0.97
Wiklund Vasomotor Symptom Subscale score								
3 mo	–0.47 (–1.18 to 0.24)	0.098	0.15 (–0.57 to 0.87)	0.88	–0.23 (–0.94 to 0.48)	0.28	–2.60 (–3.74 to –1.46)	<0.001
6 mo	0.20 (–0.52 to 0.91)	0.90	0.80 (0.07 to 1.52)	0.062	0.72 (–0.01 to 1.44)	0.057	–1.78 (–2.80 to –0.76)	<0.001
12 mo	0.10 (–0.68 to 0.88)	0.83	0.44 (–0.34 to 1.22)	0.43	0.57 (–0.21 to 1.35)	0.27	–1.77 (–2.79 to –0.75)	<0.001
Treatment effect over all follow-up time points	–0.06 (–0.67 to 0.55)	0.48	0.46 (–0.15 to 1.08)	0.30	0.35 (–0.26 to 0.96)	0.41	–2.05 (–3.02 to –1.08)	<0.001

* All analyses were adjusted for age (continuous), body mass index (kg/m^2, continuous), hysterectomy (yes or no), previous use of hormone therapy (yes or no), menopausal status (menopause transition vs. postmenopausal), and randomization arm (4-arm without hormone therapy vs. 5-arm with hormone therapy). Estimates of difference in mean change from baseline vs. placebo, along with *P* values and 95% CIs, from mixed-model analysis that used data from baseline and all 3 follow-up time points (total, n = 349 for adjusted analyses).

Discussion

In this large, randomized, double-blind trial, none of the 3 herbal treatments had clinically meaningful effects on any of the primary outcomes. As expected, hormone therapy resulted in a clinically important decrease in vasomotor symptom frequency and Wiklund scores throughout 1 year of treatment.

At least 5 randomized, placebo-controlled trials of black cohosh and menopausal symptoms have been published (16–20). All were short-term (≤12 weeks), and most were small, typically randomly assigning 30 to 60 women per group. Among 5 trials that examined frequency of hot flashes (16–19, 21), the only trial reporting a positive effect for black cohosh also found no effect of conjugated equine estrogen versus placebo (18); this result raises concerns about the validity of the findings. When menopause rating scales were used, 2 trials reported a positive effect of black cohosh (16, 18), 3 reported no difference from placebo (17, 19, 21) , and 1 did not report main trial effects (20). The only trial that investigated a multibotanical containing black cohosh found no differences in vasomotor symptom frequency or menopause scale scores com-

Table 3. **Difference in Adjusted Mean Change in Frequency of Hot Flashes and Night Sweats and Wiklund Menopause Symptom Scale Scores between Intervention and Placebo Groups***

Variable	Black Cohosh Group (n = 80)		Multibotanical Group (n = 76)		Multibotanical plus Soy Counseling Group (n = 79)		Conjugated Equine Estrogen with or without Medroxyprogesterone Acetate Group (n = 32)	
	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo	Difference in Mean Change vs. Placebo (95% CI)	*P* Value vs. Placebo
Hot flashes per day								
3 mo	–0.59 (–1.43 to 0.26)	0.174	0.30 (–0.56 to 1.15)	0.50	–0.34 (–1.19 to 0.50)	0.42	–3.55 (–5.24 to –1.86)	0.0002
6 mo	–0.26 (–1.19 to 0.67)	0.59	0.55 (–0.40 to 1.50)	0.26	0.32 (–0.62 to 1.26)	0.51	–2.89 (–4.52 to –1.26)	0.001
12 mo	–0.28 (–1.16 to 0.60)	0.53	–0.05 (–0.93 to 0.84)	0.92	0.32 (–0.56 to 1.20)	0.48	–3.15 (–4.84 to –1.47)	<0.001
Treatment effect over all follow-up time points	–0.38 (–1.13 to 0.37)	0.33	0.27 (–0.49 to 1.03)	0.49	0.10 (–0.65 to 0.85)	0.80	–3.20 (–4.82 to –1.58)	<0.001
Night sweats per day								
3 mo	–0.38 (–0.72 to –0.04)	0.030	0.11 (–0.23 to 0.46)	0.52	–0.18 (–0.53 to 0.16)	0.29	–0.98 (–1.40 to –0.56)	<0.001
6 mo	–0.23 (–0.57 to 0.11)	0.182	0.23 (–0.12 to 0.57)	0.198	0.00 (–0.34 to 0.34)	1.00	–0.98 (–1.40 to –0.56)	<0.001
12 mo	0.08 (–0.30 to 0.47)	0.67	0.12 (–0.27 to 0.51)	0.54	0.16 (–0.23 to 0.54)	0.43	–0.60 (–1.08 to –0.13)	0.015
Treatment effect over all follow-up time points	–0.18 (–0.48 to 0.12)	0.25	0.15 (–0.15 to 0.46)	0.32	–0.01 (–0.31 to 0.29)	0.95	–0.85 (–1.26 to –0.45)	<0.001
Wiklund Menopause Symptom Subscale score								
3 mo	–0.15 (–0.47 to 0.17)	0.23	–0.04 (–0.36 to 0.28)	0.57	–0.10 (–0.42 to 0.22)	0.35	–0.87 (–1.29 to –0.46)	<0.001
6 mo	0.07 (–0.26 to 0.41)	0.86	0.35 (0.01 to 0.68)	0.068	0.25 (–0.08 to 0.58)	0.23	–0.37 (–0.90 to 0.16)	0.028
12 mo	–0.03 (–0.40 to 0.33)	0.83	0.14 (–0.23 to 0.51)	0.54	0.03 (–0.34 to 0.40)	0.99	–0.49 (–0.93 to –0.05)	0.013
Treatment effect over all follow-up time points	–0.04 (–0.33 to 0.26)	0.64	0.15 (–0.15 to 0.44)	0.45	0.06 (–0.23 to 0.35)	0.90	–0.58 (–0.98 to –0.18)	<0.001

* All analyses were adjusted for age (continuous), body mass index (kg/m^2, continuous), hysterectomy (yes or no), previous use of hormone therapy (yes or no), menopausal status (menopause transition vs. postmenopausal), and randomization arm (4-arm without hormone therapy vs. 5-arm with hormone therapy). Estimates of difference in mean change from baseline vs. placebo, along with *P* values and 95% CIs, from mixed-model analysis that used data from baseline and all 3 follow-up time points (total, n = 349 for adjusted analyses).

pared with placebo (22). Thus, the totality of the evidence does not consistently support a short-term effect of black cohosh on menopausal symptoms.

Effects of herbal products, such as black cohosh, may be sensitive to dose, extraction method, plant type, and coadministration of other herbs. The total daily triterpene glycoside dose in our black cohosh product was 5 mg, comparable to the 2 to 4 mg found in Remifemin (Schaper & Brümmer GmbH, Salzgitter, Germany), the most widely used product. Like Remifemin, our black cohosh (fingerprinted and verified to be *Actaea racemosa*) (7) was standardized to 27-deoxyactin. Remifemin is an isopropyl alcohol extract, whereas the products we tested are ethanol extracts. The implications of these different extraction techniques are unknown.

The literature on the other ingredients in the multibotanical is limited. Randomized trials have shown no improvement in vasomotor symptoms with dong quai (23) or Siberian ginseng (24). We are unaware of any study that has examined how the other components of the multibotanical affect vasomotor symptoms, although similar formulas are prescribed by naturopathic clinicians (7) and sold as over-the-counter supplements.

We reviewed 16 randomized clinical trials that tested whole soy or soy isoflavone supplements for vasomotor symptoms (25–40). Most were 12-week trials (range, 4 to

52). Eight studies found statistically significant improvements in at least 1 menopause symptom measure (26–28, 31, 33, 35, 37, 39). The magnitude of benefit was a 25% to 55% decrease in frequency or severity of menopause symptoms. Only 3 short-term studies evaluated dietary soy and vasomotor symptoms (38–40); 1 found a statistically significant improvement in vasomotor symptoms after 12 weeks of a soy- and flax-enriched diet (39).

To our knowledge, ours is the longest and largest placebo-controlled, double-blind trial to date, and we included both placebo and hormone therapy as benchmark comparison groups. Findings from our study indicate that trials longer than 12 weeks are necessary to evaluate the sustainability of effects. Adherence and retention were high. We included women in the menopausal transition, and women with 2 or more vasomotor symptoms per day (compared with the 7 to 8 required in Food and Drug Administration–monitored drug trials [13]), because women with fewer symptoms are a key target for herbal therapies. Our findings were similar for women with 7 or more versus fewer than 7 symptoms per day. Although we found no evidence of significant side effects, only a larger and longer trial could provide reassurance in this regard. Our results are generalizable to white, relatively well-educated women who have an average of at least 2 vasomotor symptoms per day. We conducted independent testing of the herbal products; no contaminants were found, and key constituents were present in the amounts specified by the manufacturers (7).

An important question in a trial that does not find statistically significant treatment effects is whether the negative findings were due to a true lack of clinically important effects or a lack of statistical power. The confidence intervals for our primary outcome of symptoms per day can be used to determine the effect size that can be "ruled out" by our results (**Table 2**). Over all 3 follow-up time points, one can rule out reductions beyond 1.5 symptoms per day from black cohosh and 1.0 symptom per day from the multibotanical treatments. Whether these are clinically important effects is debatable, but we would argue that most women would not think so. It is important to emphasize that these are the largest possible effects that are consistent with our data. Our best estimates of effect are far less; the average reduction in symptoms per day over the entire follow-up period was less than 0.01 symptom per day, combining results for the 3 herbal groups, all of whom received black cohosh.

Treatments used in naturopathic practice motivated our choice of interventions. The whole-person approach used by most naturopathic physicians differs significantly from the treatments selected for our study, and this might have affected response to therapy. Time spent with the patient on counseling about diet, exercise, and emotional issues related to menopause; dose revisions; and additional supplements are important aspects of the naturopathic strategy for managing menopausal symptoms. We could not replicate this approach.

In summary, there is a pressing need for safe and effective interventions for vasomotor symptoms. Regrettably, this trial and the totality of the evidence indicates that black cohosh used in isolation, or in a multibotanical product, has little potential to play an important role in relief of vasomotor symptoms.

Table 4. **Women with Adverse Events, Mean Adherence, and Reasons for Withdrawal or Discontinuation of Medication Use by Treatment Group over 12 Months of Follow-up**

Variable	Black Cohosh Group (*n* = 80)	Multibotanical Group (*n* = 76)	Multibotanical plus Soy Counseling Group (*n* = 79)	Conjugated Equine Estrogen with or without Medroxyprogesterone Acetate Group (*n* = 32)	Placebo Group (*n* = 84)
Adverse events					
Menstrual disorders, *n (%)*	10 (13)	8 (11)	14 (18)	19 (59)*	17 (20)
Breast discomfort, *n (%)*	0	1 (1)	2 (3)	5 (16)†	3 (4)
Gastrointestinal upset, *n (%)*	12 (15)	11 (14)	8 (10)	4 (13)	13 (15)
Headache, *n (%)*	12 (15)	8 (11)	12 (15)	6 (19)	16 (19)
Fatigue, asthenia, or malaise, *n (%)*	12 (15)	7 (9)	12 (15)	6 (19)	8 (10)
Myalgia or arthralgia, *n (%)*	11 (14)	9 (12)	9 (11)	1 (3)	10 (12)
Adherence					
Average medications taken, %	88	80	87	87	82
Reasons for study medication discontinuation or study withdrawal, *n (%)*					
No symptom relief	1 (1.3)	5 (6.6)	2 (2.5)	1 (3.1)	1 (1.2)
Other symptoms	1 (1.3)	1 (1.3)	–	4 (12.5)	2 (2.4)
Change in health status	–	–	4 (5.1)	1 (3.1)	3 (3.6)
Other	5 (6.3)	3 (3.9)	4 (5.1)	2 (6.3)	5 (6.0)

* $P < 0.001$ vs. placebo.
† $P = 0.04$ vs. placebo.

From Group Health Center for Health Studies, the University of Washington, and Fred Hutchinson Cancer Research Center, Seattle, Washington; and Bastyr University, Kenmore, Washington.

Acknowledgments: The authors acknowledge all those who contributed significantly to this work, especially the 351 dedicated women who participated in this study. In particular, they thank Linda Palmer, RN, and Jill Seymour, RN, ARNP, for their outstanding work with study participants; David Carrell and Nirmala Sandhu for their excellent programming and data management; Sheree Miller, PharmD, Lead Pharmacist, Investigational Drug Services, University of Washington Medical Center, for packaging and assuring blinding of study medications; and the late Natalie Koether, JD, Pure World, Inc., South Hackensack, New Jersey, for providing black cohosh. The authors also thank the members of the Data and Safety Monitoring Committee: Drs. Gregory Burke (*Chair*), Ellen Gold, Susan Johnson, and Barry Storer.

Grant Support: This project was funded by the National Institute on Aging and the National Center for Complementary and Alternative Medicine (grant R01AG17057) and by an administrative supplement from the National Institute on Aging (Alternative Therapies For Menopause—A Randomized Trial: Herbal Alternatives Quality Control Supplement).

Potential Financial Conflicts of Interest: None disclosed.

Requests for Single Reprints: Katherine M. Newton, PhD, Center for Health Studies, Group Health Cooperative, 1730 Minor Avenue, Suite 1600, Seattle, WA 98101; e-mail, newton.k@ghc.org.

Current author addresses and author contributions are available at www.annals.org.

References

1. **Anderson GL, Limacher M, Assaf AR, Bassford T, Beresford SA, Black H, et al.** Effects of conjugated equine estrogen in postmenopausal women with hysterectomy: the Women's Health Initiative randomized controlled trial. JAMA. 2004;291:1701-12. [PMID: 15082697]
2. **Rossouw JE, Anderson GL, Prentice RL, LaCroix AZ, Kooperberg C, Stefanick ML, et al.** Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results From the Women's Health Initiative randomized controlled trial. JAMA. 2002;288:321-33. [PMID: 12117397]
3. **Eisenberg DM, Davis RB, Ettner SL, Appel S, Wilkey S, Van Rompay M, et al.** Trends in alternative medicine use in the United States, 1990-1997: results of a follow-up national survey. JAMA. 1998;280:1569-75. [PMID: 9820257]
4. **Oldendick R, Coker AL, Wieland D, Raymond JI, Probst JC, Schell BJ, et al.** Population-based survey of complementary and alternative medicine usage, patient satisfaction, and physician involvement. South Carolina Complementary Medicine Program Baseline Research Team. South Med J. 2000;93:375-81. [PMID: 10798505]
5. **Newton KM, Buist DS, Keenan NL, Anderson LA, LaCroix AZ.** Use of alternative therapies for menopause symptoms: results of a population-based survey. Obstet Gynecol. 2002;100:18-25. [PMID: 12100799]
6. **Keenan NL, Mark S, Fugh-Berman A, Browne D, Kaczmarczyk J, Hunter C.** Severity of menopausal symptoms and use of both conventional and complementary/alternative therapies. Menopause. 2003;10:507-15. [PMID: 14627858]
7. **Newton KM, Reed SD, Grothaus L, Ehrlich K, Guiltinan J, Ludman E, et al.** The Herbal Alternatives for Menopause (HALT) Study: background and study design. Maturitas. 2005;52:134-46. [PMID: 16186076]
8. **Rapp SR, Espeland MA, Shumaker SA, Henderson VW, Brunner RL, Manson JE, et al.** Effect of estrogen plus progestin on global cognitive function in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA. 2003;289:2663-72. [PMID: 12771113]
9. **Shumaker SA, Legault C, Rapp SR, Thal L, Wallace RB, Ockene JK, et al.** Estrogen plus progestin and the incidence of dementia and mild cognitive impairment in postmenopausal women: the Women's Health Initiative Memory Study: a randomized controlled trial. JAMA. 2003;289:2651-62. [PMID: 12771112]
10. **Beresford SA, Thompson B, Feng Z, Christianson A, McLerran D, Patrick DL.** Seattle 5 a Day worksite program to increase fruit and vegetable consumption. Prev Med. 2001;32:230-8. [PMID: 11277680]
11. **Wiklund I, Holst J, Karlberg J, Mattsson LA, Samsioe G, Sandin K, et al.** A new methodological approach to the evaluation of quality of life in postmenopausal women. Maturitas. 1992;14:211-24. [PMID: 1508061]
12. **Wiklund I, Karlberg J.** Evaluation of quality of life in clinical trials. Selecting quality-of-life measures. Control Clin Trials. 1991;12:204S-216S. [PMID: 1663856]
13. Center for Drug Evaluation and Research. Guidance for Industry: Estrogen and Estrogen/Progestin Drug Products to Treat Vasomotor Symptoms and Vulvar and Vaginal Atrophy Symptoms—Recommendations for Clinical Evaluation. 2003. Accessed at www.fda.gov/cder/guidance/5412dft.pdf on 27 July 2007.
14. **Frankenfeld CL, Patterson RE, Horner NK, Neuhouser ML, Skor HE, Kalhorn TF, et al.** Validation of a soy food-frequency questionnaire and evaluation of correlates of plasma isoflavone concentrations in postmenopausal women. Am J Clin Nutr. 2003;77:674-80. [PMID: 12600860]
15. U.S. Food and Drug Administration. COSTART: Coding Symbols for Thesaurus of Adverse Reaction Terms. 95. Springfield, VA: U.S. Department of Commerce, National Technical Information Service, 1995.
16. **Wuttke W, Seidlova-Wuttke D, Gorkow C.** The Cimicifuga preparation BNO 1055 vs. conjugated estrogens in a double-blind placebo-controlled study: effects on menopause symptoms and bone markers. Maturitas. 2003;44 Suppl 1:S67-77. [PMID: 12609561]
17. **Jacobson JS, Troxel AB, Evans J, Klaus L, Vahdat L, Kinne D, et al.** Randomized trial of black cohosh for the treatment of hot flashes among women with a history of breast cancer. J Clin Oncol. 2001;19:2739-45. [PMID: 11352967]
18. **Stoll W.** Phytopharmacon influence atrophic vaginal epithelium: double-blind study of cimicifuga vs. estrogenic substances. Therapeutikon. 1987;1:23-31.
19. **Frei-Kleiner S, Schaffner W, Rahlfs VW, Bodmer CH, Birkhauser M.** Cimicifuga racemosa dried ethanolic extract in menopausal disorders: a double-blind placebo-controlled clinical trial. Maturitas. 2005;51:397-404. [PMID: 16039414]
20. **Osmers R, Friede M, Liske E, Schnitker J, Freudenstein J, Henneicke-von Zepelin HH.** Efficacy and safety of isopropanolic black cohosh extract for climacteric symptoms. Obstet Gynecol. 2005;105:1074-83. [PMID: 15863547]
21. **Pockaj BA, Gallagher JG, Loprinzi CL, Stella PJ, Barton DL, Sloan JA, et al.** Phase III double-blind, randomized, placebo-controlled crossover trial of black cohosh in the management of hot flashes: NCCTG Trial N01CC1. J Clin Oncol. 2006;24:2836-41. [PMID: 16782922]
22. **Verhoeven MO, van der Mooren MJ, van de Weijer PH, Verdegem PJ, van der Burgt LM, Kenemans P, et al.** Effect of a combination of isoflavones and *Actaea racemosa Linnaeus* on climacteric symptoms in healthy symptomatic perimenopausal women: a 12-week randomized, placebo-controlled, double-blind study. Menopause. 2005;12:412-20. [PMID: 16037756]
23. **Hirata JD, Swiersz LM, Zell B, Small R, Ettinger B.** Does dong quai have estrogenic effects in postmenopausal women? A double-blind, placebo-controlled trial. Fertil Steril. 1997;68:981-6. [PMID: 9418683]
24. **Wiklund IK, Mattsson LA, Lindgren R, Limoni C.** Effects of a standardized ginseng extract on quality of life and physiological parameters in symptomatic postmenopausal women: a double-blind, placebo-controlled trial. Swedish Alternative Medicine Group. Int J Clin Pharmacol Res. 1999;19:89-99. [PMID: 10761538]
25. **Washburn S, Burke GL, Morgan T, Anthony M.** Effect of soy protein supplementation on serum lipoproteins, blood pressure, and menopausal symptoms in perimenopausal women. Menopause. 1999;6:7-13. [PMID: 10100174]
26. **Murkies AL, Lombard C, Strauss BJ, Wilcox G, Burger HG, Morton MS.** Dietary flour supplementation decreases post-menopausal hot flushes: effect of soy and wheat. Maturitas. 1995;21:189-95. [PMID: 7616867]
27. **Albertazzi P, Pansini F, Bonaccorsi G, Zanotti L, Forini E, De Aloysio D.** The effect of dietary soy supplementation on hot flushes. Obstet Gynecol. 1998;91:6-11. [PMID: 9464712]
28. **Crisafulli A, Marini H, Bitto A, Altavilla D, Squadrito G, Romeo A, et al.** Effects of genistein on hot flushes in early postmenopausal women: a randomized, double-blind EPT- and placebo-controlled study. Menopause. 2004;11:400-4. [PMID: 15243277]

29. **Penotti M, Fabio E, Modena AB, Rinaldi M, Omodei U, Vigano P.** Effect of soy-derived isoflavones on hot flushes, endometrial thickness, and the pulsatility index of the uterine and cerebral arteries. Fertil Steril. 2003;79:1112-7. [PMID: 12738504]
30. **Burke GL, Legault C, Anthony M, Bland DR, Morgan TM, Naughton MJ, et al.** Soy protein and isoflavone effects on vasomotor symptoms in peri- and postmenopausal women: the Soy Estrogen Alternative Study. Menopause. 2003; 10:147-53. [PMID: 12627040]
31. **Faure ED, Chantre P, Mares P.** Effects of a standardized soy extract on hot flushes: a multicenter, double-blind, randomized, placebo-controlled study. Menopause. 2002;9:329-34. [PMID: 12218721]
32. **Van Patten CL, Olivotto IA, Chambers GK, Gelmon KA, Hislop TG, Templeton E, et al.** Effect of soy phytoestrogens on hot flashes in postmenopausal women with breast cancer: a randomized, controlled clinical trial. J Clin Oncol. 2002;20:1449-55. [PMID: 11896091]
33. **Han KK, Soares JM Jr, Haidar MA, de Lima GR, Baracat EC.** Benefits of soy isoflavone therapeutic regimen on menopausal symptoms. Obstet Gynecol. 2002;99:389-94. [PMID: 11864664]
34. **St Germain A, Peterson CT, Robinson JG, Alekel DL.** Isoflavone-rich or isoflavone-poor soy protein does not reduce menopausal symptoms during 24 weeks of treatment. Menopause. 2001;8:17-26. [PMID: 11201510]
35. **Scambia G, Mango D, Signorile PG, Anselmi Angeli RA, Palena C, Gallo D, et al.** Clinical effects of a standardized soy extract in postmenopausal women: a pilot study. Menopause. 2000;7:105-11. [PMID: 10746892]
36. **Quella SK, Loprinzi CL, Barton DL, Knost JA, Sloan JA, LaVasseur BI, et al.** Evaluation of soy phytoestrogens for the treatment of hot flashes in breast cancer survivors: A North Central Cancer Treatment Group Trial. J Clin Oncol. 2000;18:1068-74. [PMID: 10694559]
37. **Upmalis DH, Lobo R, Bradley L, Warren M, Cone FL, Lamia CA.** Vasomotor symptom relief by soy isoflavone extract tablets in postmenopausal women: a multicenter, double-blind, randomized, placebo-controlled study. Menopause. 2000;7:236-42. [PMID: 10914616]
38. **Dalais FS, Rice GE, Wahlqvist ML, Grehan M, Murkies AL, Medley G, et al.** Effects of dietary phytoestrogens in postmenopausal women. Climacteric. 1998;1:124-9. [PMID: 11907915]
39. **Brzezinski A, Adlercreutz H, Shaoul R, Rosler A, Shmueli A, Tanos V et al.** Short-term effect of phytoestrogen-rich diet on postmenopausal women. Menopause. 1997;4:89-94.
40. **Baird DD, Umbach DM, Lansdell L, Hughes CL, Setchell KD, Weinberg CR, et al.** Dietary intervention study to assess estrogenicity of dietary soy among postmenopausal women. J Clin Endocrinol Metab. 1995;80:1685-90. [PMID: 7745019]

Current Author Addresses: Dr. Newton: Center for Health Studies, Group Health Cooperative, 1730 Minor Avenue, Suite 1600, Seattle, WA 98101.
Dr. Reed: Obstetrics and Gynecology, Harborview Medical Center, P.O. Box 359865, Seattle, WA 98104.
Dr. LaCroix: Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, Washington 98109.
Mr. Grothaus and Ms. Ehrlich: Group Health Center for Health Studies, 1730 Minor Avenue, Suite 1600, Seattle, WA 98101.
Dr. Guiltinan: Bastyr University, 3670 Stone Way North, Seattle, WA 98103.

Author Contributions: Conception and design: K.M. Newton, S.D. Reed, A.Z. LaCroix, L.C. Grothaus, K. Ehrlich, J. Guiltinan.
Analysis and interpretation of the data: K.M. Newton, S.D. Reed, A.Z. LaCroix, L.C. Grothaus, J. Guiltinan.
Drafting of the article: K.M. Newton, S.D. Reed, A.Z. LaCroix, L.C. Grothaus, J. Guiltinan.
Critical revision of the article for important intellectual content: K.M. Newton, S.D. Reed, A.Z. LaCroix, L.C. Grothaus, K. Ehrlich, J. Guiltinan.
Final approval of the article: K.M. Newton, S.D. Reed, A.Z. LaCroix, K. Ehrlich.
Provision of study materials or patients: S.D. Reed.
Statistical expertise: A.Z. LaCroix, L.C. Grothaus.
Obtaining of funding: K.M. Newton, S.D. Reed, A.Z. LaCroix.
Administrative, technical, or logistic support: K. Ehrlich.
Collection and assembly of data: K.M. Newton, L.C. Grothaus, K. Ehrlich.

Appendix Table. **Consolidated Standards of Reporting Trials (CONSORT) Requirements for Controlled Trials of Herbal Interventions***

Criteria	Black Cohosh: Single Herb Product	Multibotanical Product		
		Black Cohosh	Alfalfa	Boron Citrate
Latin binomial name	*Actaea rasemosa*	*Actaea rasemosa*	*Medicago sativa*	NA
Botanical authority				NA
Family name	Ranuculaceae	Ranuculaceae	Fabaceae	NA
Common names	Black bugbane, black snakeroot, bugwort, rattleroot, rattletop, rattleweed, macrotys	Black bugbane, black snakeroot, bugwort, rattleroot, rattletop, rattleweed, macrotys	Alfalfa, lucerne, purple medik, trefoil	Boron
Proprietary product name (i.e., brand name) or extract name; manufacturer	CimiPure (Naturex, Avignon, France)	†	†	†
Is product authorized (licensed, registered) in United States?	Yes, trademarked	No trademark or registration	No trademark or registration	No trademark or registration
Parts of plant used to produce the product or extract	Rhizome and root	Root	Leaf	NA
Type of product used (e.g., raw, fresh, or dry, extract), type and concentration of extraction solvent, ratio of herbal drug to extract	70% ethanol extract	Water and alcohol 4:1 extract	No solvents 4:1 extract	NA
Method of authentication of raw materials		GC	TLC	HPLC
Lot number of the raw material	M008250	SB01-0160	220100-50	021390910
State whether a voucher specimen (i.e., retention sample) retained; if so, where it is kept or deposited, and reference number	Yes, voucher specimen 51680, deposited at the New York Botanical Garden	No—past time of retention	No—past time of retention	No—past time of retention
Product dosage per capsule	80 mg	50 mg	100 mg	20 mg
Content (e.g., as weight, concentration; may be given as a range where appropriate) of all quantified herbal product constituents, both native and added, per dosage unit form	Per capsule: 80 mg of black cohosh, 510 mg of rice flour			
Added materials (binders, fillers, and other excipients)	Rice flour	None	None	None
Standardization	Triterpene glycosides	None	None	Boron
Product's chemical fingerprint and methods used	HPLC, CE	HPLC, CE	HP-TLC	HPLC, CE
For standardized products, quantity of active/marker constituents per dosage unit form	2.5%	NA	NA	NA
Amount detected	Triterpene glycosides, 3.125%	Triterpene glycosides, 4.4%	L-canavanine Alfalfa juice, 20% extract, 1 mg/g Alfalfa leaf, 4:1 extract, 3 mg/g	0.92 mg
Was a sample of the product (i.e., retention sample) retained? If so, where it is kept or deposited?	Yes			

* All analyses were preformed by ConsumerLab.com. Specifics of special testing/purity testing are as follows (abbreviations used in tables defined at end of footnote): 1. Chlorinated pesticide screens were tested on the black cohosh and the multibotanical finished products, evaluated by GC/MS. The detection limit level was set at 50 parts per billion. The following were tested and were not found: pentacholorobenze, tetracholoroaniline, hexacholorobenzene, α-benzene hexachloride (BHC), pentachloronitrobenzene, lindane, β-BHC, heptachlor, pentachloroaniline, δ-BHC, aldrin, pentachlorothioanisole, heptachlor, epoxide, γ-chlordane, endosulfan I, α-chlordane, dieldrin, endrin, endrin aldehyde, endosulfan sulfate, dichloro-diphenyl-trichloroethane, dichlorodiphenyldichloroethylene, dichloro-diphenyl-dichloroethane, endosulfan II, methoxychlor, and endrine ketone. 2. Black cohosh, and the multibotanical were tested for heavy metals by inductively coupled plasma–mass spectroscopy. The black cohosh product contained <0.06 μg of lead, <0.06 μg of cadmium, and 0.97 μg of arsenic. The multibotanical finished product contained <0.49 μg of lead, <0.0714 μg of cadmium, and 1.16 μg of arsenic. 3. Alfalfa was tested for L-canavanine, an antinutrient, by using HP-TLC. The alfalfa leaf and juice extract raw material had 1 mg/g of L-canavanine in the juice extract (20% extract) and 3 mg/g in the leaf (4:1 extract). 4. Screening for estrogenic drug substances was detected by GC/MS. The following were tested and not found: diethylstilbestrol, estradiol, estrone, estriol, ethynyl estradiol, and tamoxifen. CE = capillary electrophoresis; GC = gas chromatography; HPLC = high-performance liquid chromatography; HP-TLC = high-performance thin-layer chromatography; MS = mass spectroscopy; NA = not available; TLC = thin-layer chromatography.

† Progyne (Progena, Albuquerque, NM, Lot 1051504).

‡ Per capsule: 50 mg of black cohosh, 100 mg of alfalfa, 1 mg of boron citrate, 50 mg of chaste tree, 100 mg of dong quai, 50 mg of false unicorn, 50 mg of licorice, 100 mg of oats, 100 mg of pomegranate, 100 mg of siberian ginseng; no filler or other constituents.

Appendix Table—Continued

Multibotanical Product						
Chaste Tree	**Dong Quai**	**False Unicorn**	**Licorice**	**Oats Straw**	**Pomegranate**	**Siberian Ginseng**
Vitex agnus-castus	*Angelica sinensis*	*Chamaelirium luteum*	*Glycyrrhiza glabra*	*Avena sativa*	*Punica granatum*	*Eleutherococcus senticosus*
Verbenaceae	Apiaceae	Liliaceae	Fabaceae	Poacaea	Lythraceae	Araliaceae
Chaste berry, Monk's pepper, Abraham's balm, chaste lamb-tree, safe tree, Indian-spice, wild pepper	Dong quai, dang gui, tang-kuei	Starwort, helonias root, blazing star root, devil's bit, false unicorn	Lacrisse, sweet licorice, licorice root	Oats	Pomegranate	Ginseng
†	†	†	†	†	†	†
No trademark or registration	No trademark or registration	No trademark or registration	No trademark or registration	No trademark or registration	No trademark or registration	No trademark or registration
Berry	Root	Root	Root	Straw	Fruit	Root
Dry powder	Water and alcohol 4:1 extract	Water and alcohol 4:1 extract	Water and alcohol 4:1 extract	Water and alcohol 10:1 extract	Dry powder	Water and alcohol 0.8% extract
GC	GC	TLC	TLC	GC	GC	GC
38699C	SB01-0031	14C1-6	20012450	SB01-0136C-J	L5029P	SG001006C
No—past time of retention	No—past time of retention	No—past time of retention	No—past time of retention	No—past time of retention	No—past time of retention	No—past time of retention
50 mg	100 mg	50 mg	50 mg	100 mg	100 mg	100 mg
‡	‡	‡	‡	‡	‡	‡
None	None	None	None	None	None	None
None	None	None	None	None	None	Eleutherosides B and E
HPLC, CE	HPLC, CE	HPLC, CE	HPLC, CE	Not tested	HP-TLC	HPLC, CE
NA	NA	NA	NA	NA	NA	0.8%
Agnuside, 0.024%	Ligustilide, <0.001%; ferulic acid, 0.016%	Diosgenin (free) Not detected	Glycyrrhizic acid, 14%	Not tested	Polyphenolics Not detected	Eleutherosides B and E, 1.6%
Yes—for both products, a retention sample is being stored at the Group Health Center for Health Studies						

JOURNAL OF NATURAL PRODUCTS

Subscriber access provided by LEHMAN COLL

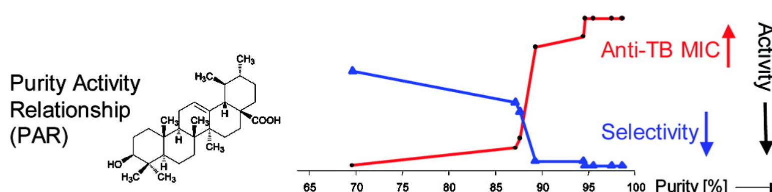
Article

Purity#Activity Relationships of Natural Products: The Case of Anti-TB Active Ursolic Acid

Birgit U. Jaki, Scott G. Franzblau, Lucas R. Chadwick, David C. Lankin, Fangqiu Zhang, Yuehong Wang, and Guido F. Pauli

J. Nat. Prod., **2008**, 71 (10), 1742-1748 • DOI: 10.1021/np800329j • Publication Date (Web): 18 September 2008

Downloaded from http://pubs.acs.org on December 4, 2008



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of Natural Products is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Purity−Activity Relationships of Natural Products: The Case of Anti-TB Active Ursolic Acid

Birgit U. Jaki,[†] Scott G. Franzblau,[†] Lucas R. Chadwick,[‡,§] David C. Lankin,[‡] Fangqiu Zhang,[†,⊥] Yuehong Wang,[†] and Guido F. Pauli*[,†,‡]

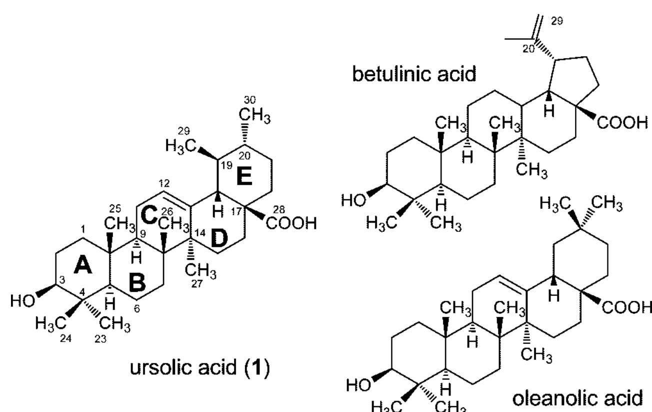
Institute for Tuberculosis Research and Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, Illinois 60612-7231

Received June 3, 2008

The present study explores the variability of biological responses from the perspective of sample purity and introduces the concept of purity−activity relationships (PARs) in natural product research. The abundant plant triterpene ursolic acid (**1**) was selected as an exemplary natural product due to the overwhelming number yet inconsistent nature of its approximate 120 reported biological activities, which include anti-TB potential. Nine different samples of ursolic acid with purity certifications were obtained, and their purity was independently assessed by means of quantitative ^{1}H NMR (qHNMR). Biological evaluation consisted of determining MICs against two strains of virulent *Mycobacterium tuberculosis* and IC_{50} values in Vero cells. *Ab initio* structure elucidation provided unequivocal structural confirmation and included an extensive ^{1}H NMR spin system analysis, determination of nearly all *J* couplings and the complete NOE pattern, and led to the revision of earlier reports. As a net result, a sigmoid PAR profile of **1** was obtained, demonstrating the inverse correlation of purity and anti-TB bioactivity. The results imply that synergistic effects of **1** and its varying impurities are the likely cause of previously reported antimycobacterial potential. Generating PARs is a powerful extension of the routinely performed quantitative correlation of structure and activity ([Q]SAR). Advanced by the use of primary analytical methods such as qHNMR, PARs enable the elucidation of cases like **1** when increasing purity voids biological activity. This underlines the potential of PARs as a tool in drug discovery and synergy research and accentuates the need to routinely combine biological testing with purity assessment.

Natural products are evolutionary shaped molecules with a profound impact on human health.[1−3] Nature's biosynthetic engine produces innumerate secondary metabolites with distinct biological properties that make them valuable as health products or as structural templates for drug discovery. Synthetic variation of structure and [quantitative] correlation of structure and activity ([Q]SAR) are widely used tools in drug discovery,[4] placing the elucidated structure and its biological potency in the center of contemplation. A common finding in the natural products literature, in particular with regard to well-established natural compounds, is that the same compound is reported to act on a myriad of targets, with very different potential, and with an inconsistent activity pattern. While the unique specificity of each individual assay and variability in the performance of the bioassays are widely accepted as potential explanations, the influence of sample purity has not been examined in a systematic manner.

Typically, when a substance is pharmacologically evaluated, the assumption is made that the sample represents a single chemical entity (SCE) or a defined mixture of known chemical entities, such as in the case of stereoisomers. Conversely, pharmacopoeias and agencies worldwide have established rigorous limits for the amounts of unacceptable impurities. According to guidelines of the International Conference on Harmonization (ICH),[5] the reported impurity thresholds in new drug applications (NDAs) are often as low as 0.05−0.03%, even for enantiomeric impurities. The relevance of minor constituents cannot be overlooked when assigning pharmacologically active principles in materials of complex origin, such as in parallel (bio)synthesis and natural products. Whenever bioactive materials require isolation from a complex matrix, they are most likely to retain residual complexity even in a refined ("pure") stage. Thus, knowledge of both known and unknown impurities becomes increasingly relevant in drug discovery and bioactive natural products.



The pentacyclic plant triterpene ursolic acid (**1**, ua) exemplifies the overall situation since it appears ubiquitously in plants, has a large number of known congeneric analogues, and has a remarkable wide array of reported biological functions. A literature search using the NAPRALERT database[6] revealed 120 different bioactivities ascribed to **1** alone. Upon closer inspection, several of the reported activities are inconsistent. For example, while some report antibacterial activity[7−12] against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, others find **1** to be inactive against the same organisms[7,9,12−15] or even observe bacterial growth stimulation.[10] Anticytotoxic[16] and cytotoxic activity[17] have been reported against HEPG-2 cells. Furthermore, **1** has been reported as being both active and inactive in anti-inflammatory (*in vivo*, external, mouse),[18−21] cell differentiation induction (leuk-M1),[22,23] lipoxygenase-5-inhibition,[24,25] antiulcer (*in vivo*, rat),[26,27] antiyeast (*Candida albicans*),[7,11,13,15,28] and cytotoxicity (leuk P388)[29−31] assays. Due to its ubiquitous nature, **1** had been isolated from very different chemotaxonomic matrixes. This most likely resulted in very different impurity profiles of the final isolates, which were subsequently tested in the corresponding biological

* Corresponding author. Tel: (312) 355-1949. Fax: (312)-355-2693. E-mail: gfp@uic.edu.
[†] Institute for Tuberculosis Research.
[‡] Department of Medicinal Chemistry and Pharmacognosy.
[§] Current address: Cherry Instruments, 451 W. Wrightwood Ave., #306, Chicago, IL 60614.
[⊥] Current address: Sanofi Pasteur Limited, 1755 Steeles Ave. West, Toronto, Ontario M2R 3T4, Canada.

10.1021/np800329j CCC: $40.75 © 2008 American Chemical Society and American Society of Pharmacognosy
Published on Web 09/18/2008

Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data of **1**[a]

position[b]	δ_C [ppm]	δ_H [ppm]	integral	multiplicity	J [Hz] (coupled proton)
1a=ax	40.27	1.41[c]	1H	m[c]	2.7 (H-2ax), ~13 (H-1eq)[c]
1b=eq		0.88	1H	m[c]	2.7 (H-2ax), 3.4 (H-2eq), ~13 (H-1ax)[c]
2a=eq	28.81	1.41[c]	1H	m[c]	6.4 (H-3), 3.4 (H-1eq), ~13 (H-2 ax)[c]
2b=ax		1.42[c]	1H	m[c]	9.4 (H-3), 2.7 (H-1ax), ~13 (H-2 eq)[c]
3	80.12	2.997	1H	dd	6.4 (H-2eq), 9.4 (H-2ax)
4	40.35				
5	56.85	0.535	1H	dd	1.9 (H-6eq), 11.8 (H-6ax)
6a=ax	19.94	1.415[c]	1H	dd/m[c]	11.8 (H-5), 9.3 (H-7ax)
6b=eq		1.315[c]	1H	dd/m[c]	1.9 (H-5), 2.9 (H-7ax), 8.6 (H-7eq)
7a=ax		1.322	1H	ddd	2.9 (H-6eq), 8.6 (H-7eq), 9.3 (H-6ax)
7b=eq		1.141[c]	1H	d/m[c]	8.6 (H-7ax)
8	41.16				
9	49.14	1.320	1H	dd	5.7 (H-11eq), 11.9 (H-11ax)
10	38.56				
11a=ax	24.86	1.706	1H	ddd	3.8 (H-12), 10.8 (H-11eq), 11.9 (H-9)
11b=eq		1.729	1H	ddd	3.8 (H-12), 5.7 (H-9), 10.8 (H-11ax)
12	126.88	5.052	1H	pseudo-t	3.8 (H-11ax, H-11eq)
13	139.91				
14	43.66				
15a=ax	29.84	1.709	1H	ddd	4.1 (H-16eq), 13.6 (H-16ax), 14.1 (H-15eq)
15b=eq		0.888	1H	ddd	4.4 (H-16ax), 3.3 (H-16eq), 14.1 (H-15ax)
16a=ax	25.84	1.787	1H	ddd	4.4 (H-15eq), 13.1 (H-16eq), 13.6 (H-15ax)
16b=eq		0.901	1H	ddd	4.1 (H-15ax), 13.1 (H-16ax), 3.3 (H-15eq)
17	49.18				
18	54.35	2.014	1H	dd	1.8 (H-20), 11.4 (H-19)
19eq	40.69	1.14[c]	1H	dd/m[c]	6.6 (H_3-29), 11.4 (H-18)
20	40.48	0.83[c]	1H	dd/m[c]	1.8 (H-18), 6.2 (H_3-30)
21a=eq	32.33	1.30[c]	1H	ddd	1.3 (H-22ax), 2.9 (H-22eq), 13.0 (H-21ax)
21b=ax		1.34[c]	1H	ddd	2.2 (H-22eq), 14.0 (H-22ax), 13.0(H-21eq)
22a=eq	38.38	1.54[c]	1H	dt	2.2 (H-21ax), 2.9 (H-21eq), 12.9 (H-22ax)
22b=ax		1.51[c]	1H	dt	1.3 (H-21eq), 14.0 (H-21ax), 12.9 (H-22eq)
23	29.65	0.800	3H	s	
24	17.40	0.591	3H	s	
25	17.07	0.736	3H	s	
26	18.66	0.642	3H	s	
27	25.14	0.899	3H	s	
28	181.50				
29	22.84	0.756	3H	d	6.6 (H-19)
30	18.75	0.669	3H	d	6.2 (H-20)

[a] Solvents: $CDCl_3$ (950 μL) and DMSO (50 μL); 500/125 MHz. [b] All assignments were confirmed by gHSQC, gHMBC, and gCOSY maps; for numbering refer to structure drawing of **1**. [c] Signal pattern remains partially unclear due to severe signal overlap and higher order effects, basic multiplicities given under first-order assumptions.

assays. More generally, the high natural abundance of **1** in vascular plants raises doubts regarding the plausibility of it being a true panacea. Prompted by frequent reports[6] and the authors' own observations pointing at **1** as (a member of) the underlying anti-TB active principle, the goal of the present study was to study the influence of purity on antimycobacterial activity.

On the basis of the above, it can be hypothesized that previously investigated preparations of **1** represent a chemically heterogeneous group of samples, for which the presence of an SCE[32,33] does not necessarily apply. In order to test this hypothesis, the present study takes the following three-pronged approach to providing experimental evidence: (i) acquire a significant number of samples of **1**, isolated from various natural sources, determine their individual purity, and unambiguously confirm their structure; (ii) use state-of-the-art methods to determine their statistically significant biological activity against a target, for which significant literature reports exist; and (iii) establish PARs by correlating the level of purity with the observed biological activity. For the purpose of this study, a combination of quantitative antituberculosis and mammalian cell cytotoxicity assays was chosen. Although the innate susceptibility of mycobacterial strains to **1** was not expected to be significantly different, parallel evaluation employed two isogenic strains of *M. tuberculosis*, $H_{37}Rv$ and the GFP-carrying strain $H_{37}Rv$GFP (see Experimental Section), and two independent bioassays for the purpose of being able to recognize variability in drug susceptibility.

There are numerous reports on antimicrobial activities of **1**,[7−14,23,34,35] and triterpene aglycones have attracted particular attention as potential new leads in anti-TB drug discovery.[36−38] Accordingly, the (im)purity profile and the antimycobacterial and cytotoxic activities of nine ursolic acid samples (ua-01 to ua-09, Table 3), most of them representing compendial-quality reference materials with a certified purity of 81.00−99.57% obtained from various commercial sources, were investigated. Both impurity profiling and detailed structure elucidation were carried out by qualitative (1D and 2D NMR) and qHNMR spectroscopy, respectively.

Results and Discussion

***Ab Initio* Structure Assignment.** Despite the routine availability of high-field NMR instrumentation, such as the 500 MHz instrument used in this study or even higher field strengths, the complete analysis of the NMR spectra of triterpenes such as **1** remains a challenge in particular due to the lack of ^{1}H NMR dispersion of the signals of the tetra- and pentacyclic CH skeleton. The presence of higher order spin systems, severe signal overlap, and complex long-range coupling impede the structure elucidation process and made selective NOE experiments as well as spectra simulation necessary to solve the coupling pattern. For the present study, *ab initio* structural confirmation was performed on these molecules with the aid of ^{1}H, (APT)/^{13}C, COSY, HMQC, and HMBC spectra. The relative configuration of the stereogenic centers of the highly complex pentacyclic ring system of **1** was established by a series of selective 1D double-pulsed field gradient NOE (dpfgNOE) experiments. Although the structure of **1** has often been derived, existing reports are typically limited to carbon shifts and rather incomplete proton assignments as exemplified in ref 39.

Table 2. Purity and Bioactivity of the Nine Investigated Samples of **1** Sorted by Their Anti-TB Selectivity against Strain $H_{37}Rv$

sample	purity [%]			anti-TB MIC [μg/mL] (±SD)		cytotoxicity IC_{50} [μg/mL] (±SD)	anti-tb selectivity index (SI)	
	declared	qHNMR	Δ	$H_{37}Rv$	$H_{37}Rv$GFP	Vero cells	$H_{37}Rv$	$H_{37}Rv$GFP
ua-08	81.00	69.66	11.34	65 (±6)	30 (±2)	18 (±1)	0.28	0.61
ua-02	98.9	87.13	11.77	88 (±16)	27 (±3)	18 (±2)	0.21	0.68
ua-09	99.57	87.67	11.90	100 (±19)	31 (±5)	19 (±2)	0.19	0.62
ua-05	90.0	89.29	0.71	220 (±16)	66 (±20)	20 (±2)	0.08	0.28
ua-07	96.5	94.83	2.04	233 (±6)	n.a.	18 (±2)	0.08	n.a.
ua-04	98.6	94.65	3.81	>256[a]	64 (±24)	19 (±2)	<0.07	<0.29
ua-06	98.5	95.52	2.98	>256[a]	68 (±8)	19 (±2)	<0.07	<0.28
ua-01	98.6	97.48	1.12	>256[a]	65 (±21)	19 (±2)	<0.07	<0.29
ua-03	98.6	98.64	−0.04	>256[a]	98 (±26)	18 (±2)	<0.07	<0.19
betulinic acid				>128				
oleanolic acid				100				

[a] The highest test concentration was 256 μg/mL, which was used to create Figure 1.

Table 3. qHNMR Impurity Profiles of the Nine Investigated Samples of **1** (ua-01 to ua-09)

sample	impurity #	content [%]	assignment	sample	impurity #	content [%]	assignment
ua-01	imp 1	0.41	oleanolic acid	ua-06	imp 1	0.63	u[a]
	imp 2	1.17	u		imp 2	0.45	u
	imp 3	0.31	u		imp 3	2.16	u
	imp 4	0.13	u		imp 4	1.42	u
	imp 5	0.03	u		imp 5	0.03	u
	imp 6	0.45	u				
	imp 7	0.09	betulinic acid				
ua-02	imp 1	9.30	oleanolic acid	ua-07	imp 1	1.22	oleanolic acid
	imp 2	0.16	u		imp 2	0.40	u
	imp 3	2.06	u		imp 3	0.22	u
	imp 4	2.42	u		imp 4	0.63	betulinic acid
	imp 5	0.08	u		imp 5	1.86	u
	imp 6	0.44	u		imp 6	1.38	u
	imp 7	0.02	u		imp 7	0.16	u
	imp 8	0.30	U		imp 8	0.41	u
ua-03	imp 1	0.18	oleanolic acid	ua-08	imp 1	26.07	oleanolic acid
	imp 2	0.35	u		imp 2	0.06	u
	imp 3	0.21	u		imp 3	0.39	u
	imp 4	0.06	u		imp 4	0.63	u
	imp 5	0.45	betulinic acid		imp 5	7.18	u
	imp 6	0.13	u		imp 6	6.73	u
ua-04	imp 1	2.53	oleanolic acid	ua-09	imp 1	5.38	oleanolic acid
	imp 2	0.88	u		imp 2	1.47	u
	imp 3	0.43	u		imp 3	2.18	u
	imp 4	0.39	betulinic acid		imp 4	1.06	u
	imp 5	0.55	u		imp 5	1.84	betulinic acid
	imp 6	0.34	u		imp 6	1.08	u
	imp 7	0.77	u		imp 7	0.57	u
	imp 8	0.17	u		imp 8	0.42	u
					Imp 9	0.06	u
ua-05	imp 1	2.31	oleanolic acid				
	imp 2	0.80	u				
	imp 3	0.27	u				
	imp 4	0.15	u				
	imp 5	0.40	betulinic acid				
	imp 6	0.57	u				
	imp 7	3.71	u				
	imp 8	2.34	u				
	imp 9	0.16	u				

[a] Unassigned impurities (u) were calculated as isomers of **1**.

As summarized in Table 1 the ^{13}C NMR spectra revealed a total of 30 carbon resonances, which could be identified as seven methyl, nine methylene, and seven methine groups, along with seven quaternary carbons, one of which represents a carboxylic acid. Analysis of the COSY and selective 1D dpfgNOE spectra revealed the presence of five almost isolated ^{1}H spin systems, i.e., one spin system for each of the five rings A−E of the triterpene skeleton: spin system #1 of ring A (H-1ax, H-1eq, H-2eq, H-2ax, and H-3), #2 of ring B (H-5, H-6a, H-6b, H-7a, H-7b), #3 of ring C (H-9, H-11a, H-11b, H-12), #4 of ring D (H-15ax, H-15eq, H-16ax, H-16eq), and #5 of ring E (H-18, H-19eq, H_3-29, H_3-30, H-20, H-21a, H-21b, H-22ax, H-22eq). The carbon skeleton was assembled using the HMBC map. In particular, correlations from H_3-23, H-7ax, and H-7eq to C-5 established the connection of the spin systems A and B via the C-5 bridgehead; correlations from H-1ax and H-1eq to C-25, C-10, CH-9, and C-5 determined the fragment that connects the ^{1}H spin systems A and C. Correlations between the olefinic proton H-12 and both C-13 and CH-18 connect spin systems C and E, while E and D are connected by a fragment determined by correlations from H-22ax and H-22eq to C-28, C-17, and CH_2-16 and from H-18 to C-17, C-28, and CH_2-16. The spin systems B, C, and D are connected by a fragment, which was established by correlations from H-11ax, H-11eq, and H-9 to C-8; H-7ax and H-7eq to C-8 and C-14; and H_3-27 to C-8, C-14, and CH_2-15. Positioning of the methyl groups at C-4 was proven by HMBC correlations from H-3ax to CH_3-23 and CH_3-24, from H_3-24 to CH_3-23, and from H_3-23 to CH_3-24. The methyl groups CH_3-25 and CH_3-26 were assigned on the basis of correlations between the axial H-9 and CH_3-25 and CH_3-26. The methyl group CH_3-27 was located by correlations from H-15ax and H-15eq to CH_3-27 as well as from H_3-27 to C-8 and C-13. The vicinal CH_3 groups CH_3-29 and CH_3-30 were assigned with the aid of HMBC cross-peaks, correlating protons H-18, H-19, H-20, and H_3-30 with carbon CH_3-29, and H_3-29, H-19, H-20, H-21ax, and H-21eq with carbon CH_3-30. Finally, the carboxyl group C-28 was placed following correlations from H-22ax, H-22eq, H-16ax, and H-16eq to C-28.

Due to the severe overlap of proton signals, selective 1D NOE (selNOE) experiments and molecular modeling were essential in solving the coupling pattern of the spin systems and to derive the relative configuration. Selective excitation of the signal of H-3 and consideration of the proton distances in an energy-minimized model led to the signal assignment and the establishment of the coupling pattern of H-5 and H_3-23eq as well as the signal assignment of H-1ax. Determination of coupling constants of the C-1 and C-2 methylene protons remains partially unclear due to higher order effects and signal overlap. Selective excitation of the signal H-12 led to the unambiguous assignment of the signals of H-11ax, H-11eq, and H-9. With the help of the selective pulse experiments it was possible to distinguish between the signals of H-11ax and H-11eq by creating a line-fitted subspectrum of spin system C, followed by spectral simulation and iteration. NOEs were detected from H-12 to two methyl groups. As only H_3-26 and H_3-30 are within a distance of 3.5 Å and below, the reported assignments of CH_3-30 and CH_3-29 need to be revised. The four signals between δ 1.709 and 1.787 could be resolved by creation of line-fitted spectra of all three signals and subtraction of the line-fitted signals of H-11ax and H-11eq, which were known from the NOE irradiation of H-12. The result was a spectrum of the signals of H-15ax and H-16eq. The signals for H-15eq and H-16eq, which both overlap

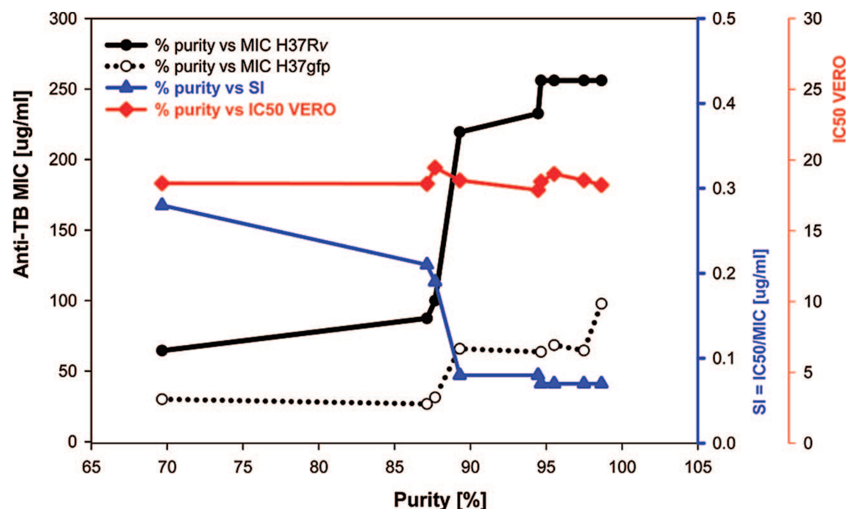


Figure 1. Purity–activity relationship (PAR) of ursolic acid. The graphic illustrates the correlation between sample *im*purity and antimycobacterial activity and proves, in turn, that there is no correlation between antimycobacterial activity and purity. This leads to the assumption that the antimycobacterial activity is not caused by the single pure compound ursolic acid, but can be related to synergic effects. Cytotoxic effects against Vero cells seem to be widely sample purity-independent. Together with the very low selectivity indices, this indicates that the cytotoxicity can be assigned to ursolic acid itself.

with methyl group signals, could, therefore, be generated by spectra simulation and optimization of the whole spin system D. SelNOE experiments with excitation of the signals of H-22ax and H-22eq, and in combination with measurements of proton distances in the force-field model, allowed the assignment, relative configuration, and the determination of coupling constants of H-21ax and H-21eq. SelNOE of H-7ax enabled the resolution of its coupling pattern and stereochemical assignment of spin system 2. Finally, excitation and spin system analysis of H-18 exhibited the dd nature of its signal, which is caused by a coupling to H-19eq, and a long-range coupling to H-20 (Table 1.).

Impurity Profiling. A combination of HPLC, routine ^{1}H NMR and, when appropriate, ^{13}C NMR represent the most frequently used methods for the purity determination of natural compounds. However, because all chromatographic methods depend on a method of detection, **1** is a problematic candidate for HPLC assays, as it lacks a UV chromophore (HPLC-DAD) and is poorly ionizable (GC- and LC-MS). While other methods such as ELSD or RI (no gradient elution!) principally allow the HPLC purity assays of **1**, UV-detection methods are still frequently used as standard methods. In order to overcome the inherent limitations of chromatography, the present study generated nonchromatographic impurity profiles of all samples by means of quantitative ^{1}H NMR (qHNMR),[33,40,41] in combination with the establishment of the above-mentioned structural dossier.

A spectral processing concept for optimizing the quantitative ^{1}H NMR spectra was developed. The best line shape (=lowest $\omega_{1/2}$) was achieved with the Lorentzian–Gaussian transformation (LG, Gaussian factor of 0.02 was better than 0.05). The best signal-to-noise (S/N) resulted from exponential multiplication (EM), the worst from LG with a Gaussian factor 0.20. For the ursolic acid samples, the optimum choice was an LG with a Gaussian factor of 0.05 and a line broadening of 0.3, which resulted in S/N between that of EM and GM. The digital resolution was increased by adding two equivalent numbers of zeros at the end of the FID data set (double zero fill). As a result, the purity of all except one of the nine ursolic acid samples was found to be notably lower than declared (Table 2), while one sample (ua-03) was slightly more pure by qHNMR than labeled ($\Delta = 0.04\%$). In three out of the nine cases the purity measured by qHNMR was found to deviate by 11–12% from the declared values.

Using qHNMR, five to nine different impurities could be determined in the samples, including oleanolic acid, which was detected in eight of the samples, and betulinic acid, which was detected in six of the samples. The identities of oleanolic and betulinic acids were verified by the following compound-specific marker signals: a doublet of a doublet split with $J = 3.4$ and 14.3 Hz at δ 2.760 (H-18) for oleanolic acid, and a doublet split with $J = 2.0$ Hz at δ 4.615 (H_2-29) for betulinic acid (Table 3).

Biological Activity. Besides providing the results of the purity evaluation, Table 2 also summarizes the antimycobacterial and cytotoxic bioactivities of the nine samples of **1**. These data led to the establishment of PARs for the anti-TB activity, the cytotoxicity, and the anti-TB selectivity of **1** (Figure 1). As expected, there were only moderate differences in the susceptibility of the two mycobacterial strains to **1** (Table 2). However, curve shape and progression demonstrate that activity and purity were *not* proportional, nor are they correlated in a linear or logarithmic fashion. Overall, an *inverse* correlation between the antimycobacterial activity and the sample purity of **1** was observed. When extrapolating the sigmoid curve in Figure 1 toward "100% purity", **1** exhibits an MIC of $\gg$256 and $\gg$100 μg/mL against $H_{37}Rv$ and $H_{37}Rv$GFP, respectively, which means that it is essentially inactive. Interestingly, at the other end of the curve, those samples with the lowest purity demonstrated the highest anti-TB activity (lowest MIC) with values as low as 27 μg/mL.

In contrast to the anti-TB properties, all samples of **1** show virtually identical cytotoxicity against Vero cells, with IC_{50} values in a very narrow range between 17.9 and 19.5 μg/mL (Table 2). Thus, while the cytotoxic activity appears to be attributable to **1** as the main component, there is no quantitative correlation of dose and response between the two. Moreover, because cytotoxicity IC_{50}'s are below anti-TB MICs throughout, the anti-TB selectivity indices (SIs) were determined to be <1 (i.e., nonselective) for both *M. tuberculosis* isogenic strains ($H_{37}Rv$ and $H_{37}Rv$GFP). Moreover, because cytotoxicity was almost identical in all nine samples, the curve representing anti-TB *selectivity* essentially represents the *inverse* of the anti-TB *activity* (Figure 1).

Quantitative Correlation of Chemistry and Biology. The above evidence supports the hypothesis that neither the antimycobacterial nor the cytotoxic activity of ursolic acid isolates is caused by **1** alone: The SCE, ursolic acid, cannot explain the sigmoid anti-TB and almost constant cytotoxicity curves in the PARs plotted in Figure 1. The latter represent a new form of quantitative correlation between chemical (purity) and biological (potency) parameters and

are additive to the definitive structural assignments that are required for establishing [Q]SARs. As a matter of fact, knowledge of PARs can be considered a prerequisite for the establishment of [Q]SARs. While PARs are *quantitative* by nature, they provide unique *qualitative* information about the SCE character[32,33,41] of a biologically active agent. Transforming this qualitative information into structural information is a separate task. In the case of ursolic acid, this turned out to be a quite challenging endeavor, reaching well beyond the scope of the current study. However, the following summarizes the numerous attempts that were made to chemically and spectroscopically characterize the "active fraction" of the impure samples of **1**.

One prerequisite for further study was the use of a loss-free separation technique, avoiding selective loss of sample constituents by irreversible adsorption, such as in silica-based LC. This made countercurrent chromatography (CCC) the method of choice.[42−44] Representing one of the least pure but most active materials, sample ua-08 was chosen as a model for further purification of the anti-TB active principle under bioassay guidance. Using high-resolution CCC methodology and applying various members of the HEMWat family of two-phase CCC solvent systems,[42,45,46] 10−50 mg aliquots of ua-08 (MIC 65 μg/mL) were separated under various HSCCC conditions (V_{tot} 120 mL, S_f ~0.55−0.75, normal and reverse phase) and fractions monitored by TLC and ^{1}H NMR. Under any of the chosen conditions and unlike analytes of typical behavior,[42,45−47] **1** always eluted as a relatively broad band, even when acid (0.1% TFA) was added to the solvent system in order to avoid the formation of pH-dependent species of **1**. At the same time, the combined fractions free of **1** showed no anti-TB activity (MIC > 128 μg/mL vs $H_{37}Rv$, mostly in four combined fractions), while MICs were between 25 and 58 μg/mL in the active part of the combined fractions that contained the bulk of **1**. The purities of all combined fractions were assessed by qHNMR and confirmed that the inactive, much more hydrophilic and lipophilic impurities had been successfully removed. The biological profiles of the obtained fractions suggest that none of the impurities had anti-TB activity (MICs > 128 μg/mL) nor were potent cytotoxic agents, while one or more must have synergistic potency, fostering a selective antimycobacterial effect. The combined qHNMR and anti-TB evaluation indicated that fractions containing **1** with ≤70% purity were more active (MICs < 50 μg/mL) than the more enriched fractions (≥85% of **1**, MICs > 70 μg/mL). This not only was in line with the observed PARs of the panel of reference materials (Figure 1) but also supported the preliminary conclusion that the residual complexity and specific composition of enriched fractions play a role in their antimycobacterial susceptibility.

Preliminary experiments showed no involvement of betulinic acid (five-membered, isopropylidene-substituted E-ring isomer of **1**) or oleanolic acid ([C-29, C-30]-20,20-dimethyl isomer of **1**) in synergistic anti-TB activity of **1**. The further specific exploration of synergy in ursolic acid samples using the isobole method, as recently demonstrated for the anti-TB ethnobotanical *Oplopanax horridus*,[48] would depend on the actual isolation of sufficient quantities of known impurities and was beyond the scope of this study.

A compelling parallel exists between the presently observed essential inactivity of **1** against mycobacteria and the report of its solubilizing properties for allelochemicals of *Calamintha ashei* and *Ceratiola ericoides*:[49] While **1** itself was found to be inactive, its presence enhanced the allelochemical potency of a number of lipophilic monoterpenes. Both the relatively weak (high critical micelle concentration [CMC]) surfactant properties and its potential to interact with membrane transport mechanisms were discussed as a potential explanation.[49] Another related interesting finding was the recent discovery of self-assembling cannabinomimetics, in which the agents, *N*-alkylamides, partition between the target and various levels of aggregates.[50] As a result, the compounds exhibit differential receptor affinity as a function of concentration. Despite the reported high CMC of **1**,[49] the formation of macromolecular aggregates and potential involvement of the impurities might offer an alternative explanation for the observed PARs of **1**. We are presently investigating whether polymorphism, a well-know source of altered pharmacology, is involved.

In summary, the anti-TB activity remained inseparable from **1** as one chemical entity and could also not be enriched in fractions that contained other chemical entities, in particular congeneric triterpenes. Taking into account the qHNMR-based PARs for anti-TB and cytotoxic activity established above, these findings support the hypothesis that one or more of the reported biological activities of ursolic acid are related to synergistic effects between ursolic acid and its common impurities, or to the impurities themselves. The observed biological effects root in nonclassical phenomena that involve complex chemical−biological interactions, such as synergy, and secondary/tertiary chemical structures, such as macromolecular and physicochemical properties that involve structural arrangements beyond the single molecule level.

A recent SAR study[51] of C-30-substituted cinnamate ester analogues of **1**, betulinic acid, and oleanolic acid concluded that the introduction of a *p*-coumarate moiety increases the anti-TB activity by 2-, 8-, and 8-fold, from MICs of 12.5, 50, and 50 μg/mL, respectively. The observed potencies and perceived potential of triterpene cinnamate esters as anti-TB drug leads in this semisynthetic study[51] are in apparent contrast to the results of the present work. However, keeping in mind that the purities of underivatized and modified products were not investigated, PARs might provide the missing link to the understanding of the underlying mechanisms.

Potential of qHNMR-Based PARs. With respect to natural products and related research involving samples of complex composition, the results of this study suggest that purity should be routinely investigated for all isolates, whenever their biological activity is deemed to be of sufficient interest as to warrant further studies. This particularly applies to drug discovery screening candidates evolving from complex matrices. Examples comprise, but are not limited to, natural products from terrestrial, marine, and microbial organisms, as well as to products of parallel and combinatorial synthesis. Since potency and selectivity are the two primary desired attributes of a hit in a screening program geared toward drug discovery, the implications of overlooking purity-related effects can be profound. The case of **1** exemplifies the importance of combining state-of-the-art structure elucidation or dereplication with the determination and report of sample purity, whenever feasible, together with the biological data. The data also underline the great potential qHNMR has in providing distinctive information about the nature and amount of impurities with virtually no additional effort.[33] qHNMR is the method of choice for purity evaluation and establishment of PARs. The latter can offer new insights when working with materials that require purification from complex matrices such as products of combinatorial or parallel (bio)synthesis and natural products, including dietary supplements.

Furthermore, the case of **1** demonstrates that the complexity of biologically active natural products extends beyond their valuable structural diversity: Whenever active principles are elucidated chemically, it is important to consider "hidden" mechanisms that might involve (apparent) SCEs and their interactions with (residually) complex natural matrices. The establishment of PARs provides a powerful tool for recognizing such "hidden" connections, in particular when employing nonchromatographic methodology such as qHNMR in the purity dimension. PAR by qHNMR carries the advantage of allowing detection of even those instances where highly complex or unknown chemical interactions are an integral part of a bioactive principle and represent concealed assets of SCE-dominated evaluation strategies. Because of their nuclear perspective[40] and ability to function well even under flawed molecular

weight assumptions,[32] qHNMR-based PARs provide an independent rationale for exploratory drug discovery efforts and can be implemented early in any workflow aimed at the discovery of bioactive principles. In addition, PARs have promise for the cross-validation of compendial reference materials.

Experimental Section

Sample Material. Ursolic acid (**1**) was obtained from nine different commercial sources (details are available upon request from the authors). All samples came with a certificate of analysis including purity assignments, which were based on HPLC assays.

Structure Elucidation and Impurity Profiling. Prior to analysis the samples were dried thoroughly over P_4O_{10} *in vacuo* to eliminate variations from residual water. Samples ranging from 4 to 11 mg were dissolved in 50 μL of DMSO (99.9% isotopic purity), and $CDCl_3$ (99.8% isotopic purity) was added to give a final volume of 1000 μL, corresponding to a filling height of 50 mm in 5 mm NMR tubes. The NMR spectra were recorded using a Bruker AMX 500 instrument. Chemical shifts (δ in ppm) were referenced to the residual $CDCl_3$ signals at δ 7.240 and 77.00, respectively, and couplings constants (J) are given in Hz. For all NMR experiments, off-line data analysis was performed using the NUTS software package, Acorn NMR Inc. The 1D digital resolution was better than 0.4 Hz, equivalent to 0.0008 ppm (32K real data points, 12 ppm spectral width), in the ^{1}H, and 0.7 Hz, equivalent to 0.006 ppm (32K real data points, 240 ppm spectral width), in the ^{13}C domain.

For (im)purity profiling, ^{1}H NMR spectra were measured with 512 scans to yield spectra suitable for a quantitative evaluation (qHNMR).[40,41] The precision of the detection of minor impurities present at ca. 1% abundance was better than 2%. All acquisition parameters were selected in agreement with quantitative NMR conditions[40,41] and without broadband ^{13}C decoupling, as this only very recently reported qHNMR methodology[33] was not available at the commencement of this study.

In the second step, a spectral processing concept for optimizing the quantitative ^{1}H NMR spectra was developed. The first objective was the determination of the optimal window function and parameters. Therefore, a study was designed to compare the $\omega_{1/2}$ (width [Hz] at half-height) of the reference signal ($CDCl_3$ singlet at 7.240 ppm) with the achieved signal-to-noise ratio when using different window functions and parameters as follows: (i) Exponential multiplication (EM), (ii) Gaussian multiplication (GM), (iii) Lorentzian−Gaussian resolution enhancement (LG) with Gaussian factor 0.05, (iiii) LG with Gaussian factor 0.20. The line-broadening value was set at 0.01−2.5 in steps of 0.1. As expected, the best line shape (=lowest $\omega_{1/2}$) was achieved with the LG (Gaussian factor 0.02 better than 0.05). The S/N resulted from the EM, the worst from LG with a Gaussian factor 0.20. For the ursolic acid samples, the optimum choice was an LG with a Gaussian factor of 0.05 and a line broadening of 0.30, which resulted in S/N between that of EM and GM. The digital resolution was increased by adding two equivalent numbers of zeros at the end of the FID data set (double zero fill). To improve integration, the baseline of the spectrum was corrected, broad water as well as other −OH and exchangeable proton signals were eliminated by repeated simulation and subtraction from the uneven baseline, and, finally, a baseline flattening was applied by nth ($n < 10$)-order polynomial correction. The doublet at δ 2.033 of the main component **1** served as a reference signal set to an arbitrary integral value of 100. 2D COSY spectra were consulted to aid in the assignment of the impurities.

Antimycobacterial Assays. *M. tuberculosis* $H_{37}Rv$ ATCC 27294 ($H_{37}Rv$) was obtained from the American Type Culture Collection (Rockville, MD). The fluorescent (GFP) strain $H_{37}Rv$-pFPCA1 ($H_{37}Rv$GFP) was constructed in the Institute for Tuberculosis Research (ITR), University of Illinois at Chicago.[52] $H_{37}Rv$ was cultured 100 mL of Middlebrook 7H9 broth (Difco, Detroit, MI) supplemented with 0.2% (v/v) glycerol (Sigma Chemical Co., Saint Louis, MO), 10% (v/v) OADC (oleic acid, albumin, dextrose, catalase; Difco), and 0.05% (v/v) Tween 80 (Sigma), a culture medium referred to as 7H9GC-T80. $H_{37}Rv$GFP was cultured identically, except that kanamycin (30ug/mL) was added. The cultures were incubated in 300 mL nephelometer flasks on a rotary shaker (New Brunswick Scientific, Edison, NJ) at 150 rpm and 37 °C until they reached an optical density of 0.4−0.5 at 550 nm. The bacteria were washed and suspended in 20 mL of phosphate-buffered saline and passed through an 8 μm pore size filter to eliminate bacterial clumps. The filtrates were aliquoted and stored at −80 °C.

Kanamycin sulfate (KM) and rifampin (RMP) were obtained from Sigma. The chemicals were solubilized according to the manufacturers' recommendations. Stock solutions were filter sterilized (0.22 μm pore size) and stored at −80 °C. The 7H12 media consisted of Middlebrook 7H9 broth supplemented with 0.1% casitone (Difco), 0.1% palmitic acid (5.6 mg/mL free acid in ethanol, Sigma), 10% albumin (50 mg/mL in water, Sigma), and 0.1% catalase (4 mg/mL in water, Sigma).

Minimum inhibitory concentrations (MIC) of the ursolic acid samples were determined using the microplate Alamar blue assay (MABA)[53] and the green fluorescent protein microplate assay (GFPMA).[52] Testing was performed in black, clear-bottomed, 96-well microplates (black view plates; Packard Instrument Company, Meriden, CT) in order to minimize background fluorescence. Initial sample dilutions were prepared in dimethyl sulfoxide, and subsequent 2-fold dilutions were performed in 0.1 mL of 7H12 media in the microplates.

The inocula were initially diluted in 7H12 media to achieve approximately 2×10^5 cfu/mL, and 0.1 mL was added to individual wells. Wells containing compounds only were used to detect autofluorescence of compounds. Additional control wells consisted of bacteria only (B) and medium only (M). Plates were incubated at 37 °C.

For the MABA assays, at day 7 of incubation of plates inoculated with $H_{37}Rv$, 20 μL of Alamar Blue solution (Trek Diagnostic Systems, Cleveland, OH) and 12.5 mL of 20% Tween 80 were added to all the wells, and plates were reincubated at 37 °C for 24 h. Fluorescence was measured in a Victor II multilabel fluorometer (Perkin-Elmer Life Sciences Inc., Boston, MA) in bottom-reading mode with excitation at 530 nm and emission at 590 nm. For the GFPMA assay, fluorescence was measured directly with excitation at 485 nm and emission at 535 nm on day 7 of incubation of plates inoculated with $H_{37}Rv$GFP. For both MABA and GFPMA, a background subtraction was performed on all wells using the mean of triplicate M wells. Percent inhibition was defined as 1 − (test well FU/mean FU of triplicate B wells) × 100. The lowest drug concentration effecting an inhibition of 90% was considered the MIC. Mean, standard deviation (SD), and coefficient of variation (CV = 100 × mean/SD) of seven replicates each were calculated for all assays.

Cytotoxicity Assay. Cytotoxic activity of compounds was determined for Vero cells (kidney, African green monkey), which had been exposed to 16−0.25 μg/mL test compounds for 72 h. The assay (n = 7) was performed using the CellTiter 96 aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI). The IC_{50} is defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells.

Chromatographic Separation. Sample ua-08, the sample with the lowest detected purity (69.66%) and the lowest MIC (64.57 μg/mL MIC $H_{37}Rv$), was subjected to further separation by means of HSCCC. A 200 mg sample, which was an ursolic acid sodium salt, was dissolved in 50 mL of $CHCl_3$ (containing 0.05 mL of TFA 0.01%), 50 mL of H_2O was added, and the two-phase solution was transferred to a separation funnel. After 15 min reaction time, the $CHCl_3$ phase (lower phase) was washed with water (upper phase) × 3. The lower phases were collected and combined. The last separation of the two phases was done overnight to get a clean two-phase system. $CHCl_3$ was evaporated to yield the free ursolic acid.

The sample was further fractionated by HSCCC with a Pharma-Tech Research Corp. instrument (CCC1000) in tail-to-head mode using various members of the HEMWat family of two-phase solvent systems.[46] The solvent system consisting of n-hexanes−EtOAc−MeOH−0.01% TFA in H_2O (6:4:5:5) was determined to be most suitable. For bioassay-controlled separation, a 33 mg sample of ua-08 (dissolved in equal amounts of upper and lower phase, 2 mL) was injected; 245 fractions were collected and recombined into 14 fractions, which were all subjected to mycobacterial testing ($H_{37}Rv$, MABA). The separation was monitored qualitatively by silica TLC, developed with EtOAc−n-hexanes (5:5) and using 2% anisaldehyde−2% H_2SO_4 in MeOH as detection reagent, as well as ^{1}H NMR of selected fractions.

Acknowledgment. The expert NMR support of Dr. R. Kleps, Research Resources Center at UIC, Chicago, and Dr. M. Westler, NMR-FAM, University of Wisconsin, Madison, WI, is gratefully acknowledged. The authors are thankful to Drs. N. Farnsworth and G. Cordell of UIC, Chicago, and Dr. N. H. Fischer, Denton (TX), for fruitful discussions, and to M. L. Quinn-Beattie, NAPRALERT at UIC, Chicago, for her invaluable help with the complex literature search.

Finally, the support of W.-W. Zhao and Dr. S.-N. Chen of UIC, Chicago, is gratefully acknowledged.

References and Notes

(1) Newman, D. J.; Cragg, G. M.; Snader, K. M. *Nat. Prod. Rep.* **2000**, *17*, 215–234.
(2) Newman, D. J.; Cragg, G. M.; Snader, K. M. *J. Nat. Prod.* **2003**, *66*, 1022–1037.
(3) Newman, D. J.; Cragg, G. M. *J. Nat. Prod.* **2007**, *70*, 461–477.
(4) Lill, M. A. *Drug Discovery Today* **2007**, *12*, 1013–1017.
(5) U.S. Food and Drug Administration. Guidance for Industry. Q3A Impurities in New Drug Substances. http://www.fda.gov/cber/gdlns/ichq3a.htm#v (accessed May 31, 2008).
(6) Farnsworth, N. R. The NAPRALERT database. Available on-line through the Scientific and Technical Network (STN) of Chemical Abstracts Service, Columbus, OH, 2007.
(7) Braghiroli, L.; Mazzanti, G.; Manganaro, M.; Mascellino, M. T.; Vespertilli, T. *Phytother. Res.* **1996**, *10*, S86−S88.
(8) Mathuram, V.; Bhahmadhayalaselvam, A.; Jaffar Hussain, A.; Bhima Rao, R.; Patra, A. *J. Ind. Chem. Soc.* **1998**, *75*, 262–264.
(9) Richards, R. M.; Durham, D. G.; Liu, X. *Planta Med.* **1994**, *60*, 471–473.
(10) Takechi, M.; Tanaka, Y. *Phytochemistry* **1993**, *34*, 675–677.
(11) Panizzi, L.; Catalano, S.; Miarelli, C.; Pioni, P. l.; Campeol, E. *Phytother. Res* **2000**, *14*, 561–563.
(12) Kuroyanagi, M.; Ebihara, T.; Tsukamoto, K.; Fukushima, S.; Ishizeki, C.; Satake, M. *Screening of antibacterial constituents of crude drugs and plants*; 5th Symposium on development and application of naturally occuring drug materials; Japan, 1984.
(13) Haraguchi, H.; Kataoka, S.; Okamoto, S.; Hanafi, M.; Shibata, K. *Phytother. Res.* **1999**, *12*, 151–156.
(14) Osawa, K.; Yasuda, H.; Morita, H.; Takeya, K.; Itokawa, H. *Nat. Med.* **1997**, *51*, 365–367.
(15) Gonzales, B.; Suarez Roca, H.; Bravo, A.; Salas Auvert, R.; Avila, D. *Pharm. Biol.* **2000**, *38*, 287–290.
(16) Miura, N.; Matsumoto, Y.; Miyairi, S.; Nishiyama, S.; Naganuma, A. *Mol. Pharmacol.* **1999**, *56*, 1324–1328.
(17) Ahn, K. S.; Hahm, M. S.; Park, E. J.; Lee, H. K.; Kim, E. H. *Planta Med.* **1998**, *64*, 468–470.
(18) Manez, S.; Recio, M. C.; Giner, R. M.; Rios, J. L. *Eur. J. Pharmacol.* **1997**, *334*, 103–105.
(19) Huang, M. T.; Ho, C. T.; Wang, Z. Y.; Ferraro, T.; Lou, Y. R.; Stauber, K.; Ma, W.; Georgiadis, C.; Laskin, J. D.; Conney, A. H. *Cancer Res.* **1994**, *54*, 701–708.
(20) Huguet, A. I.; Del Carmen Recio, M.; Manez, S.; Giner, R. M.; Rios, J. L. *Eur. J. Pharmacol.* **2000**, *410*, 69–81.
(21) Yasukawa, K.; Takido, M.; Takeuchi, M.; Nakagawa, S. *Chem. Pharm. Bull.* **1989**, *37*, 1071–1073.
(22) Umehara, K.; Takagi, R.; Kuroyanagi, M.; Ueno, A.; Taki, T.; Chen, Y. J. *Chem. Pharm. Bull.* **1992**, *40*, 401–405.
(23) Liu, J. *J. Ethnopharmacol.* **1995**, *49*, 57–68.
(24) Ali, R. M.; Houghton, P. J. *Planta Med.* **1999**, *65*, 455–457.
(25) Simon, A.; Najid, A.; Chulia, A. J.; Delage, C.; Rigaud, M. *Biochim. Biophys. Acta* **1992**, *1125*, 68–72.
(26) Ma, X. H.; Zhao, Y. C.; Yin, L.; Xu, R. L.; Han, D. W.; Wang, M. S. *Yao Hsueh Hsue Pao* **1986**, *21*, 332–335.
(27) Okuyama, E.; Yamazaki, M.; Ishii, Y. *Shoyakugaku Zasshi* **1983**, *37*, 52–55.
(28) Kim, D. W.; Bang, K. H.; Rhee, Y. H.; Lee, K. T.; Park, H. J. *Arch. Pharm. Res.* **1998**, *21*, 688–691.
(29) Lee, K. H.; Lin, Y. M.; Wu, T. S.; Zhang, D. C.; Yamagishi, T.; Hayashi, T.; Hall, I. H.; Chang, J. j.; Wu, R. Y.; Yang, T. H. *Planta Med* **1988**, *54*, 308–311.
(30) Han, G. Q.; Che, C. T.; Fong, H. H. S.; Farnsworth, N. R.; Phoebe, C. H. J. *Fitoterapia* **1988**, *59*, 242–244.
(31) Numata, A.; Takahashi, C.; Miyamoto, T.; Yoneda, M.; Yang, P. *Chem. Pharm. Bull.* **1990**, *38*, 942–944.
(32) Pauli, G. F.; Jaki, B. U.; Lankin, D. C.; Walter, J. A.; Burton, I. W. *Quantitative NMR (qNMR) of Bioactive Natural Products in Bioactive Natural Products: Detection, Isolation and Structural Determination*, 2nd ed.; Colegate, S. M., Molyneux, R. J., Eds.; Taylor & Francis: New York, 2008.
(33) Pauli, G. F.; Jaki, B.; Lankin, D. *J. Nat. Prod.* **2007**, *70*, 589–595.
(34) Schühly, W.; Heilmann, J.; Calis, I.; Sticher, O. *Planta Med.* **1999**, *65*, 740–743.
(35) Isobe, T.; Noda, Y.; Ohsaki, A.; Sakanaka, S.; Kim, M.; Taniguchi, M. *Yakugaku Zasshi* **1989**, *109*, 175–178.
(36) Cantrell, C. L.; Franzblau, S. G.; Fischer, N. H. *Planta Med.* **2001**, *67*, 685–694.
(37) Copp, B. R.; Pearce, A. N. *Nat. Prod. Rep.* **2007**, *24*, 278–297.
(38) Newton, S. M.; Lau, C.; Wright, C. W. *Phytother. Res.* **2000**, *14*, 303–322.
(39) Weiss, R.; Seebacher, W. *Magn. Reson. Chem.* **2002**, *40*, 455–457.
(40) Pauli, G. F. *Phytochem. Anal.* **2001**, *12*, 28–42.
(41) Pauli, G. F.; Jaki, B.; Lankin, D. *J. Nat. Prod.* **2005**, *68*, 133–149.
(42) Berthod, A.; Friesen, J. B.; Inui, T.; Pauli, G. F. *Anal. Chem.* **2007**, *79*, 3371–3382.
(43) Ito, Y. *J. Chromatogr. A* **2005**, *1065*, 145–168.
(44) Berthod, A. *Countercurrent Chromatography: The Support-free Liquid Stationary Phase*, 1st ed.; Elsevier: Amsterdam, 2002; Vol. 38.
(45) Friesen, J. B.; Pauli, G. F. *J. Agric. Food Chem.* **2008**, *56*, 19–28.
(46) Friesen, J. B.; Pauli, G. F. *J. Liq. Chromatogr. Relat. Technol.* **2005**, *28*, 2877–2808.
(47) Friesen, J. B.; Pauli, G. F. *J. Chromatogr. A* **2007**, *1151*, 51–59.
(48) Inui, T.; Wang, Y.; Deng, S.; Smith, D.; Franzblau, S.; Pauli, G. F. *J. Chromatogr. A* **2007**, *1151*, 211–215.
(49) Tanrisever, N. *Plant Germination and Growth Inhibitors from Ceratiola ericoides and Calamintha ashei*; Louisiana State University: Baton Rouge, 1986.
(50) Raduner, S.; Bisson, W.; Abagyan, R.; Altmann, K. H.; Gertsch, J. *J. Nat. Prod.* **2007**, *70*, 1010–1015.
(51) Tanachatchairatana, T.; Bremner, J. B.; Chokchaisiri, R.; Suksamrarn, A. *Chem. Pharm. Bull.* **2008**, *56*, 194–198.
(52) Changsen, C.; Franzblau, S. G.; Palittapongarnpim, P. *Antimicrob. Agents Chemother.* **2003**, *47*, 3682–3687.
(53) Collins, L.; Franzblau, S. *Antimicrob. Agents Chemother.* **1997**, *41*, 1004–1009.

NP800329J