Characterization and Formulation of a Black Cohosh Root Extract (BCE) Lot to be Used in Rodent Toxicology Studies

Esra Mutlu[1], Tim A. Cristy[2], Jessica A. Pierfelice[2], Jon C. Andre[2], Billie A. Stiffler[2], Brian L. Burback[2], and Suramya Waidyanatha[1]

[1]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, NC; [2]Battelle, Columbus, OH

Abstract

Black cohosh (*Actaea racemosa*) is sold as a dietary supplement for the treatment of menstrual and menopausal symptoms in women. Although it has widespread human exposure, there is limited safety data in rodents and humans. The National Toxicology Program (NTP) is investigating the toxicity of BCE in rodents. The objective of this work was to screen commercially available lots sold as BCE to select a unformulated product for use in NTP studies. Multiple lots of unformulated and formulated products sold as BCE, standard reference materials of BCE and potential adulterants (Chinese, red, and yellow cohosh) were analyzed using a combination of non-targeted and targeted analytical techniques. Authenticity was confirmed by chemical fingerprinting and DNA barcoding. Based on the results, a lot was selected for further characterization. The constituents of the selected lot (actein, 0.47%; 27-deoxyactein, 2.09%; ferulic acid, 0.04%; isoferulic acid, 0.70%; caffeic acid, 0.28%; cimiracemoside C, 2.13%; 26-deoxycimicifugoside, 0.08%; magnoflorine, 0.01%) were determined by LC-MS-MS. Other analyses included moisture content (5.6%), ash (5.8%), and nutritional content (fat, carbohydrate and protein). The lot was also analyzed for contaminants (heavy metals, ≤476ppb; pesticides, ≤0.3ppm; mycotoxins, ≤50ppb; microbial content, <10 CFU/g).

A method for formulation of BCE in 0.5% methylcellulose was developed and a formulation analysis method was validated to quantify isoferulic acid. Analytical method was linear (r ≥0.99) and accurate and formulations were homogeneous (% relative error, RE ≤ ±10 and % relative standard deviation, RSD ≤ ±5). The formulations were stable for multiple markers (actein, 27-deoxyactein, isoferulic acid and cimiracemoside C) up to 42 days with % RE ≤ ±20 of day 0. These data demonstrate that the BCE lot is suitable and can be formulated to be used in rodent toxicological studies.

Background and Objectives

- BCE has a long history of use for treatment of menstrual and menopausal symptoms in women.
- BCE was nominated to the National Toxicology Program (NTP) for toxicity testing due to widespread exposure and to lack of adequate data supporting its safety or toxicity.
- BCE can be purchased in a variety of forms such as pills, teas, and tinctures, and may be blended with other botanicals.

•The objective of this work was

- **To screen commercially available formulated and unformulated BCE products, standard reference material of BCE (XRM), and potential adulterants such as other cohosh species vouchered botanical reference materials (VBRMs) by non-targeted and targeted analyses and identify a suitable unformulated product for use in NTP toxicity studies.**
- **Develop methods to prepare and analyze formulations and stability for testing.**

Lot Selection and Characterization Approach

Phase I: Lot Selection

Procurement of 18 Lots → Screening of 18 BCE Lots, 1 XRM, 4 VBRMs, 8 supplements, and 23 Standards → Principle Component Analysis (PCA) → Authentication of the Lot (DNA Barcoding and HP-TLC) → Selection of the Lot for Further Characterization

Phase II: Lot Characterization

Further Characterization of the Lot → Analyses for Characterization:

1. Nutritional Analysis
2. Contaminant Analysis
3. LC-Q-TOF-MS/MS
4. Quantitation of Marker Constituents by HPLC-MS/MS
5. PIXE Analysis
6. Bulk Stability

Phase III: Formulations

1. Formulation Development and Analysis
2. Formulation Stability

HP-TLC: High Performance-Thin Layer Chromatography
LC-Q-TOF-MS/MS: Liquid Chromatography-Quad-Time of Flight-Tandem Mass Spectrometry
HPLC-MS/MS: High Performance Liquid Chromatography-Tandem Mass Spectrometry
PIXE: Proton (particle)-Induced X-Ray Emission

Sample Preparation

400 mg of powdered BCE (bulk lots, XRM, and VBRMs) → Extract with hano : (80:20) → HPLC-MS/MS

20 mg Remifemin® tablets (equivalent to 400 mg of BCE) → Ground by mortar and pestle → Extract with hano : (80:20)

Same sample preparation was used for non-targeted and targeted analyses in Phase I and II, .
HPLC-CAD: High Performance Liquid Chromatography-Charged Aerosol Detection
HPLC-UV: High Performance Liquid Chromatography-Ultraviolet Detection

Phase I. Screening Multiple Lots

- Multiple lots of herbal products sold as BCE, XRM, VBRMs, supplements, and standards of cohosh constituents were analyzed for terpenes and polyphenols by HPLC-UV and CAD.

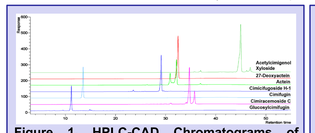

Figure 1. HPLC-CAD Chromatograms of Standards of Cohosh Constituents

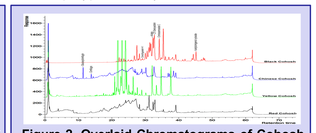

Figure 2. Overlaid Chromatograms of Cohosh Species References (VBRMs)

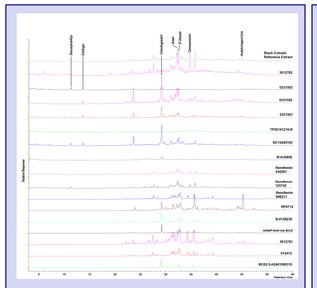

Figure 3. Triterpene Glycosides Screening in BCE lots and XRM by HPLC-CAD

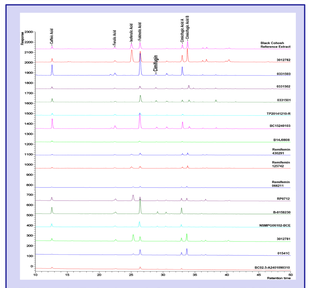

Figure 4. Polyphenol Screening in BCE lots and XRM by HPLC-UV

Sample	Lot	Caffeic Acid	Ferulic Acid	Isoferulic Acid
BCE XRM	00030148-005	0.0589	0.0507	0.172
BC Extract	3012782	0.0791	0.0494	0.407
Remifemin©	066211	0.0242	0.0232	0.0527

Table 1. Comparison of Polyphenols in Representative Lots of BCE by Weight Percent

Sample	Lot	Cimifugin	Glucosyl-cimifugin	Cimicifugoside H-1	Actein	27-Deoxy-actein	Cimiracemoside C	Acetylcimigenol Xyloside
BCE XRM	00030148-005	ND	ND	0.0587	1.05	0.292	0.351	0.0753
BC Extract	3012782	ND	ND	0.172	1.96	0.758	0.704	0.0701
Remifemin©	066211	ND	ND	0.0333	0.627	0.549	0.286	0.0119

Table 2. Comparison of Triterpene Glycosides in Representative Lots of BCE by Weight Percent

- The weigh percent was used as an estimated value for profiling and general comparison .
- The weight percent was calculated based on a single point solvent standard.
- Only one representative BCE lot and one supplement lot were presented in the above tables
- Based on the analyses above (Figures1-4), Lot 3012782 was most similar to the XRM.
- Most other lots appear to not be BCE and most likely adulterated with other cohosh species.
- Remifemin® tablets were very similar to Lot 3012782.

Phase I. Principal Component Analysis

- Multiple lots of herbal products sold as BCE were analyzed by HPLC-CAD.
- XRM, VBRMs from several cohosh species (black cohosh, Chinese cohosh, red cohosh, and yellow cohosh), and BCE lots used for screening were also analyzed.
- HPLC-CAD Chromatograms were imported into SpecAlign by aligning the peaks by retention times.
- The comma-separated values (CSV) files from SpecAlign were imported into the Eigenvector Research Solo (Manson, WA) version 8.5.1 chemometrics software and statistical analysis was performed, including principal component analysis (PCA).

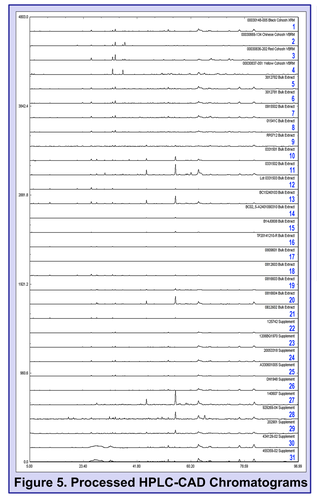

Figure 5. Processed HPLC-CAD Chromatograms

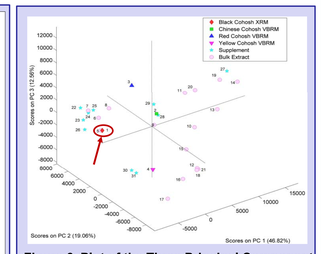

Figure 6. Plot of the Three Principal Component Analysis

Based on a combination of the screening analysis and the PCA,

- ***Lot No. 3012782 (5) was found to be most similar to the BCE XRM (1) hence was selected for authentication (DNA Barcoding and HP-TLC).***
- Bulk Lot Nos. 3012781 *(6)*, 0915502 *(7)*, and 01541C *(8)* and five supplements including Lot Nos. 125742 *(22)*, 1206BQ1970 *(23)*, 20053318 *(24)*, A330601005 *(25)*, and DN1948 *(26)* were also similar to the XRM *(1)*.

Phase I. Authentication by DNA Barcoding

Sample	NSF AuthenTechnologies	MEI
Lot # *3012782*	Confirmed the presence of *Actaea racemosa*; no other *Actaea* species were identified	Greater than 96% *Actaea racemosa*. Also found small amounts of yeast, and verbena family and balsam family components

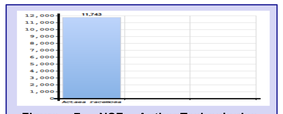

Figure 7. NSF AuthenTechnologies Species Identification Test Results

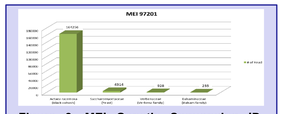

Figure 8. MEI Genetic Sequencing ID Conclusion

- The use of "specific primers" as utilized by NSF resulted in amplification of DNA and demonstrated the presence of *BCE* DNA.
- It is unclear whether the primers used were specific to *Actaea racemosa* or applicable to other members of the genus.
- The identity of the test lot was concluded to be greater than 96 percent *Actaea racemose* by MEI.
- The analysis also identified less than 3 percent sac fungi, less than 1 percent *Verbenaceae* (verbena family), and less than 0.5 percent *Balsaminaceae* (balsam family).
- The sac fungus was identified as a yeast in the *Saccharomycetaceae* family, which can be found wherever carbohydrates are plentiful.

Phase I. Authentication by HP-TLC

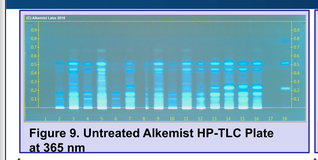

Figure 9. Untreated Alkemist HP-TLC Plate at 365 nm

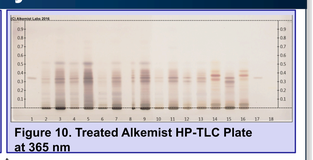

Figure 10. Treated Alkemist HP-TLC Plate at 365 nm

Lane	Sample
1	Actein – 3 µL
4	**Lot # *3012782*** – 0.5 µL
5	**Lot # *3012782*** – 2 µL
12	*Actaea racemosa* (rhizome)–2 µL
13	*Actaea racemosa* (root)–2 µL
14	*Actaea cimicifuga* (rhizome)–2 µL
15	*Actaea podocarpa* (root&rhizome)–2 µL
16	*Actaea heracleifolia* (root)–2 µL
18	Cimifugin – 3 µL

	Method Summary
Sample Preparation	0.3 g + 3 mL 50% grain ethanol; sonicate/heat @ ~50°C for ~0.5 hours
Stationary Phase	Silica gel 60, F_{254}, HP-TLC plates
Mobile Phase	Toluene:ethyl formate:formic acid (5:3:2)
Detection	Figure 1 - UV @ 365 nm Figure 2 - 10% Ethanolic sulfuric acid @ 120°C for 10 minutes, visible (ambient) light

- It was concluded the **Lot # *3012782*** is positive for BCE, *Actaea racemosa* by three different labs.
- Due to the absence of a characteristic prominent band (cimifugin) in the test lot which is present in *Actaea cimicifuga, Acteaea podocarpa, and Actaea heracleifolia*, it can be concluded that the test lot is not comprised of these species.
- Lanes 2,3, 6-11, and 17 are unrelated to analysis of test lot.

Phase II. Contaminant and Nutritional Analysis

- Based on Authentication of Lot# 3012782 (***Test Lot***) was further characterized by contaminant, nutritional and PIXE analysis.

Sample	Antimony (ppm)	Arsenic (ppm)	Cadmium (ppm)	Lead (ppm)	Mercury (ppm)	Aflatoxins (ppb)
Test Lot	<10.0	276	16.3	164	<10.0	<50.0

- Pesticides were in general <0.05 ppm. Two pesticides (chlorpropham and 2-phenylphenol) were below the lowest EPA residue tolerance levels for commodities listed for each particular pesticide.

Sample	Fat	Carbohydrates	Protein	Ash	Extractables	Moisture	Total
Test Lot	1.8 %	22.4 %	26.7 %	5.8 %	73.9 %	5.6 %	136.2 %

- The percent total estimated is potentially overestimated because the extractables likely Include some moisture, protein, carbohydrates, and fat.

Sample	Iron	Calcium	Copper	Magnesium	Manganese	Phosphorus	Potassium	Sodium	Zinc
Test Lot (mg/100g)	11.4	260	0.911	259	6.68	120	2390	30.6	1.222

- Inorganics were analyzed by Inductive Coupled Plasma Spectrometry (ICPS)

Phase II. LC-Q-TOF-MS/MS Analysis

- The scope of this analysis was to identify potential marker compounds of BCE.
- The constituent analysis was performed after extracting test lot with methanol/water followed by LC-Q-TOF-MS/MS analysis.
- Analytes were identified based on 16 commercially available known constituents of BCE from literature.

Chemical	Result
Actein	Inconclusive[a]
Allocryptopine	Inconclusive[b]
Caffeic acid	Detected
Cimiracemoside C	Detected
Ferulic acid	Detected
Formononetin	Not detected
Isoferulic acid	Detected
Kaempferol	Not detected
Phellodendrine	Likely detected[c]
Protocatechuic acid	Detected
Cimicifugoside H-1	Detected
27-Deoxyactein	Detected
26-Deoxycimicifugoside	Detected
Prim-O-glucosylcimifugin	Detected
Salsolinol	Detected
Magnoflorine	Inconclusive[d]

Table 3. Summary of the LC-Q-TOF-MS/MS Results from the Analysis of the Lot by Comparison to 16 Commercially Available Standards

Phase II. Quantitation of Marker Constituents

- Standard addition was conducted to quantify the amounts of constituents identified by LC-Q-TOF-MS/MS present in test lot by HPLC-MS/MS detection.

Chemical	Result (%)
Actein	0.47
Allocryptopine	ND
Caffeic acid	0.28
Cimiracemoside C	2.13
Ferulic acid	0.04
Isoferulic acid	0.70
Cimicifugoside H-1	ND
27-Deoxyactein	2.09
26-Deoxycimicifugoside	0.08
Prim-O-glucosylcimifugin	ND
Magnoflorine	0.01

Table 4. Standard Addition Results

- The difference observed between the weight percent calculated during profiling and the standard addition during characterization can be contributed to matrix effect and variability between methods.

Phase III. Formulation Development and Analysis

- A formulation analysis method was developed and validated ($r \geq 0.99$; precision ≤1.6%; accuracy, ≤±1.7%) to analyze BCE formulated in 0.5% methylcellulose (MC) in deionized water over the concentration range 30-300 mg/mL.
- Isoferulic acid was selected as the marker compound and analyzed by HPLC-UV.
- Formulations were gavagable with an 18-G needle and resuspendable at 300 mg/mL

Formulation Preparation and Analysis in Support of the Toxicology Study:

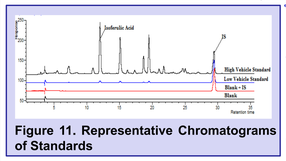

Figure 11. Representative Chromatograms of Standards

Target Concentration (mg/mL)	Batch No	Average Determined Concentration (mg/mL)	RSD	Average RE (%)
0	1	BLOQ	NA	NA
100	2	98.2	5.8	-1.8
0	3	BLOQ	NA	NA
100	4	99.0	1.1	-1.0

Table 5. Formulation Analysis Results

- BCE formulations were prepared in 0.5% MC at target concentrations of 0 and 100 mg/mL for gavage administration in support of the toxicology study.
- Formulations were analyzed using the validated analytical method prior to administration.
- The concentrations of the 100 mg/mL formulations were within 10% of target (RE%), the relative standard deviation (RSD) values were also within 10% (Table 5).
- The 0 mg/mL formulations contained no detectable BCE.

Phase III. Formulation Stability

- Stability study of BCE formulations in 0.5% MC was performed to determine the stability of known constituents isoferulic acid, actein, 27-deoxyactein, and cimiracemoside C.
- Formulations with target concentrations of 3, 30, and 100 mg/mL were prepared on six separate days and stored at 2 to 8ºC for 42, 35, 21, 14, 7, and 0 days.
- On Day 0, all formulations were analyzed by HPLC-MS detection.

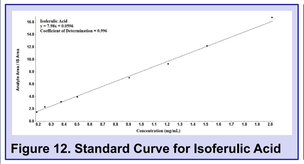

Figure 12. Standard Curve for Isoferulic Acid

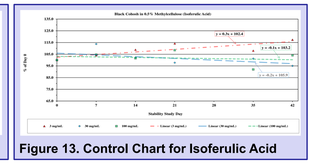

Figure 13. Control Chart for Isoferulic Acid

- Results indicated that selected known constituents of BCE are stable in formulations stored in sealed clear glass bottles in amber plastic bags for 42 days at approximately 5ºC.
- The simulated animal room study indicated no significant loss after 3 hours of dosing.

Conclusions

- Unformulated BCE bulk lots, formulated products, XRM, and potential adulterants (VBRMs), were procured
- Non-target techniques (HPLC-CAD, HPLC–UV, and PCA) were used to identify a potential test lot to support toxicology studies.
- Based on the non-targeted techniques, Lot 3012782 was most similar to BCE XRM and commonly used supplement Remifemin® .
- The lot was further authenticated by DNA barcoding and HP-TLC.
- Contaminant analysis of the test lot didn't have significant levels of metals and pesticides.
- The composition as determined by the nutritional analysis was consistent with plant material and supports an identification of BCE.
- PIXE analysis indicated that the test lot consists of 11.7% H, 69.5 % C, 15.4% O, 2.5% K, 0.3% Mg, and 0.3% Ca.
- Constituents in test lot were identified by LC-Q-TOF-MS/MS and selective constituents were quantified by standard addition (by HPLC-MS/MS).
- The lot consisted of relatively large amounts of actein, 27-deoxyactein, cimiracemocide C, and isoferulic acid, as well as containing little or no cimifugin, glucosyl cimifugin, or cimicifugoside H-1.
- Bulk stability indicated that the test lot was stable for at least 14 days at RT and below.
- Formulations were successfully prepared in 0.5% MC and were stable up to 42 days at 5ºC.
- In conclusion, BCE lot selected for testing was authenticated to be BCE. The test lot was comprehensively characterized and formulated to be used in toxicology testing in rodent models.

Acknowledgments

This work was conducted under NIEHS Contract HHSN273201400027C