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## Fukinolic Acid Derivatives and Triterpene Glycosides from Black Cohosh Inhibit CYP Isozymes, but are Not Cytotoxic to Hep-G2 Cells *In Vitro*

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### Abstract

Black cohosh (*Actaea racemosa* L. [syn. *Cimifuga racemosa* L.]) extracts (BCE) are marketed worldwide for the management of menopausal symptoms. However, recently more than 75 cases of hepatotoxicity associated with black cohosh ingestion have been reported. While these cases have not been fully substantiated for causality, the data suggest that herb-drug interactions may be involved rather than a direct hepatotoxic event. This work describes the *in vitro* inhibition of four CYP450 enzymes (1A2, 2D6, 2C9, 3A4) by black cohosh extracts and identifies the active inhibitory constituents. Ethanol extracts (75 and 80% ethanol) and a 40% isopropanol extract induced a concentration-dependent inhibition of all CYP450 isozyme activities, with median inhibitory concentrations (IC<sub>50</sub>) ranging from 21.9 µg/ml to 65.0 µg/ml. Isolation of the active chemical constituents, showed that the triterpene glycosides were weakly active (IC<sub>50</sub> 25-100 µM), while fukinolic acid and cimicifugic acids A and B strongly inhibited all CYP isozymes (IC<sub>50</sub> 1.8-12.6 µM). None of the extracts inhibited the growth of Hep-G2 cells in concentrations up to 50 µg/ml. These data suggest that BCEs are not directly hepatotoxic, but may have the potential to induce herb-drug interactions, which may in turn explain the rare cases of hepatotoxicity observed in women using multiple medications and dietary supplements, including black cohosh.

### Keywords

Black cohosh; cytochrome P450; hepatotoxicity; herb-drug interactions; menopause

### Introduction

Menopause is a universal female experience associated with disturbing symptoms such as hot flashes, night sweats, vaginal dryness, and irritability [1-5]. In addition to these acute symptoms, the incidence of chronic disease, such as cardiovascular disease, osteoporosis, and dementia are all significantly increased in the post-menopausal period [1-3]. Hormone therapy (HT; estrogen and/or progesterone) remains the gold standard for the symptomatic treatment of menopause, although since the publication of the Women's Health Initiative,

many women are searching for alternative treatments for menopause, including herbal medicines [1, 3, 5]. One herbal therapy for the management of menopausal symptoms is black cohosh, known scientifically as *Actaea racemosa* L. [3, 5-9].

Black cohosh is a coarse perennial woodland herb with large compound leaves, and a thick, knotted rhizome (root) system [3, 5]. The plant is native to North America, with a distribution from southern Canada to Georgia. There are numerous vernacular (common) names for this plant, including black snakeroot, black root, bugbane, rattle root, rattle top, rattle squawroot, and rattle-weed. Historically, black cohosh rhizomes (roots) were used as a medicine by Native Americans (Penobscot, Winnebago and Dakota), for the treatment of coughs, colds, constipation, fatigue, and rheumatism, as well as to increase breast milk production [3, 6-8]. The therapeutic use of black cohosh for these conditions has not been scientifically investigated. In 1832, a tincture of black cohosh rhizome was used for the treatment of pain and inflammation associated with endometriosis, rheumatism, neuralgia and dysmenorrhea, but again there are no clinical data to support these uses [3, 5]. More recently, extracts of black cohosh have been marketed worldwide for the management of menopausal symptoms [3-9]. The clinical efficacy of these products for the treatment of vasomotor symptoms associated with menopause has been repeatedly tested in over 15 clinical trials [reviewed in 10, 11], with positive results reported in most studies [10, 11], however the two most recent clinical trials were negative [12, 13].

Published clinical data for black cohosh shown few adverse events [3, 5-7, 10-13], including the newest trials [12, 13]. In fact, one of the most recent studies, reported no significant differences between the black cohosh treatments and placebo for any of the assessed safety parameters which included breast and endometrial safety, assessment of liver enzymes, complete blood count, or lipid profiles [12]. In particular, there was no evidence for hepatotoxicity of black cohosh during the 12-month intervention [12]. Thus, it is surprising that over the past seven years approximately 78 cases of hepatotoxicity associated with the ingestion of black cohosh containing products have been reported in the literature, as well as to regulatory agencies worldwide [8, 9]. These cases ranged in severity from minor elevations in liver enzymes to liver failure resulting in transplantation and a few deaths [8, 9]. However, as is often with dietary supplements all of the cases were poorly documented, and confounding variables included failure to identify the BC product; use of herbal mixtures with multiple ingredients in addition to BC; co-medication with synthetic drugs and dietary supplements including herbal ones; concomitant missing temporal association between BC use and development of liver disease; not specified modalities of BC treatment; failure of de-challenge after BC discontinuation; pre-existing liver diseases; insufficiently excluded other liver diseases and the possible presence of alternative liver diseases [8, 9].

Black cohosh preparations ranked eighth of all herbal supplements sold in mainstream retail outlets in 2004 [14]. However, total retail sales of black cohosh in all channels of trade are difficult to estimate, but may be as high as \$76 million (data from 2003) [14]. Considering the millions of doses of black cohosh that are used by women worldwide, the cases of hepatotoxicity are a rare occurrence, but still of significant concern. While these cases have not been fully substantiated for causality, there is a possibility that an herb-drug interaction may be involved rather than direct hepatotoxic effects due to the fact that many of these women used multiple drug and dietary supplements product and alcohol concomitantly [8, 9].

Cytochrome P450 is a superfamily of hemoproteins (heme-containing monooxygenases) of which there are approximately 50 known CYP enzymes in the human body [15-16]. Cytochrome P450 (P450 or CYP) is involved in drug metabolism, and can be divided into many subfamilies, e.g. CYP1A2, 2C9, 2C19, 2D6, 2E1, 3A4, each of which is linked to a

different set of drugs, and numerous reviews have been published on this topic [15-16]. Studies of the biochemical and enzymatic properties of these enzymes and activity have greatly enhanced the understanding of several aspects of clinical pharmacology such as pharmacokinetic variability, drug toxicity, and drug interactions. Three CYP families, namely CYP1, CYP2 and CYP3 are responsible for approximately 80% of hepatic drug metabolism, carcinogenesis and degradation of xenobiotics [16]. Thus, modulation of isozymes in all three of these subfamilies would indicate significant potential for herb-drug interactions and present the possibility for drug-induced hepatotoxicity. *In vitro* evidence has suggested that botanical dietary supplements, including black cohosh, may modulate the activity and expression of CYP isozymes [15, 17]. However, the *in vivo* findings in humans have shown weak or equivocal results [18-20].

This work reports the *in vitro* inhibition of the activities of CYP1A2, 2C9, 2D6, and 3A4 isozymes by three clinically relevant extracts of black cohosh. The active constituents responsible for these activities were identified using bioassay-guided fractionation. Furthermore, the work also assessed the effects of black cohosh extracts and pure compounds on the growth of human hepatocellular carcinoma (Hep-G2) cells *in vitro*.

## Materials and Methods

### Chemicals and Reagents

HPLC grade solvents acetonitrile and methanol (Fisher Scientific, Itasca, IL) were used for sample preparation and HPLC analysis. Reagents were obtained from Fisher Scientific and Sigma Chemical Co. (St. Louis, MO).

### Plant Materials and Extracts

Black cohosh (*Actaea racemosa* L. syn. *Cimicifuga racemosa*) roots/rhizomes were obtained from PureWorld Botanicals, Inc. (South Hackensack, NJ) and Chromadex (Santa Ana, CA). A dried 40% isopropanol extract was also prepared and dissolved in DMSO. This extract is standardized to contain 1 mg of triterpene glycosides calculated as 23-*epi*-26-deoxyactein per 20 mg of extract. For the other two extracts, the dried, milled, rhizomes of *A. racemosa* (1 kg) were exhaustively extracted with 80% or 75% ethanol, and the triterpene glycoside concentrations were determined by HPLC based on the protocols described [21, 22]. Pure actein (compound **1**) was isolated as colorless needles (MW 676, C<sub>37</sub>H<sub>56</sub>O<sub>11</sub>); 23-*epi*-26-deoxyactein (compound **2**) was also isolated as colorless needles (MW 660, C<sub>37</sub>H<sub>56</sub>O<sub>10</sub>); and cimicracemoside A (MW 632; C<sub>36</sub>H<sub>56</sub>O<sub>9</sub>) was also isolated. On the basis of <sup>1</sup>H NMR spectra and LC/MS/MS, three triterpene glycosides were confirmed as cimicracemoside A; 23-*epi*-26-deoxyactein, and actein. The fukinolic acid and the cimicifugic acids A and B were isolated and identified as previously described [23] using bioassay guided fractionation. Individual purified triterpene glycosides from black cohosh for HPLC standards were purchased from ChromaDex, Inc. (Santa Ana, CA).

### CYP Isozyme Bioassays

The extracts and pure compounds were screened for their ability to inhibit the activities of CYP1A2, 2C9, 2D6, and 3A4 isozymes using a rapid high-throughput *in vitro* fluorometric microtiter plate assay as previously described [24, 25]. The enzymes were obtained from human recombinant insect Sf9 cells and assays for the activities of four principal human cytochromes P450 metabolizing enzymes, CYP1A2, CYP2C9, CYP2D6, and CYP3A4 were performed. Two direct fluorometric assays were used. For CYP1A2, CYP2C9, and CYP2D6, 3-cyano-7-ethoxycoumarin (CEC) (White) was used as the substrate. The CEC substrate concentration for CYP2C9 was 25 μM, for CYP1A2, 5 μM was used and for CYP2C9 the CRC concentration used was 50 μM. Resorufin benzyl ether [BzRes (Burke)]

was used as the substrate for CYP3A4 at a concentration of 50  $\mu\text{M}$  [24]. The assays were conducted in 96-well microtiter plates and the substrates were prepared as homogenous suspensions in pH 7.5 buffer (potassium phosphate 75 mM) by sonication. All extracts were dissolved in either methanol or DMSO. Final solvent was less than 1%. Control wells contained vehicle solvent or sterile distilled water and NADPH ( $\alpha$ -nicotinamide adenine dinucleotide phosphate, reduced form; Sigma Chemical Co., St. Louis, MO) solution; blank wells contained distilled water or vehicle solvent and buffer solution (1:4.7 v/v mixture of 75 mM potassium phosphate in DW, pH 7.5); test wells consisted of extract or pure compound and NADPH solution. Test-blank wells consisted of the corresponding extract, pure compound and buffer solution. After substrate and inhibitor addition the plates were pre-warmed to 37°C. Incubations were initiated by the addition of 0.1 ml of pre-warmed enzyme and cofactors. Final incubation volume was 0.2 ml. The concentrations of enzymes (baculovirus/insect cell-expressed human cytochromes P450) and co-factors were as described [25]. Incubations were carried out for 30 min (CYP1A2 and CYP3A4) or 45 min (CYP2C9 and CYP2D6) and stopped by the addition of 0.1 ml of 60% acetonitrile, 40% 0.1 M Tris, pH 9. Metabolite formation was linear for these incubation times. Fluorescence per well was measured using a Synergy HT multi-detection microplate reader (Biotek, Winooski VT). The CEC metabolite, 3-cyano-7-hydroxycoumarin, was measured using an excitation wavelength of 420 nm (50-nm bandwidth) and emission wavelength of 485 nm (20-nm bandwidth). The BzRes metabolite, resorufin, was measured using an excitation wavelength of 530 nm (25-nm bandwidth) and emission wavelength of 590 nm (35-nm bandwidth). Detection of the products of either assay was linear over the range used for these assays. Data were exported and analyzed using an Excel spread sheet and IC<sub>50</sub> values were calculated by linear interpolation. For CYP1A2 the reference inhibitor compound used was furafylline, for CYP2C9 with inhibitor control was sulfaphenazole, for CYP2D6, quinidine was used and for CYP3A4 ketoconazole was the control compound. Each assay was repeated in triplicate. Median inhibitory concentrations (IC<sub>50</sub>) were only determined for extracts showing >50% inhibition. For IC<sub>50</sub> determinations, the concentration range of each extract used was 1-250  $\mu\text{g/ml}$  [25]. Extracts inducing >50% inhibition at a concentration of 50  $\mu\text{g/ml}$  were further assessed for IC<sub>50</sub>s. Only data sets yielding the highest readings without saturation were used to calculate the percent inhibition. The resultant percent inhibition calculations based on the mathematical combinations for the differences in fluorescence between the test/test-blank wells and the mean difference between each control and blank well. Control reference compounds were run with every assay and median inhibitory concentrations for the reference compounds were obtained.

## Cell Cultures

Human hepatocellular liver carcinoma cells (Hep-G2 cells (ATCC, Manassus, VA) were maintained in RPMI 1640 (Gibco BRL) containing 10% FBS (Gibco BRL), 2 mg/ml sodium bicarbonate, 1% penicillin sodium salt and 1% streptomycin sulfate [26]. All extracts and compounds were dissolved in DMSO at a concentration range of 1-100  $\mu\text{g/ml}$ , and diluted in tissue culture medium before use. Cells were plated in 96-well microassay culture plates and grown in RPMI 1640 medium with 10% FBS for 24 h prior to treatment. Tested compounds were then added to the wells to achieve final concentrations ranging from 1 to 100  $\mu\text{g/ml}$ . After treatment, all the cultures were incubated at 37°C, 5% CO<sub>2</sub> for 48 h followed by the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay as described [26].

## MTT Cytotoxicity Assay

Cells were harvested in the exponential phase from culture flask with 0.25% trypsin/0.1% EDTA (Sigma-Aldrich Chemical, St. Louis, MO). Cells were washed, centrifuged, and counted *via* hemocytometer and viability determined by 0.5% trypan blue vital dye

exclusion. Only harvested aliquots showing greater than 90% viability were used in these studies. Cells were transferred to flat-bottom 96-well microtiter plates (Costar) at a concentration of 50,000 cells per well in 0.1 ml and cultured as described above. Preliminary studies demonstrated that this concentration of cells provided appropriate confluence at the end of the assay and provided an optimal MTT absorbance value. The cells were allowed to adhere for 24 hours in 5% CO<sub>2</sub> at 37°C. Medium was then replaced and various concentrations of plant extracts were added and allowed to incubate for 72 hours. Cell viability was then determined for each concentration of extract tested by the colorimetric assay described [26]. The yellow tetrazolium salt MTT (Sigma-Aldrich Chemical, St. Louis, MO) is converted into a purple formazan product by living cells in direct proportion to cell number and activity [26]. Briefly, 5 µl of a 10 mg/ml solution of MTT in phosphate-buffered saline (0.01 mol/L of PO<sub>4</sub>, 0.85% NaCl, at pH of 7.4) was added to each well and incubated for 4 hours at 37°C. After the incubation period, 100 µL of acid isopropanol (0.04N HCl) was added to each well to dissolve the dark blue crystals. The absorbance of each well was measured by a multiple scanning spectrophotometer (Power Wave 200, Bio-Tek Instruments) at 570 nm, with a reference wavelength of 630 nm. Percent viability was determined by (absorbance of extract)/(control cells alone) × 100, where (absorbance of extract) = mean absorbance of extract wells at a given concentration and (control cells alone) = mean absorbance of control wells. Data are means from three replicate wells and three separate experiments. Microscopic inspection of wells revealed that decreased absorbance values from control wells correlated with decreased cell number and not with diminished metabolic rate [26].

### Statistical Analysis

The values for the enzyme activities were compared by analysis of variance (ANOVA) followed by Dunnett's test. Statistically significant differences were determined at  $p < 0.05$ .

### Results

All three black cohosh extracts (BCEs) inhibited the activities of the four CYP isozymes in a concentration-dependent manner (Fig. 1). The median inhibitory concentrations (IC<sub>50</sub>s) of the three BCEs ranged from 21.9 to 75 µg/ml (Table 1). The 40% isopropanol extract was slightly more active than either the 75 or 80% ethanol extracts for all CYP isozymes tested, however these results were not statistically significant. Inhibitor control compounds were within normal concentration limits for each of the CYP assays (Table 2) [27-31]. The isolated triterpene glycosides cimracemoside A, 23-*epi*-26-deoxyactein, and actein inhibited the activities of CYP2C9 and CYP3A4, with an IC<sub>50</sub> range of 16.9-33.8 µM. None of the triterpene glycosides tested inhibited CYP1A2 at concentrations up to 100 µM. Jiang *et al.* [21] reported that the total percentage of the triterpene glycosides and the phenolic constituents in a 75% ethanol and 40% isopropanol extracts of black cohosh roots and rhizomes ranged from 6.9 to 10.3% for the triterpene glycosides and from 5.2 to 5.8% for the phenolic constituents [21]. Thus, the observation that the three BCEs inhibited all four CYP isozymes similarly, may be explained by the chemistry of the extracts.

The triterpene glycosides (TGs; Fig. 2) had previously been isolated as the active constituents of black cohosh rhizomes [21, 22], and were thought to be the active constituents that inhibit the activity of CYP3A4 *in vitro*. However, the data presented here demonstrate that the TGs at best, weakly inhibit the activities of CYP isozymes, and only at higher concentrations. In addition, the TGs had no effect on CYP1A2 at concentrations up to 100 µM.

The three phenolic constituents fukinolic acid, and its derivatives cimicifugic acids A and B (Fig. 3), were significantly ( $p < 0.05$ ) more active than any of the TGs tested, and inhibited



all four CYP isozymes with an  $IC_{50}$  range of 1.8-12.6  $\mu M$ . All three of these compounds inhibited the activity of CYP 1A2 *in vitro* at very low concentrations ( $IC_{50}$  1.8-7.2  $\mu M$ ), with fukinolic acid being the most active inhibitor of CYP1A2 ( $IC_{50}$  1.8  $\mu M$ ). Interestingly, resveratrol (Fig. 4), a naturally occurring stilbene with a chemical structure similar to fukinolic acid has demonstrated similar activity with an inhibitory  $IC_{50}$  1.89  $\mu M$  in CYP 1A2 [27].

None of the extracts exhibited cytotoxicity in Hep-G2 cells up to concentrations of 50  $\mu g/ml$ , with > 90% viability at this concentration. At higher concentrations (>100  $\mu g/ml$ ) all three extracts of black cohosh inhibited cell proliferation by 89-100%.

## Discussion

In the past few years, there have been approximately 78 case reports suggesting that administration of black cohosh extracts may cause hepatotoxicity, in the form of severe acute hepatitis and fulminant liver failure [8, 9]. These case reports of hepatotoxicity are disturbing and inexplicable, as black cohosh has been used in over 2800 patients in human studies and clinical trials with a low incidence of side effects, and previously had no history of hepatotoxic effects [5-7, 8-9, 12-13]. Even the two most recent NIH-funded clinical trials report no serious adverse events [12, 13]. Previous *in vitro* testing of black cohosh extracts reported no effect on rat hepatocytes or HepG2 cells [32]. Lude *et al.* [33] demonstrated that BCEs only have a direct cytotoxic effects in HepG2 cells at concentrations of 75 mg/ml or higher. The results presented here confirm these data, as none of the extracts or pure compounds were cytotoxic in Hep-G2 cells at concentrations up to 50  $\mu g/ml$ . Furthermore, Lude *et al.* [33] also assessed microvesicular steatosis of the liver in rats treated with black cohosh and found that a very high dose of 1000 mg extract per kg body weight was needed to observe any liver toxicity. Thus, the probability that BCEs, are directly hepatotoxic appears remote, when these products are used alone in normal therapeutic doses.

However, the other possible explanation for these case reports may be due to an interaction of black cohosh extracts with the drug metabolizing CYP isozymes, especially 3A4 and 2D6. In fact, in most of the rare cases of hepatotoxicity associated with black cohosh were observed in menopausal women using black cohosh containing supplements in combination with other herbals, alcohol and/or prescription medications [8, 9]. In this work, three extracts of black cohosh inhibited the CYP isozymes 1A2, 2C9, 2D6 and 3A4 *in vitro*, in a concentration-dependent manner. Interestingly, the 40% isopropanol extract was slightly more active than the other two extracts, particularly against the 1A2 isozyme. However, these results were not statistically significant. Bioassay guided fractionation lead to the isolation of three triterpene glycosides (TGs), namely actein, 23-*epi*-26-deoxyactein and cimracemoside A. All three compounds inhibited isozymes 2C9, 2D6 and 3A4 but did not inhibit CYP1A2 in concentrations up to 100  $\mu M$ .

Previously, Tsukamoto *et al.*, [32] had identified six TGs, including 26-deoxyactein, as inhibitors of CYP3A4. The reported  $IC_{50}$  for this compound was 100  $\mu M$ , although the  $IC_{50}$  for their black cohosh extract was 27  $\mu M$ , suggesting that 26-deoxyactein was not the active constituent [32]. The data from this work support the results of Tsukamoto *et al.* [32], as the  $IC_{50}$ s for the three black cohosh extracts tested ranged from 35-48  $\mu g/ml$ . Furthermore, we have identified 23-*epi*-26-deoxyactein (Fig. 2), an isomer of 26-deoxyactein [22], that differs in structure only in the stereochemistry at C 23 position (Fig. 2), as the most active TG, with an  $IC_{50}$  28.7  $\mu M$ , which is similar to the activity of the extract. The average daily dose of a standardized black cohosh extract is 40-80 mg, and the total triterpene glycoside concentration of the extracts ranges between 2.5-10%. Thus, even at the highest concentration of 10%, a daily dose of 80 mg would deliver only 8 mg of total triterpene

glycosides, and after absorption and distribution in the body, it would be difficult to achieve sufficiently high enough concentrations of TGs to inhibit the CYP isozymes. A daily dose of 25 to 50 times higher would be needed to achieve concentrations of TGs sufficient to inhibit the activities of the CYP isozymes, if this would even be possible.

Our results further demonstrate that the phenolic constituents fukinolic acid, cimicifugic acids A and B are significantly more active than the TGs against all CYP isozymes tested, and particularly inhibited CYP1A2 at very low concentrations indicating that they are more likely to be the CYP inhibitors of black cohosh extracts. Fukinolic acid and cimicifugic acids A and B were isolated from an *n*-butanol soluble fraction of a black cohosh extracts that exhibited significant binding to the serotonin receptor 7 [34], and it has been suggested that black cohosh extracts may have a serotonergic mechanism of action [35]. It is well known that modulators of the serotonin system such as some serotonin reuptake inhibitors (SSRIs), as well as serotonin and norepinephrine reuptake inhibitors (SNRIs) inhibit CYP isozymes, including CYP3A4, 1A2 and 2D6 [36]. Thus, it is possible that fukinolic acid and cimicifugic acids A and B may inhibit the CYP isozyme activities with a mechanism similar to these drugs.

While black cohosh extracts appear to inhibit the CYP isozymes *in vitro*, corroboration of the *in vivo* results have been equivocal in healthy young human subjects [18-20]. Gurley and co-workers have performed extensive *in vivo* studies addressing the effects of various dietary supplements on CYP activities in humans [18-20]. Oral administration of a black cohosh root extract (1090 mg twice daily standardized to 0.2% triterpene glycosides) to 12 young healthy volunteers for 28 days did not produce significant changes in phenotypic markers of CYP1A2, CYP2D6, and CYP3A4, however a small (~7%) but significant inhibition of CYP2D6 was observed [18]. In the second study, involving 16 healthy young subjects (8 female), oral administration of 40 mg twice daily of a black cohosh extract (standardized to containing 0.2% triterpene glycosides) for 14 days exerted no significant effects on CYP2D6 activity [20]. No clinically relevant effect on CYP3A4 activity was observed in 19 healthy young subjects (9 females) administered an isopropanol extract of black cohosh standardized to 2.5% triterpene glycosides at a daily dose of 80 mg for 14 days [19]. Thus, in healthy young subjects of both sexes, the impact of 14-28 days of therapeutic doses of black cohosh extracts for 14-28 days on CYP isozymes appears to be minimal.

However, the adverse events reports associating the ingestion of black cohosh containing supplements with hepatotoxicity have been observed in women of peri- to post-menopausal age (39-72 years old) [8, 9]. Many of the affected women used the black cohosh containing products in therapeutic doses for longer than one month; many had multiple chronic diseases, and used multiple medications, as well as alcohol concomitantly [8, 9]. Drug-induced hepatotoxicity is most frequently observed in older women, with more than 80% of cases in women with an average age of 45-60 years [37]. The varied manifestations of liver injury include steatosis, acute and chronic hepatitis, hepatic fibrosis, zonal or diffuse hepatic necrosis, bile duct injury, veno-occlusive disease, and acute liver failure requiring liver transplantation [8, 9, 38]. Therefore, while the human data for black cohosh shows equivocal or no effect in healthy young subjects (including women), no studies have investigated the effects of black cohosh on CYP-450 isozymes in older menopausal women. It is recognized that the activities of human CYP-450 isozymes may be affected by gender, and hormonal factors such as the menopausal [39-41]. Thus, investigations using healthy subjects while informative do not reflect the true clinical situation in menopausal women with multiple pathologies using multiple prescription medications, supplements and alcohol. Thus, it would be beneficial to test black cohosh supplements in this specific population to determine effects on CYP450.

Black cohosh is an herbal medicine that has been used in the United States for over 100 years and is currently being used by women worldwide for the management of menopausal symptoms. The ever-expanding case reports of hepatotoxicity associated with BCEs even when used in recommended doses are inexplicable, but need to be seriously addressed considering the number of women using these products. Our data suggest that compounds in BCEs may inhibit CYP450 isozymes, although the concentration of the compounds needed to inhibit CYP isozymes *in vivo* would be 25 to 50 times a normal daily dose. Extrapolation of *in vitro* data to determine the safety of an herbal product based is limited as the bioavailability and distribution of the isolated compounds in the body is currently not well understood. Data suggesting that BCEs inhibit the proliferation of Hep-G2 cells at higher concentrations may imply that users of BCEs exposed to common hepatotoxins such as alcohol and medications deleterious hepatotoxic effects may not be able to repair any hepatic injury, thereby exacerbating any limited hepatotoxicity. Therefore, new human studies involving menopausal women using normal standards of care, clinically relevant BCEs, and perhaps in combination with other drugs or alcohol would be clinically significant.

As part of a previously published review [8], the United the United States Dietary Supplement Information Expert Committee has recommended that black cohosh products should be labeled to include a cautionary statement [8]. The cautionary statement: “Discontinue use and consult a healthcare practitioner if you have a liver disorder or develop symptoms of liver trouble, such as abdominal pain, dark urine, or jaundice”, was put in place to alert consumers and healthcare professionals to pay close attention to minimize potential risk [8].

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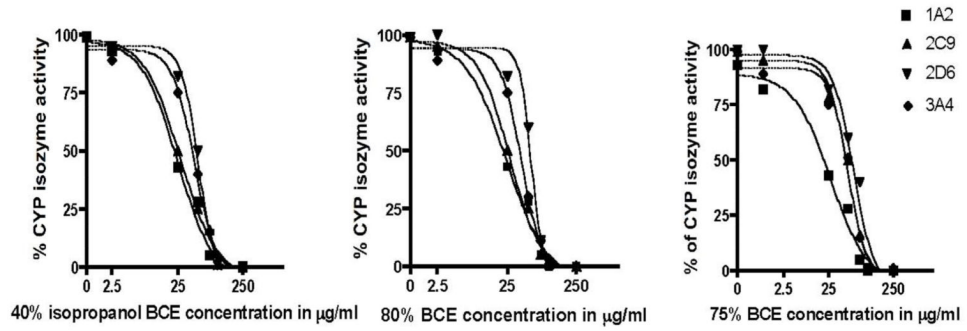
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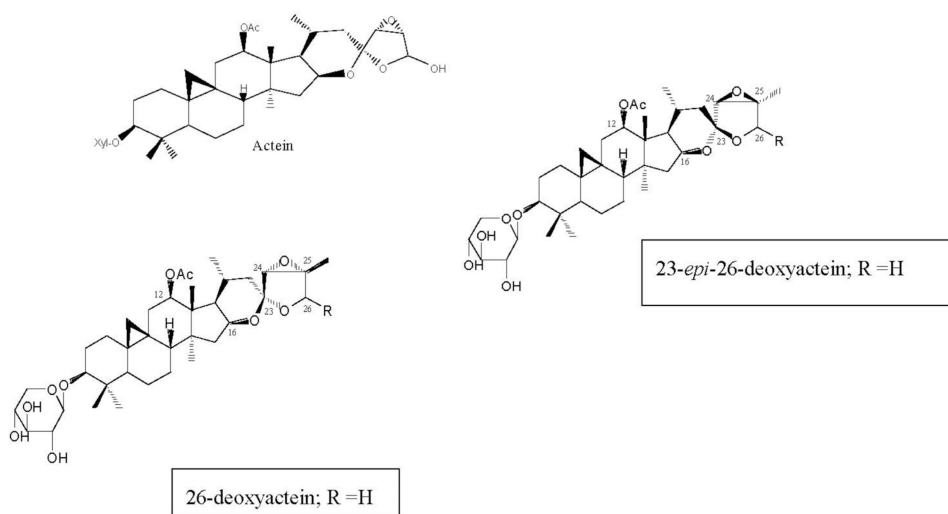


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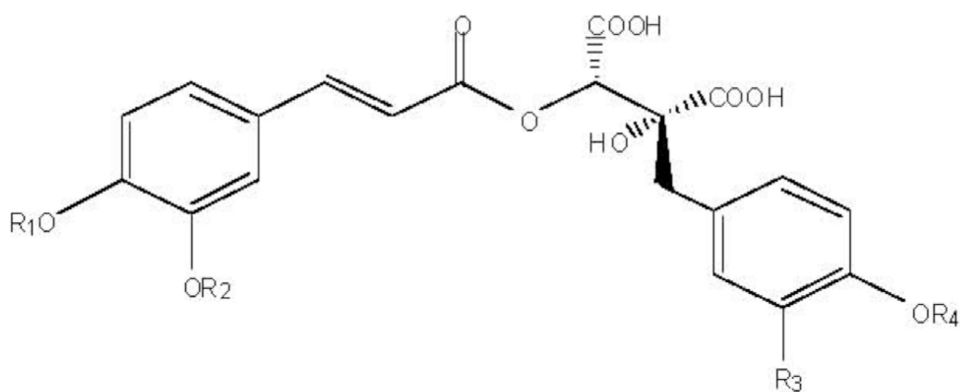
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**Fig. 1.** Concentration-dependent inhibition of CYP1A2, 2D6, 2C9 and 3A4 by a 40% isopropanol, 75% and 80% ethanol extracts of black cohosh rhizomes.



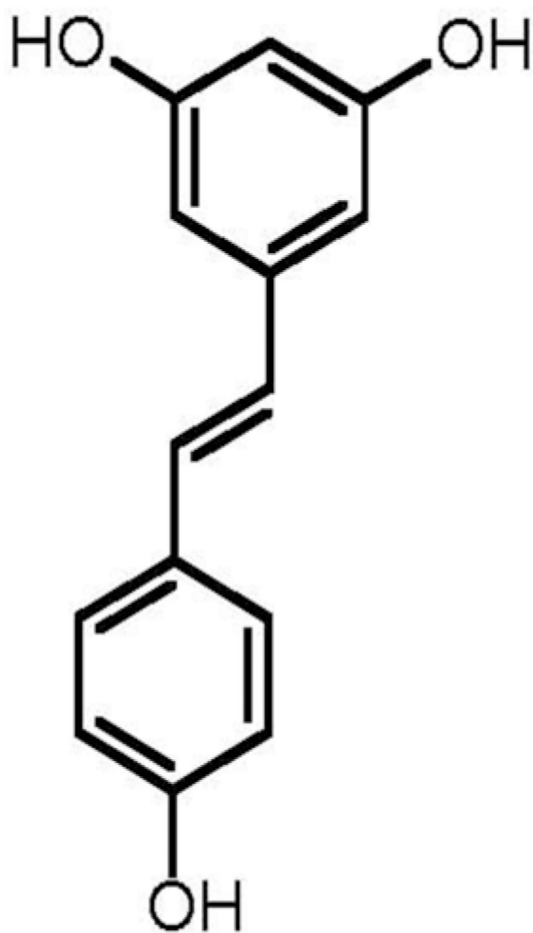
**Fig. 2.** Chemical structures of the triterpene glycosides isolated from black cohosh root extracts with inhibitory activities in CYP 2D6, 2C9 and 3A4 bioassays.



Cimicifugic acid A:  $R_1 = \text{H}$ ,  $R_2 = \text{Me}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{H}$   
 Cimicifugic acid B:  $R_1 = \text{Me}$ ,  $R_2 = \text{H}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{H}$   
 Fukinolic acid:  $R_1 = R_2 = \text{H}$ ,  $R_3 = \text{OH}$ ,  $R_4 = \text{H}$

**Fig. 3.** Chemical structures of fukinolic acid and cimicifugic acids A and B isolated from black cohosh root extracts with inhibitory activities in CYP 1A2, 2D6, 2C9 and 3A4 bioassays.





**Fig. 4.**  
Resveratrol, a naturally occurring stilbene from red wine.

**Table 1**  
**Median Inhibitory Concentrations (IC<sub>50</sub>) for Human Recombinant CYPs for Black Cohosh (BC) Extracts (μg/ml) and Pure Compounds (μM)**

Plant Extract	CYP1A2	CYP3A4	CYP2C9	CYP2D6
BC-80% ethanol	31.4	45.8	35.5	75.0
BC-75% ethanol	38.8	48.1	50.0	76.7
BC-40% isopropanol	21.2	35.3	23.6	48.2
CR-A <sup>1</sup>	na	51.3	40.5	32.2
Actein	na	35.2	25.0	51.1
Deoxyactein <sup>2</sup>	na	28.7	25.9	100.0
Fukinolic acid	1.8	7.2	7.1	5.4
CAA <sup>3</sup>	7.2	9.7	8.3	9.0
CAB <sup>4</sup>	7.35	9.8	12.5	12.6

<sup>1</sup>Cimiracemoside A.

<sup>2</sup>Deoxyactein = 23-epi-26-deoxyactein.

<sup>3</sup>CAA = Cimicifugic acid A.

<sup>4</sup>CAB = Cimicifugic acid B.

na: not active at concentrations of 1-100 μg/ml; each IC<sub>50</sub> concentration is the result of three experiments in triplicate.

**Table 2**  
**Median Inhibitory Concentrations of Reference Inhibitor Compounds for CYP 1A2, 2C9, 2D6, and 3A4 Isozyme Assays**

CYP Isozyme	Reference Compound	IC <sub>50</sub> $\mu$ M	S.D.	Literature
1A2	Furafylline	0.96	$\pm$ 0.06	0.5-6.0 $\mu$ M [27]
2C9	Sulfaphenazole	0.31	$\pm$ 0.04	0.14-0.7 $\mu$ M [28]
2D6	Quindine	0.04	$\pm$ 0.002	20-150 nM [29, 30]
3A4	Ketoconazole	0.036	$\pm$ 0.004	0.07-8.5 $\mu$ M [31]

For CYP 1A2 the reference inhibitor compound used was furafylline, for CYP 2C9 with inhibitor control was sulfaphenazole, for CYP 2D6, quindine was used and for CYP 3A4 ketoconazole was the control compound. The IC<sub>50</sub> obtained for each of the control compounds (known inhibitors) were within the limits published in the scientific literature. Each assay was repeated in triplicate.