POLYPHENOLIC CONSTITUENTS FROM BLACK COHOSH (*ACTAEA RACEMOSA*) AND RELATED SPECIES

by

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Abstract

POLYPHENOLIC CONSTITUENTS FROM BLACK COHOSH (*ACTAEA RACEMOSA*) AND RELATED SPECIES

by

Paiboon Nuntanakorn

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Black cohosh (*Actaea racemosa* L.) is an important botanical dietary supplement for the treatment of menopausal symptoms in women. Despite the documented clinical efficacy of black cohosh, little is known of its active compounds. The phytochemistry of *A. racemosa*, as well as the other three closely related species, *A. pachypoda, A. podocarpa*, and *A. rubra*, was investigated.

Activity-guided fractionation of a standardized black cohosh powdered extract resulted in the isolation of two novel polyphenolic compounds, (3*S*)-2-(3″,4″-dihydroxyphenylmethylene)-3-hydroxy-3-(3′,4′-dihydroxybenzoyl)-γ–butyrolactone (**1**) (2*R*,3*S*)-2-*O*-(3′,4′-dimethoxy-*E*-cinnamoyl)-3-hydroxy-3-[(3″,4″-dihydroxyphenyl) methyl]-butanedioic acid (**2**), together with fifteen known polyphenols, namely protocatechuic acid, protocatechualdehyde, *p*-coumaric acid, caffeic acid, methyl caffeate, ferulic acid, ferulate-1-methyl-ester, isoferulic acid, 1-isoferuloyl-β-D-glucopyranoside, fukinolic acid, cimicifugic acids A, B, D, E, and F. Compounds **1** and **2** displayed high antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free-radical assay with IC_{50} values of 26.94 and 37.40 μM, respectively. Also, compounds **1** and **2** were able to stimulate MCF-7 cell proliferation of 1.24-fold (14 μM) and 1.14-fold

(10 μM), respectively, when compared with the untreated cells. The fifteen known polyphenolic compounds were tested in the DPPH assay and displayed activities ranging from IC_{50} values of 12.91 to 321.41 μM. Fukinolic acid had the highest antioxidant activity, while *p*-coumaric acid had the lowest activity.

A reversed-phase high-performance liquid chromatography (RP-HPLC) with diode array detection (DAD) method was developed and validated to quantify eight major polyphenols in the rhizomes and roots of four North American *Actaea* species, *A. pachypoda, A. podocarpa*, *A. racemosa*, and *A. rubra*. This validated method allowed for the identification and quantification of caffeic acid, ferulic acid, isoferulic acid, fukinolic acid, cimicifugic acids A, B, E, and F. The highest levels of the eight measured polyphenols were found in *A. rubra*, the second highest in *A. podocarpa*, the third highest in *A. racemosa,* and the lowest in *A. pachypoda*. The polyphenol levels corresponded with the antioxidant activities determined by the DPPH assay.

In summary, four North American *Actaea* species were analyzed for their phytochemical profiles with a new validated HPLC-DAD method, and two novel and fifteen polyphenolic compounds were identified and isolated from *A. racemosa* roots and rhizomes.

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Introduction

1.1. Antioxidants and Human Health

Reactive oxygen species (ROS), including the hydroxyl radical ($HO^{\bullet}$), superoxide anion radical ($O_2^{\bullet-}$), peroxyl radical ($ROO^{\bullet}$), peroxynitrite ($ONOO^-$), alkoxyl ($RO^{\bullet}$), nitric oxide ($NO^{\bullet}$), and hydrogen peroxide (H_2O_2), are highly reactive free radicals produced during normal cellular respiration by activated leucocytes as part of the immune system. They are also introduced to the body by exogenous oxidants such as air pollution and cigarette smoke.[1] In living cells, these free radicals can attack all classes of biomolecules, including lipids, proteins, and nucleic acids. Oxidation-induced attack causes membrane damage, protein modification, and DNA damage. This oxidative damage is believed to play a major causative role in aging and in the pathogenesis of numerous degenerative and chronic diseases such as coronary heart disease (CHD), atherosclerosis, cancer,[2] Alzheimer's disease,[3] and rheumatoid arthritis.[4]

A well-known example of an oxidation product leading to disease is oxidized low density lipoprotein (LDL), which is a major factor in the promotion of CHD and atherosclerosis.[5-7] To protect themselves from the injurious effects of ROS, cells have developed a wide range of defense mechanisms to limit the production of ROS.[8] For example, superoxide dismutase (SOD) enzymes convert superoxide anion to hydrogen peroxide and oxygen, and catalase (CAT) enzymes mediate the breakdown of hydrogen peroxide to water and oxygen. Non-enzymatic endogenous antioxidant molecules include glutathione, bilirubin, and melatonin.[9] However, oxidative stress may occur when the balance between ROS production and defense mechanisms is disrupted, or as a result of accumulated long-term damage done by ROS. Thus, additional dietary antioxidants are

needed for diminishing the cummulative effects of oxidative damage which occur over a lifespan.[1]

Antioxidants scavenge free radicals, and can therefore be effective in preventing or suppressing disorders generated by oxidative stress. Many clinical and epidemiological studies have shown the beneficial effects of vitamin antioxidants (e.g. vitamins A, C, E, and β-carotene) in the prevention of CHD,[10,11] cancer,[12,13] and cataracts.[14]

1.2. Phytochemical Antioxidants

Plants produce a variety of antioxidants in their seeds, fruits, leaves, and roots. Beside vitamin antioxidants, the major class of plant-derived antioxidants are polyphenolic compounds, including phenolic acids, phenylpropanoids, and flavonoids.[15] Natural polyphenols occur primarily in conjugated form, with one or more sugar residues linked to hydroxyl groups.[16]

The flavonoids are a large class of compounds, ubiquitous in plants. They are important for plant normal growth, development, and defense.[17] The basic flavonoid structure is the flavan nucleus, which consists of fifteen carbon atoms arranged in three rings (C6-C3-C6), labeled as A, B and C (Table 1.1). There are over 4,000 naturally occurring flavonoids in several subclasses.[18] Among the flavonoids, flavones (e.g. apigenin, luteolin), flavonols (e.g. quercetin, myricetin, kaempferol), flavanones (e.g. hesperidine, naringenin), isoflavones (e.g. genistein, daidzeine, biochanin A, formononetin), flavan-3-ols (e.g. catechin, epicatechin, epigallocatechin), and anthocyanidins (e.g. delphidin, cyanidin) are commonly found in fruits and vegetables, as well as in beverages such as tea and red wine (Table 1.1).

Flavonoids have been reported to elicit anticancer, antibacterial, antiplatelet, antiischemic, antiallergic, and anti-inflammatory activities.[19-25] Along with these activities, flavonoids have also been shown to inhibit the activities of several oxidative enzymes, including lipoxygenase and cyclooxygenase, monooxygenases, xanthine oxidase, NADH-oxidase, phospholipase A_2, and protein kinases.[26-31] The biological activities of flavonoids are generally thought to be the result of their antioxidant properties.[32]

The antioxidant activity of flavonoids varies considerably due to their different backbone structures and functional groups.[15,18,33] Increases in the antioxidant activity among flavonoids have been attributed to three functional groups: *i*) the presence of an *ortho*-3′,4′-dihydroxy moiety in the B ring, as in catechin, luteolin, and quercetin, which confers higher stability to the radical form and participates in electron delocalization; *ii*) the presence of a 2,3-double bond in conjugation with the 4-oxo function in the C ring, which is responsible for electron delocalization in the B ring (e.g. quercetin); and *iii*) the additional presence of a 3,5-dihydroxy moiety with 4-oxo function in the A and C rings are required for maximum radical scavenging potential, such as in quercetin. Thus, flavonols and flavones containing an *ortho*-3′,4′-dihydroxy group in the B ring are highly active, with flavonols being more potent than the corresponding flavones due to the presence of the 3-hydroxl group.[33] Because of their structural similarity to estrogen, some flavonoids are able to bind to the estrogen receptor and possess estrogenic or antiestrogenic activity.[34,35] The phenolic hydroxyl group in position 4′ in ring B and 7 in ring A are essential for the estrogen-like activity of flavonoids.[36]

Phenylpropanoid derivatives with C6-C3 structures, including hydroxycinnamic acids, phenylpropenes, coumarins, and chromones, are an important group of low-molecular-weight polyphenols.[37] The typical phenylpropanoids are hydroxycinnamic acid derivatives (e.g. caffeic acid, ferulic acid, and chlorogenic acid), which are widely distributed in the plant kingdom. Found in plant foods such as fruits, vegetables, cereals, and beverages, they generally occur as esters of organic acids, as glycosides, or bound to cell wall polymers.[38] The hydroxycinnamic acids have been reported to exhibit antibacterial, antiinflammatory, antiatherosclerotic, anticancer, antioxidant and neuro-protective properties.[38-44] These properties are associated with natural antioxidants which possess multiple activities involving free radical scavenging, as well as inhibitory actions on specific enzymes that induce the formation of free radicals and lipid hydroperoxides.[45] Antioxidant activity is governed by chemical structure; the activity increases as the number of hydroxyl groups increases on the benzene rings. The *ortho* substitution of an electron donor moiety, such as a methoxy group, increases the stability of the phenoxyl radical and improves the activity.[46,47] The structures of certain common flavonoids and phenylpropanoids are shown in Table 1.1.

1.3. DPPH Free-Radical Scavenging Assay

Numerous methods have been used to evaluate antioxidant activities of natural compounds in plant extract or biological system with varying results.[38,48,49] Among the methods that have been developed to estimate antioxidant activities, the assay based on the scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH) is quick, simple, inexpensive, and reproducible. The DPPH method is widely used to determine antioxidant activity of

pure compounds as well as natural plant extracts.[46,50-53] The results obtained in this assay are in general agreement with those derived by lipid peroxidation assay in bulk oils.[54,55]

DPPH is a stable free radical, and a good indicator of radical scavenging capacity. It has an absorption peak at 515 nm, which is lost when reduced by antioxidants (Figure 1.1). To evaluate the antioxidant activity of specific compounds or extracts, they are allowed to react with an ethanolic solution of DPPH (purple color). After reacting with antioxidant compounds, DPPH is reduced to 1,1-diphenyl-2-picrylhydrazine, and; is color changes to yellow. Antioxidant activity is measured through monitoring the decrease in absorbance at 515 nm. The antioxidant activity is calculated from a calibration curve of DPPH absorbance at 515 nm vs. the concentration of the test compound reacted with DPPH.

1.4. North American *Actaea* Species

The genus *Actaea* (Ranunculaceae) is comprised of twenty-eight species of herbaceous perennial plants of Northern temperate distribution. The entire genus *Cimicifuga* was reclassified into the genus *Actaea* in 1998 after extensive morphological and genomic DNA sequence studies.[56] Most species are more than one meter tall with dense elongate racemes or panicles of multistaminate actinomorphic flowers. The leaves of all species are large, compound, with three, nine or many leaflets. All species are found in woodlands.[57] There are nineteen species of *Actaea* in Eastern Asia, eight in North America and one in Europe. Of the North American species, five are of eastern distribution, and three are of western distribution. The western North American species appear rarely in the ethnobotanical literature.[58] The North American species are summarized in Table 1.2. Northeastern American species include *Actaea podocarpa* DC.

(syn. *C. americana* Michx.), *A. cordifolia* DC. (syn. *C. rubifolia* Kearney), *A. racemosa* L. (syn. *C. racemosa* (L.) Nutt.), *A. pachypoda* Ell. (syn. *A. alba* (L.) Mill.), and *A. rubra* (Ait) Willd. (syn. *A. arguta* Nutt.). They share many morphological similarities with only minor differences. For example, white cohosh (*A. pachypoda* Ell.) and red cohosh (*A. rubra* (Ait) Willd.) are genetically and morphologically similar, differing only by pedicel thickness after anthesis, and sometimes in the color of their berries.[56] Both have fleshy berries while black cohosh has dried follicles. American bugbane (*A. podocarpa* DC.) differs from black cohosh only in that it has three ovaries per flower and black cohosh (*A. racemosa* L.) has a single ovary per flower.[58] Black cohosh is the most commonly known American species.

The rhizomes and roots of black cohosh have been used by Native Americans for a variety of ailments, including malaise, gynecological disorders, diarrhea, sore throat, and rheumatism.[59] In Europe, it has been used for the treatment of menopausal symptoms for over 40 years. Numerous clinical trials have indicated that black cohosh preparations have a beneficial effect on the treatment of menopause.[60,61] Black cohosh is best known in the United States and Europe as a potent alternative herbal medicine for the treatment of menopausal symptoms, and there has been a dramatic increase in consumption. In 1998, for example, the black cohosh herbal product sales grew 511% increase over 1997 sales.[62] Though black cohosh is grown on a small scale for landscaping purposes, commercial-scale cultivation is limited. Most of the supply for the medicinal markets is collected from the wild, where its range overlaps with closely related *Actaea* species, such as *A. pachypoda*, *A. rubra*, and *A. podocarpa*.[58] If collectors misidentify the plants, this mistake could lead to serious consequences including altering therapeutic effects, and

the risk of toxicity such as gastroenteritis. Populations of black cohosh have declined or disappeared in some states due to high collection pressure,[63] and some black cohosh product companies have been looking for an alternative plant source, including a related Asian *Actaea* species, *A. cimicifuga* L. (syn. *Cimicifuga foetida* L.). Thus, it is likely that the black cohosh products on the market may contain related species, although they claim to be solely comprised of black cohosh. Since the mechanism of action of black cohosh is not fully understood, more studies must be done. Chemical investigations of this plant may help to expedite these studies.

This research has three major aims. The first one is to investigate polyphenolic compounds in black cohosh. The next aim is to test the activity of isolated compounds through the DPPH and the MCF-7 cell proliferation assays. The final purpose is to develop and validate an analytical method for identification and quantification of polyphenols in *Actaea* species. This study should provide a better understanding of phytochemicals in black cohosh, the primary chemical profiles among the North American *Actaea* species, as well as an analytical method for quality control purposes for black cohosh extracts.

Table 1.1. Chemical structures of certain common flavonoids and phenylpropanoids

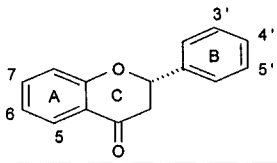
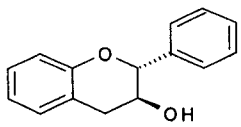
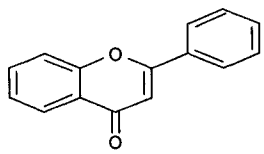
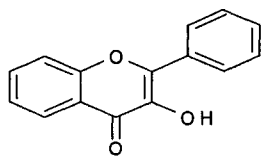
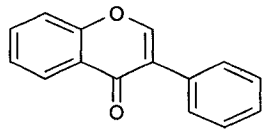
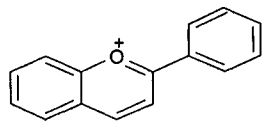
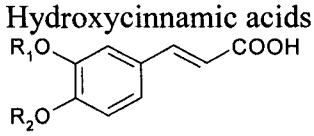
Structural formula	Representative compounds	Substitutions 5 7 3' 4' 5'
Flavanone 	Hesperidin Naringenin	OH OH OH OMe H OH OH H OH H
Flavan-3-ol 	Catechin Gallocatechin	OH OH OH OH H OH OH OH OH OH
Flavone 	Apigenin Luteolin	OH OH H OH H OH OH OH OH H
Flavonol 	Kaempferol Quercetin	OH OH H OH H OH OH OH OH H
Isoflavone 	Daidzein Genistein Formononetin	H OH H OH H OH OH H OH H H OH H OMe H
Anthocyanidin 	Delphinidin Cyanidin	OH OH OH OH OH (3=OH) OH OH OH OH H (3=OH)
Hydroxycinnamic acids 	Caffeic acid Ferulic acid	$R_1 = R_2 = H$ $R_1 = Me, R_2 = H$

Table 1.2. North American *Actaea* species

Current Name	Synonym	Common name	Distribution
Actaea podocarpa DC.	*Cimicifuga americana* Michx.	American bugbane	Georgia, Kentucky, Maryland, North Carolina, Pennsylvania, South Carolina, Tennessee, Virginia, West Virginia
A. arizonica (S. Watson) J. Compton	*C. arizonica* S. Watson	Arizona bugbane	Arizona
A. elata (Nutt.) Prantl	*C. elata* Nutt.	Tall bugbane	British Columbia, Oregon, Washington
A. laciniata (S. Watson) J. Compton	*C. laciniata* S. Watson	Mount Hood bugbane, cut-leave bugbane	Oregon, Washington
A. cordifolia DC.	*C. rubifolia* Kearney	Appalachian bugbane	Illinois, North Carolina, Pennsylvania, Tennessee, Virginia
A. racemosa L.	*C. racemosa* (L.) Nutt.	Black cohosh, black snakeroot	Northeastern America
A. pachypoda Ell.	*A. alba* (L.) Mill.	Doll's eye, white cohosh, white baneberry	Central to Eastern America
A. rubra (Ait) Willd.	*A. arguta* Nutt.	Red baneberry, Snakeberry	Central and Northeastern America

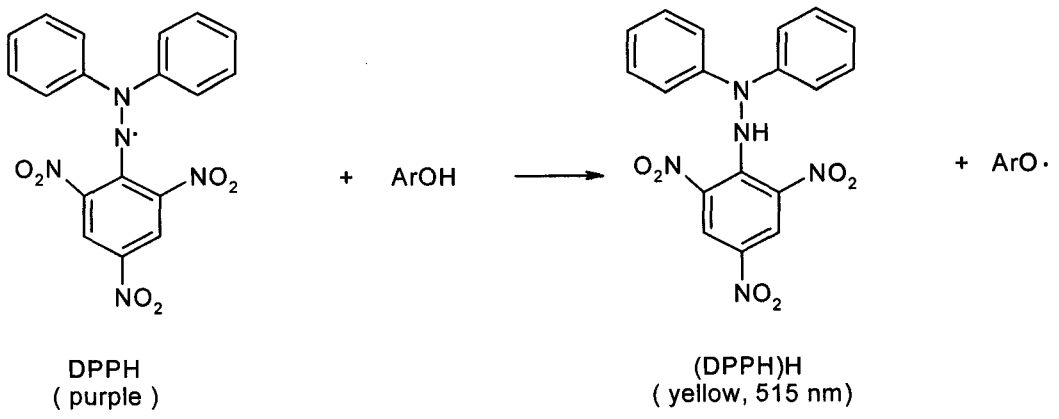


Figure 1.1. Scavenging of DPPH free radical by an antioxidant (ArOH)

Chapter 2

Black cohosh: A Review

2.1. Introduction

Botanical dietary supplements, also called botanical nutraceuticals or herbals, can be defined as plant-derived materials with medicinal benefits aimed at disease prevention or treatment. The market of botanical dietary supplements in the United States had a period of explosive growth during the mid 1990's.[64] The demand for dietary supplements is driven by a variety of factors including a growing trend to self-medicate, mistrust in conventional synthetic medicine, and the perception that natural is healthy, and that plant products are safe.[65]

Many women who reach menopause experience one or more symptoms such as hot flashes, dysmenorrhea, depression, mood swings, headache, vertigo, sleeping disorders, vaginal dryness, and joint pain. Currently, hormone-replacement therapy (HRT) with estrogens alone or combined with progesterone is the most common pharmacological treatment for relief of menopausal symptoms. However, HRT has unpleasant side effects such as irregular bleeding and promotion of endometrial or breast cancer.[66] Thus, HRT is not recommended for women with high breast cancer risk or for breast cancer patients. This problem has led to an increased interest in alternative methods of relief. One of the alternative methods is the consumption of over-the-counter botanical dietary supplements. In the year 2000 the retail sales of botanical dietary supplements in the United States surpassed $15 billion, with sales of products for menopause accounting for approximately $600 million.[67] One of the herbal products for menopause is the black cohosh, ranked nineth among all herbal preparations in U.S. sales in 2002.[68]

Numerous studies have shown the beneficial effect of black cohosh extract on the treatment of menopausal symptoms.[60,69,70] Black cohosh extracts have exhibited various biological activities including anticancer,[71-73] anti-inflammatory,[74] and antioxidant.[75] A mechanism of action of black cohosh has not been fully determined, but it is believed to be the result of complex synergistic action of its components.[61] A considerable amount of work has been conducted on the potential of black cohosh extract to display estrogenic activity. Early studies in animal studies and estrogen receptor binding assays showed the estrogenic-like effect of black cohosh extract.[76,77] Investigators also reported the isolation of the estrogenic isoflavone formononetin from black cohosh extract,[77,78] and proposed that black cohosh acts through an estrogenic pathway. However, this hypothesis has not been supported by other authors who have not observed estrogenic activity of black cohosh extracts,[79-81] and the contradictory evidence of the presence of formononetin.[82-84] Some investigators have proposed that the action of black cohosh with respect to menopausal symptoms may involve the central nervous system, possibly the serotonergic pathway. Burdette *et al.* reported that the black cohosh extract can bond to the serotonin receptors.[85] Thus, further research is needed to determine the mechanism of action of black cohosh extracts in the alleviation of menopausal hot flashes.

2.2. Classification and Nomenclature

Black cohosh [*Actaea racemosa* L. (synonym: *Cimicifuga racemosa*)] is a native North American plant in the buttercup family (Ranunculaceae) that is also known as black snakeroot, squawroot, rattleroot, rattleweed, bugbane, or cohosh. Black cohosh was first classified by Linnaeus as *Actaea racemosa* L., and published in *Species Plantarum* (1753). It was later reclassified by Pursh in the genus *Cimicifuga* after a temporary

placement in the genus *Macrotys*.[58] Recently, however, the entire genus *Cimicifuga* was placed back into *Actaea* after extensive morphological and genomic DNA sequence studies by Compton and colleagues in 1998.[56] Thus, black cohosh is, once again, *Actaea racemosa* L. the name first used more than two centuries ago.

2.3. Botanical Description

Black cohosh grows in shady, rich soil in woods from Maine to Ontario and Wisconsin, and south to Georgia.[86] Black cohosh is an erect, smooth-stemmed perennial, growing from 1-3 meters in height. The large, alternate, tri-pinnately compound tooth-edged leaves are borne on short, clasping petioles. The terminal leaflet is three-lobed. The long, wand-like, white flowers are borne on a terminal branching spike-like raceme. The main feature of the flowers is their numerous showy stamens, consisting of slender filaments with white anthers. Four or five white, small concave sepals are larger than the nearly inconspicuous, stamen-like petals. It begins blooming in May in the southern part of its range, continuing to flower into September in more northern regions.[87] The fruit is a dry, oval, ribbed follicle splitting along a ventral suture with eight to ten triangular brown seeds in two rows.

2.4. Use of Black Cohosh

The use of black cohosh has a long tradition. Native Americans used the rhizomes and roots to treat a variety of ailments, including malaise, kidney disorders, gynecological disorder, diarrhea, sore throat, and rheumatism.[59] It was also used by eclectic physicians in the United States for relief of pain associated with acute rheumatism, inflammatory condition, chorea, and neuralgia, as well as conditions that affect women's reproductive organs including menstrual problems, infertility, and relief

of labor pains. Black cohosh was an official drug in the *U.S. Pharmacopeia* from 1820-1926.[88] Black cohosh was introduced in Germany in the late nineteenth century, as a natural hormonal agent for treating premenstrually, dysmenorheically, and menopasually caused neurovegetative symptoms. In 1989, the Commission E, an expert panel charged by the German government to address herbal products, approved black cohosh as a non-prescription medicine for the treatment of climacteric ailments such as hot flashes, heart palpitations, nervousness, vertigo, sleep disturbance, and depression. Black cohosh preparations have become increasingly popular as dietary supplements in the US and Europe during the past several decades. A variety of black cohosh preparations, including isopropanolic and ethanolic extracts, are widely available. In 1996, nearly 10 million retail units of black cohosh preparations were sold monthly in Germany, Australia, and the United States.[68]

2.5. Chemical Constituents

The phytochemical constituents of the Asian *Actaea* species have been well studied. *A. simplex* (DC) Wormsk. (*Cimicifuga simplex* (DC.) Wormsk.), *A. dahurica* Franch (*C. dahurica* Maxim.), *A. cimicifuga* L. (*C. foetida* L.), and *A. heracleifolia* (Kom.) J. Compton (*C. heracleifolia* Kom.) have been used as anti-inflammatory, antipyretic and analgesic agents in traditional Japanese and Chinese medicine.[89] Triterpenoid glycosides, phenylpropanoid derivatives, and carbazone alkaloids appear to be characteristic of these plants.[90,91] An increasing interest in black cohosh has led a number of phytochemists and natural product researchers to investigate its phytochemical properties.[92-97] The chemical constituents of black cohosh can be classified into three groups: triterpenoid glycosides, phenylpropanoid derivatives, and flavonoids.

A) **Triterpenoid glycosides**: These compounds are the main phytochemical constituents in the rhizomes and roots of black cohosh. Highly oxygenated 9,19-cyclolanostane-type triterpenoids, linked with a monosaccharide unit, such as xylopyranoside or arabinoside, are commonly found in plants of the genus *Actaea*. To date, more than 43 triterpenoid glycosides have been isolated from the rhizomes and roots of black cohosh (Table 2.1). According to Kusano *et al.*,[90] triterpenoid glycosides in black cohosh can be classified into 6 subclasses, based on the chemical structures of their aglycone skeleton. Besides triterpenoid glycoside, the aglycone moieties of cimicifugenin type compounds such as actein, deaceylactein, and 23-epi-26-deoxyactein have been reported as acetylacteol, acteol, and 27-deoxyacetylacteol, respectively.

B) **Phenylpropanoid derivatives**: The majority of the phenylpropanoids in black cohosh are caffeic acid derivatives which may be present in free acid form, or in ester-linked dimers with other phenolic acids such as fukiic acid or piscidic acid. Caffeic acid derivatives, including caffeic acid, ferulic acid, and isoferulic acid, have been isolated from the roots and rhizomes of this plant (Table 2.2). They can all form ester-linked dimers with fukiic acid to yield fukinolic acid, cimicifugic acids A and B or with piscidic acid to yield cimicifugic acids E and F. Fukiic acid and piscidic acid have been isolated from the Asian species of *Actaea*,[91] but so far there are no reports from North American black cohosh. In addition, ester-linked dimers of caffeic acid derivatives (cimiracemate A-D), which are not derived from fukiic acid or piscidic acid, have been also reported.[92] Cimiracemate A is derived from isoferulic acid and the structurally related coniferyl ferulate. Cimiracemate A has been previously isolated from rhizome mixtures of the Asian *Actaea* species.[91]

C) **Flavonoids**: Among these compounds, isoflavone formononetin, was believed to responsible for the estrogenic activity of black cohosh, because of its affinity to estrogen receptors.[77] However, the isoflavone formononetin is primarily found in the Fabaceae family, and recent studies have shown that black cohosh extract does not contain formononetin.[82,83] Some investigators believed that the reports of formononetin in black cohosh were due to improper taxonomic identification of the starting material or adulteration with plant parts other than the roots and rhizomes. Other studies have reported flavonoids, such as kaemferol, biochanin A, and genestin 4′-methyl ether, present in black cohosh.[98]

In addition to these three groups of compounds, tannins, resins, and fatty acids have also been reported from the rhizomes and roots of black cohosh.[99]

2.6. Mechanism of Action

The mechanism of action by which black cohosh reduces hot flashes is not well understood. Early studies showed an estrogenic-like effect of black cohosh extracts in animal studies and an estrogen receptor bioassay.[76,77] Investigators suggested that black cohosh contained three synergistically-acting compounds able to reduce serum luteinizing hormone (LH) levels and bind to estrogen receptors. They believed triterpenoid glycosides were responsible for the reduction in LH levels, and that the isoflavone formononetin may bind to the estrogen receptors. A mechanism by which black cohosh acts through estrogen receptors has been proposed. However, this hypothesis has failed to convince other investigators for two reasons. First, more recent studies have reported a lack of estrogenic activity in the animal studies, cell binding, and cell proliferation assays (described in detail in the next section).[100-102] Second, as

mentioned above, formononetin has not been found in black cohosh extracts.[82] In addition, recent analyses of the commercial product Remifemin and wild black cohosh from 13 different locations in the eastern United States did not show detectable levels of formononetin.[83]

However, some investigators still suspect that black cohosh constituents may bind to an unidentified ERγ receptor in humans due to the selective ER modulator (SERM)-like activity of black cohosh extracts.[103-105] Research to date suggests that it is unlikely that there is a direct estrogenic effect but that black cohosh may work through a central nervous system (CNS)-mediated mechanism.

It is generally accepted that estrogens play a major role in the etiology of hot flashes because they are experienced in those periods when blood levels of estrogen are low. However, the frequency and severity of hot flashes show a poor correlation with serum estrogen levels. This suggests that other mechanisms in the nervous system may play a role.[106] Besides norepinephrine, which is the primary neurotransmitter responsible for lowering the thermoregulatory set point, serotonin (5-hydroxytryptamine or 5-HT) is another key neurotransmitter involved in inducing hot flashes. Estrogen withdrawals are associated with decreased blood serotonin levels[107] and also with up-regulation of serotonin receptors in the hypothalamus.[108] Stimulation of certain serotonin receptors (e.g. 5-HT_{2A}) may change the set point temperature, activating some autonomic functions to cool down the body, and thereby cause an increase in skin temperature and sweating, resulting in hot flashes.[109] In some studies, a decrease in hot flashes in postmenopausal women prescribed with selective seretonin reuptake inhibitors (SSRIs), including velafaxine, paroxetin, and fluxetin, for treatment of depression has been observed.

Loprinzi *et al.*[110] reported a decrease in 50% frequency and severity of hot flashes of 28 breast cancer survivors prescribed with daily 25 mg of velafaxine. Paroxetin reduced hot flash frequency by 67% and the severity of hot flashes by 75% at a dose of 10 mg daily for one week, followed by 20 mg daily for four weeks.[111] In other clinical trials, patients taking 20 mg daily of fluxetin experienced a 50% reduction in hot flashes compared with a 36 % reduction for those using a placebo.[112]

Extracts of the Asian *Actaea* species have been reported in the literature to have serotonergic activity. One study reported that a component of the water extract of *Cimicifuga foetida* (syn. *A. cimicifuga*) was able to bind the 5-HT_{1A} receptor,[113] and another study found that a methanolic extract of *Cimicifuga* rhizomes inhibited 5-HT-induced diarrhea in mice.[114] Some investigators proposed that the mechanism of action of black cohosh may be involved in the serotonergic pathway. Burdett *et al.*[85] reported the presence of compounds strongly bound to three 5-HT receptor subtypes. In their study, the isopropanolic extract was screened for the ability to bind with ten serotonin receptor subtypes. The strongest bond was found to be with 5-HT_{1A}, 5-HT_{1D}, and 5-HT_7. Subtypes 5-HT_{1A} and 5-HT_7, which are located in the hypothalamus, might be involved in hormonal regulation of hot flashes. These investigators conducted additional experiments to distinguish the relative binding potency of black cohosh alcoholic extracts (40% isopropanol, 60% ethanol, and 100% methanol) to the 5-HT_{1A} and 5-HT_7 subtypes. The methanolic extract exhibited a higher binding potency compared with the other two extracts. In addition, they showed that the methanolic extract functioned as a mixed competitive ligand of 5- HT_7 receptor. This study gave primary evidence of the serotonergic activity of black cohosh, providing support for this alternative mechanism

for hot flashes reduction. Further studies need to be conducted to determine if black cohosh acts through the serotonergic pathway.

Several investigators have proposed a third hypothesis, i.e. black cohosh may act through binding to the dopaminergic receptors,[115] although the effect of estrogen on the dopaminergic system is poorly understood.[116] This hypothesis is based on indirect evidence and is not supported by binding assay studies. De Leo *et al.*[117] investigated the effect of dopamine infusion on plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL) after acute and chronic estrogenic withdrawal in women. They found that the dopamine infusion inhibited the plasma levels of LH, and PRL, but not those of FSH. Similar results were reported by Matsubara *et al.*[116] Black cohosh extracts showed results on endocrinal hormones (LH, FSH, PRL) in animal studies similar to the results in women treated with dopamine.[76,77,118] These results lead to conclude that the biological activity on endocrinal hormone levels may be due to the presence of dopaminergic compounds in black cohosh. Johnson *et al.*[119] have reported that a dopaminergic agonist can also cause a significant decrease in proliferation of MCF-7 cells. Black cohosh extract has an inhibitory growth effect on estrogenic-sensitive MCF-7 cells, possibly due to the presence of dopaminergic compounds in the plant.[73,120] The stimulation of MCF-7 cells growth in one study[121] could be due to the loss of dopaminergic compounds and a predominance of fukinolic acid, which can stimulate the proliferation of MCF-7 cells.[97] In addition, dopamine can increase estrogen expression *in vitro*.[122] Thus, the estrogenic activity of black cohosh extract could be due to the presence of dopaminergic compounds in the plant.[76] Recently, Jarry *et al.* reported direct evidence of dopaminergic activity of black cohosh extract in a dopamine receptor ligand-binding

assays.[105] But more studies need to be done to support this hypothesis, as the structure of the purified active compounds has not been elucidated.

In summary, the mechanism of action of black cohosh has not been fully understood, but it likely involves the nervous system through CNS, via serotonergic, or dopaminergic pathways. However, the fact that black cohosh acts through estrogenic pathways cannot be excluded, if a yet-to-be identified ERγ receptor indeed exists.

The study of the mechanism of action of black cohosh is complicated by the fact that investigators have used different black cohosh extracts, which may vary in their compositions, and may lead to different pharmacological effects. The concentration of active constituents of black cohosh could vary depending on a number of factors, including extraction method, growing season, harvesting, exsiccation procedure, and the method of preservation and storage of crude drugs. For example, Kong *et al.*[123] demonstrated the effect of extraction solvents on the extracted amounts of the triterpenoid glycosides actein, 23-epi-26-deoxyactein, and cimicifugoside from *C. foetida* L. Among five extraction solvents, including ethanol, methanol, methanol-water (8:2), ethanol-water (7:3) and water, they found that ethanol and methanol can extract some unknown compounds which overlapped with the quantified peaks in HPLC chromatograms, making it difficult to quantify the three main triterpenoid glycosides accurately. Thus, further work was done with only three extraction solvents. They found that methanol-water (8:2) yielded higher amounts of the three triterpenoid glycosides than ethanol-water (7:3) and water. Therefore, quality control is very important in the identification and characterization of black cohosh extacts. Chemical fingerprints of plant extracts can provide qualitative and quantitative information. However, the thorough studies of the

chemical composition of black cohosh are important to understand the bioactive constituents responsible for its therapeutic effects.

2.7. Biological Activities

2.7.1. Estrogenic activity

It was once believed that black cohosh acts through estrogenic-like activity. The putative estrogenic activity of black cohosh has been extensively studied *in vivo* using animal models, and *in vitro* using cell binding or cell proliferation assays. The results among different studies are contradictory.

Jarry and Harnischferger[76] studied the effect of black cohosh extracts on the serum concentration of LH and FSH in ovariectomized rats. High levels of two hormones are associated with hot flashes.[124] They found that a dichloromethane extract concentrated the triterpenoid glycosides and depressed plasma LH levels but not FSH. Jarry *et al.*[77] further characterized the effect of the active constituents of black cohosh and their possible mechanisms of action in the estrogen receptor binding assay. These authors identified three active principles in a methanol extract: (i) compounds that do not bind to estrogen receptors but suppress LH levels; (ii) compounds that bind to estrogen receptors and suppress LH levels; and (iii) compounds that bind to estrogen receptors, but do not suppress LH levels. One of these compounds was identified as the isoflavone formononetin, which was shown to bind to estrogen receptors without reduction of LH levels in ovariectomized rats. Similarly, Duker *et al.* [118] found that the serum LH levels in ovariectomized rats was reduced by lipophilic but not hydrophilic black cohosh extracts. More studies have also found the estrogenic activity of black cohosh extracts.[121,125,126] Harnischfeger and Cillien[125] reported that components of the butanol and chloroform

subfraction from an alcoholic black cohosh extract were able to bind to estrogen receptors. Liu *et al.*[126] also found that the ethanolic black cohosh extract stimulated *in vitro* proliferation of estrogen-receptor-sensitive human breast cancer cells, MCF-7, and increased the uterine weight of immature female mice. Wober *et al.*[127] also reported estrogenic activity of black cohosh by measuring alkaline phosphatase in Ishikawa cells, an endometrial cancer cell line. An ethanolic extract showed a weaker estrogenic effect than that of an isopropanolic extract. Furthermore, Kruse *et al.*[97] studied the estrogenic activity of individual compounds isolated from black cohosh in MCF-7 proliferation assay. They found that fukinolic acid had a stimulating effect on the proliferation rate of MCF-7 cells.

However, many more studies have reported lack of estrogenic activity of black cohosh. Einer-Jensen *et al.*[100] investigated the estrogenic activity of an aqueous ethanolic black cohosh extract on uterine growth in immature mice and on vaginal cornification in ovariectomized rats. They found no estrogenic activity as evidenced by increase in uterine weight and number of cornified vaginal cells as compared to a positive control group treated with estradiolbenzoate. Liu *et al.*[101] demonstrated that a methanolic black cohosh extract did not bind to purified ERα and ERβ and did not increase the activity of estrogen-dependent alkaline phosphatase in Ishikawa cells. Freudenstein *et al.*[102] evaluated the safety of a standardized isopropanolic black cohosh extract on the stimulation of estrogen-dependent mammary gland cells induced with 7,12-dimethyl [a] anthracene in ovariectomized rats. There were no statistical differences in tumor number and size between the black cohosh treatment group and the control group, while the estrogen-treated group showed a significant increase in both number and size of the

tumors. These results showed a lack of mammary tumor-stimulating effect of black cohosh, indicating the absence of estrogenic activity. Furthermore, in an ER binding assay, Beck *et al.*[79] found no estrogenic activity of a standardized black cohosh extract. These results are consistent with a study by Klein *et al.*,[80] who used a methanolic black cohosh extract. Moreover, Lupu and coworkers[81] also demonstrated that an aqueous methanolic black cohosh extract did not regulate the expression of estrogen-regulated genes of the ER-positive cell lines, MCF-7 and T47D, as well as the ER-negative cell line MDA-MB-231 using the RNase protection assay. This group observed that black cohosh extract did not induce transcriptional activation of estrogen-responsive elements in the ERE-luciferase reporter assay, nor contain estrogenic activity, as determined by the Ishikawa cell assay. Furthermore, Zhang *et al.*[128] investigated the estrogenic activity of an ethanolic black cohosh extract as well as the triterpenoid glycosides actein, 26-deoxyactein, and cimiracemoside A in medaka fish. After ten days of treatment, the estrogenic activities were determined by measuring levels of plasma steroids, 17β-estradiol-induced liver vitellogenin, and aromatase activity, an indicator of the ability of gonads to convert testosterone to estradiol. These investigators found that neither the black cohosh extract nor individual triterpenoid glycosides caused any change in estrogenic activity compared to the control. Thus, they concluded that black cohosh did not exhibit estrogenic activity.

While these studies showed a lack of estrogenic activity of black cohosh, other studies have reported that black cohosh extracts have selective ER modulator (SERM)-like activity, resulting in a positive effect on bone and blood vessels, with no effect on the breast and endometrium. Seidlova-Wuttke *et al.*[129] investigated the estrogenic effects of

black cohosh extracts on bone, fat, and uterus of ovariectomized rats treated with 33 mg black cohosh extract per day over 3 months. They found that the black cohosh significantly reduced bone mineral density loss and paratibial fat deposits. There was no effect on uterine weight or gene expression of E2-regulated genes. They concluded that black cohosh exhibited SERM activity in the bone and in the fatty tissue, but not in the uterus of ovariectomized rats. Further work by the same group showed the SERM effect of black cohosh in the hypothalamus by inhibiting LH secretion, as well as an effect in bone tissue, shown by the osteoporosis-preventing effect, but no effect was found in the uterus of ovariectomized rat.[103] Wuttke *et al.*[104] also showed the SERM effect of black cohosh in the bone and in the vagina, but without estrogenic effects in the uterus of postmenopausal women. The results of these studies further support the idea that the mode of action of black cohosh may be to act through estrogenic activity. Moreover, Jarry *et al.*[105] proposed that black cohosh binds to an ERγ type, which was recently discovered in fish.[130] The group of Hawkins found that the isopropanolic black cohosh extract displaced estradiol from binding sites in the human endometrium cytosol preparation, but did not displace estradiol from either ERα or ERβ. This suggests that a third ER type, namely ERγ, may exist in the uterine cytosol. The unidentified ER may help understand why some studies showed a lack of estrogenic activity of black cohosh extracts in the ER cell-binding assay. However, further studies must be conducted this to prove this hypothesis. Thus, the mechanism of action of black cohosh acting via estrogenic activity still remains unclear.

2.7.2. Anticancer activity

One of the side effects of HRT is cancer promotion, especially endometrial or breast cancer. To evaluate the safety of black cohosh, several authors have studied the stimulation effect of black cohosh on estrogen-sensitive breast cancer MCF-7 cells and tumor induction in animal models. Bodinet and Freudenstein[120] examined an isopropanolic black cohosh extract on the growth effect of MCF-7 cells. The extract did not stimulate MCF-7 growth but instead exerted inhibitory effects on cellular proliferation. Niblen and Freudenstein[131] tested the effect of isopropanolic black cohosh extract on the transplantable endometrial adenocarcinoma cells in rats and they found that black cohosh did not stimulate the growth of the tumor cells. A study by Einbond *et al.*[73] also demonstrated the inhibitory effect of an ethyl acetate fractioned obtained from a aqueous methanolic extract on the growth of two cell binding assay, MCF-7, and MDA-MB-453. Black cohosh extract induced cell cycle arrest at G1 with a concentration of 30 μg/mL, and at G2/M with a concentration of 60 μg/mL in MCF-7 cells. This suggests that the extract contains a mixture of components with more active (or more abundant) components causing G1 arrest and the less active causing G2/M arrest. Triterpenoid glycosides including actein, 23-epi-26-deoxyactein, and cimiracemoside A, isolated from black cohosh also inhibited growth of MCF-7 cells and induced cell cycle arrest at G1. Furthermore, Hostanska *et al.*[72] reported similar results to those reported by Einbond *et al.*'s study. Honstanska *et al.* found that an isopropanolic and ethanolic black cohosh extracts inhibited growth of the estrogen–receptor-positive MCF-7 and estrogen–receptor-negative MDA-MB231 breast cancer cells caused by induction of apoptosis. Hostanska *et al.*[132] further investigated the two major fractions—the

triterpenoid glycosides and the cinnamic acid ester—from an isopropanolic black cohosh extract on the growth of MCF-7 cells. Both fractions inhibited cell growth by inducing apoptosis. The cinnamic acid ester fraction is more potent than the triterpenoid glycosides fraction. The lack of proliferation effect of black cohosh on the estrogen–receptor-sensitive MCF-7 and estrogen–receptor-negative MDA-MB breast cancer cells, *in vitro* and *in vivo*, suggests a favorable safety profile for use in women with or without a history of breast cancer. However, a long-term study still has to be conducted to confirm this biological effect.

2.7.3. Anti-inflammatory activity

The rhizomes of Asian species of *Actaea*, including *A. dahurica* Franch and *A. heracleifolia* (Kom.) J. Compton have been used frequently as antipyretic, analgesic, and anti-inflammatory agents in traditional Japanese and Chinese medicine, while black cohosh rhizomes are traditionaly used in North America against rheumatism.[89] This suggests that the rhizomes of these plants contain compounds, which exert anti-inflammatory activity. Loser *et al.*[133] reported that cinnamic acid derivatives isolated from the rhizome of black cohosh have anti-inflammatory effect. In this study, fukinolic acid and cimicifugic acid A showed high activity by measuring inhibition enzyme neutrophil elastase, which contributes to the destruction of basal membranes during inflammation. Some studies have reported the anti-inflammatory effect from the Asian *Actaea* species. Sakai *et al.*[134] showed that ferulic acid had the inhibitory effect on murine macrophage inflammatory protein-2 (MIP-2) production in a murine macrophage RAW264.7 cell lines. MIP-2 is associated in the inflammation process. They also reported that ferulic acid and isoferulic acid, isolated from *A. heralcleifolia*, have shown

the anti-inflammatory effect against the production of MIP-2 in response to respiratory syncytial virus infection.[135] Taking all data from these studies, phenylpropanoid derivatives in plants of the genus *Actaea* are, at least in part, responsible for the pharmacological anti-inflammatory activity, which leads this plant to be used as an antipyretic, analgesic, antirheumatic, and anti-inflammatory agent in traditional medicine.

2.7.4. Antioxidant activity

In the Burdette *et al.* study,[75] the methanolic extract as well as nine polyphenolic compounds isolated from black cohosh, were examined for antioxidant activity using the DPPH free-radical scavenging assay, and their capacity to prevent menadione-induced DNA damage in cultured S30 breast cancer cells as determined by single-cell gel electrophoresis. All nine polyphenols exhibited the free radical scavenging antioxidant activity, and, in particular, methyl caffeate, caffeic acid, ferulic acid, cimiracemate A, cimiracemate B, and fukinolic acid, were found to reduce menadione-induced DNA damage in cultured S30 breast cancer cells. This study suggests that these are other mechanisms by which black cohosh might reduce cancer and inflammation caused by tissue damage done by ROS.

2.8 Safety

In Germany, the Commission E approved the used of black cohosh for menopausal symptomsa and has found no evidence of serious side effects, contraindications, or drug interactions. Most commonly reported side effects are mild gastric complaints. Overdoses may causes dizziness, headaches, impaired vision, nausea, and vomiting.[136] Because of a lack of long-term data, black cohosh is not recommended for women who are pregnant or breast feeding.[137] *In vitro Salmonella* microsome assays

(Ames test) showed no evidence of a mutagenic potential of an isopropanolic extract of black cohosh and no chemical or organ toxicities were observed in female Wistar rats given very high doses—up to 5,000 mg—of black cohosh extract granulate per kilogram body weight for 26 weeks.[60]

The Commission E has recommended that black cohosh be taken for only a six-month period. However, the reason for this limitation on black cohosh is to ensure that women continue to have regular physical examinations at six-month intervals, rather than concerns about long-term safety of black cohosh. Although no studies have been done to date on the safety of the long-term use of black cohosh, findings of short-term studies using high doses of the herb indicate that it may be considered safe for long-term use.[60]

A single case study reported that a female patient developed a seizure after taking a combination of black cohosh, chaseberry, and evening primrose oil, but no clear cause-and -effect relationship was documented.[138]

Recently, there have been some reports suggesting a link between black cohosh consumption and hepatotoxicity. A 47-year-old woman used black cohosh alone for one week for symptoms related to menopause. She developed jaundice and liver enzyme abnormalities and the outcome was a liver transplant. Histological examination of her explant liver confirmed severe hepatitis, typically found in severe immunological reactions, and not a direct toxic injury. The report indicated that the dose of black cohosh did not exceed the dosage recommended on the package. However, there has been no conclusion on the causal link between black cohosh and the incident of hepatitis.[139] In the Lotos *et al.* case study, they reported that a 52-year-old woman had an acute liver failure after taking a combination of herbal preparations for three months. The herbal

preparations contained *Nepeta hederacea* (ground ivy), *Hydrastis canadensis* (golden seal), *Ginkgo biloba*, *Avena sativa* (seed oat), and black cohosh. There is no evidence to support a link between black cohosh and acute liver failure in this case report because it is not possible to determine the individual ingredient, or mixture of ingredients, that resulted in acute liver failure.[140] Nonetheless, case reports of hepatotoxicity should be taken into consideration for the patients who use black cohosh to relieve symptoms related to menopause.

Table 2.1. Triterpenoid glycosides reported from of *A. racemosa*

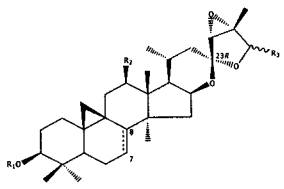
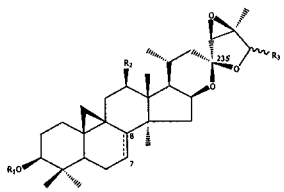
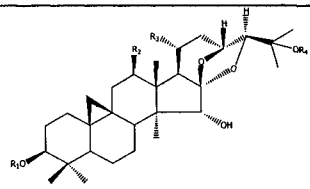
Group	Chemical structure	Compound	Ref.
1. Cimicifugenin type	23*R*-isomer 	**Actein** (R_1 = xyl, R_2= OAc, R_3 = OH)	94
		Deacetylactein (R_1 = xyl, R_2= OH, R_3 = OH)	141
		26-Deoxyactein (R_1 = xyl, R_2= OAc, R_3 = H)	94
		Cimiracemoside O (R_1 = 4′-acetyl-xyl, R_2 = OAc, R_3 = OH, Δ^7)	93
		Cimiracemoside P (R_1 = xyl, R_2 OAc, R_3 = carbonyl)	93
		26-Deoxycimicifugoside (R_1 = xyl, R_2 OAc, R_3 = H, Δ^7)	93
		2′-Acetylactein (R_1 = 2′-acetyl-xyl, R_2 = OAc, R_3 = OH)	93
	23*S*-isomer 	**23-Epi-26-deoxyactein** (previously 27-deoxyactein; R_1 = xyl, R_2= OAc, R_3 = H)	94
		Cimiracemoside I (R_1 = xyl, R_2 = R_3 = H, Δ^7)	93
		Cimiracemoside N (R_1 = ara, R_2 = OAc, R_3 = H)	93
		27-Deoxy-acetylacteol (R_1 = H, R_2= OAc, R_3 = H)	141
2. Cimigenol type		**25-*O*-Methylcimigenol-3-*O*-β-D-xylopyranoside** (R_1 = xyl, R_2 = H, R_3 = R_4 = Me)	142
		Cimiracemoside A (R_1 = ara, R_2 = H, R_3 = CH_2OH, R_4 = H)	95
		Cimiracemoside B (R_1 = xyl, R_2 =H, R_3 = CH_2OH, R_4 = H)	95
		Cimiracemoside C (cimicifugoside M; R_1 = ara, R_2 =H, R_3 = Me, R_4 = H)	94, 95
		Cimiracemoside D (R_1 = ara, R_2 =OAc, R_3 = Me, R_4 = H)	95
		12β-Hydroxy-cimigenol-3-*O*-β-D-xylopyranoside (R_1 = xyl, R_2 = OH, R_3 = Me, R_4 = H)	95

Table 2.1. (continued) Triterpenoid glycosides reported from of *A. racemosa*

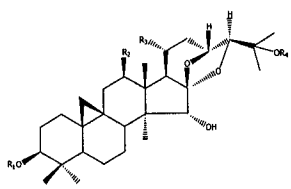
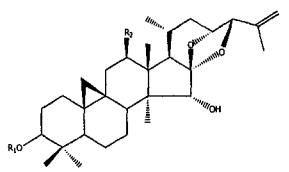
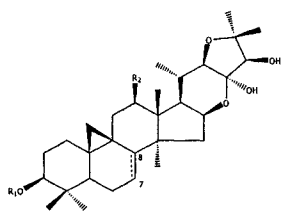
Group	Chemical structure	Compound	Ref.
2. Cimigenol type (continued)		**12β-Hydroxy-cimigenol-3-*O*-α-L-arabinopyranoside** (R_1 = ara, R_2 = OH, R_3 = Me, R_4 = H)	95
		25-*O*-Acetyl-cimigenol-3-*O*-β-D-xylopyranoside (R_1 = xyl, R_2 =H, R_3 = Me, R_4 = Ac)	93
		25-*O*-Acetyl-cimigenol-3-*O*-α-L-arabinopyranoside (R_1 = ara, R_2 =H, R_3 = Me, R_4 = Ac)	142
		12β,21-Dihydroxy-cimigenol-3-*O*-α-L-arabinopyranoside (R_1 = ara, R_2 = OH, R_3 = CH_2OH, R_4 = H)	142
		Cimicifugoside (cimigenol-3-*O*-β-D-xylopyranoside; R_1 = xyl, R_2 = H, R_3 = Me, R_4 = H)	96, 143
		**Cimiracemoside J** (R_1 = ara, R_2 = OAc)	93
		Cimiracemoside K (R_1 = xyl, R_2 = OAc)	93
		25-Anhydrocimigenol-3-*O*-α-L-arabinopyranoside (R_1 = ara, R_2 = H)	93
		25-Anhydrocimigenol-3-*O*-β-D-xylopyranoside (R_1 = xyl, R_2 = H)	93
3. Cimiacerogenin type		**Cimiracemoside F** (cimiracemoside A; R_1 = xyl, R_2 = OAc, Δ^7)	89, 95
		Cimiracemoside G (R_1 = ara, R_2 = OAc, Δ^7)	95
		Cimiracemoside H (R_1 = xyl, R_2 = OAc)	95
		(22*R*,23R,24R)-12β-Acetoxy-16β:23:22,25 -diepoxy-23,24-dihydroxy-9,19-cycoanostan-3β-yl-α-L-arabinopyranoside (R_1=ara, R_2 = OAc)	142
		Cimiaceroside A (R_1 = xyl, R_2 = H, Δ^7)	89

Table 2.1. (continued) Triterpenoid glycosides reported from of *A. racemosa*

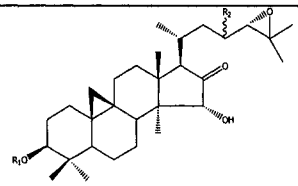
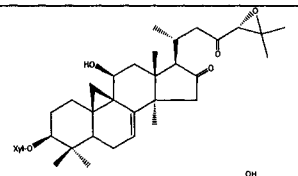
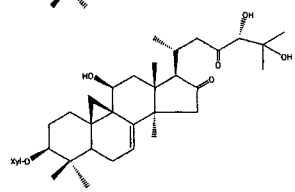
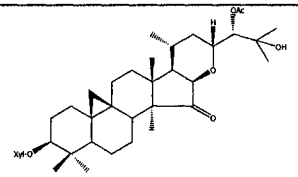
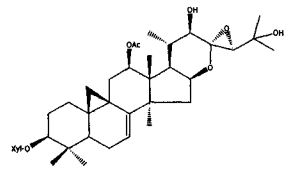
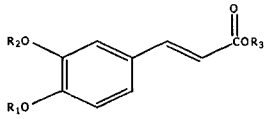
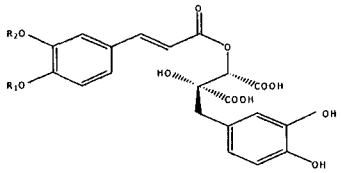
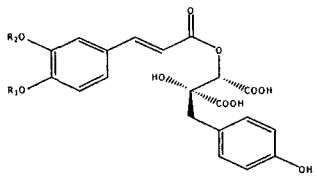
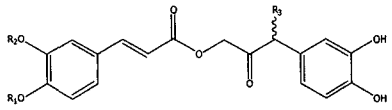
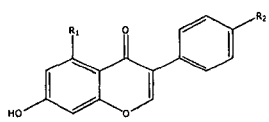
Group	Chemical structure	Compound	Ref.
4. Shengmanol type		**23-*O*-Acetylshengmanol-3-*O*-β-D-xylopyranoside** (R_1 = xyl, R_2 = α-OAc)	89
		23-*O*-Acetylshengmanol-3-*O*-α-L-arabinopyranoside (R_1 = ara, R_2 = α-OAc)	93
		Cimiracemoside L (R_1 = 4′-acetyl-ara, R_2 = β-OAc)	93
		Cimiracemoside M (R_1 = 4′-acetyl-xyl, R_2 = β-OAc)	93
		Shengmanol-3-*O*-α-L-arabinopyranoside (R_1 = ara, R_2 = α-OH)	144
5. 16,23-Diketo type		**Cimicifugoside H-1**	93
		**Cimicifugoside H-2**	93
6. Miscellaneous		**Cimiracemoside E**	95
		**Actaeaepoxide-3-*O*-β-D-xylopyranoside**	143

Table 2.2. Phenylpropanoids and flavonoids reported from *A. racemosa*

Class	Chemical structure	Compound	Ref.
Phenylpropanoid derivatives	Caffeic acid derivatives 	**Caffeic acid** (R_1 = H, R_2 = H, R_3 = H)	75, 97
		Methyl caffeate (R_1= H, R_2 = Me, R_3 = Me)	75, 97
		Ferulic acid (R_1 = H, R_2 = Me, R_3 = H)	75, 97
		Isoferulic acid (R_1 = Me, R_2 = H, R_3 = H)	75, 97
	Fukiic acid ester derivatives 	**Fukinolic acid** (R_1 = H, R_2 = H)	75, 97
		Cimicifugic acid A (R_1 = H, R_2 = Me)	75, 97
		Cimicifugic acid B (R_1 = Me, R_2 = H)	75, 97
	Piscidic acid ester derivatives 	**Cimicifugic acid E** (R_1 = H, R_2 = Me)	75, 97
		Cimicifugic acid F (R_1 = Me, R_2 = H)	75, 97
	Cimiracemate 	**Cimiciracemate A** (R_1 = Me, R_2 = H, R_3 = H)	92
		Cimiciracemate B (R_1 = H, R_2 = Me, R_3 = H)	92
		Cimiciracemate C (R_1 = Me, R_2 = H, R_3 = OMe)	92
		Cimiciracemate D (R_1 = H, R_2 = Me, R_3 = OMe)	92
Flavonoids		**Formononetin** (R_1=OH, R_2 = OMe)	77
		Biochanin A (R_1 = OH, R_2 = OMe)	98
		Genistein (R_1 = OH, R_2 = OH)	98

Chapter 3

Two New Polyphenolic Constituents from *Actaea racemosa*

3.1. Introduction

Previous chemical investigations on black cohosh reported the isolation of two principal groups of compounds, triterpenoid glycosides and polyphenolic derivatives.[89,92-95,97,142,143,145] Highly oxygenated 9,19-cyclolanostane-type triterpenoids, linked to one monosaccharide unit such as xylopyranoside or arabinoside, are common triterpenoid glycosides found in black cohosh roots and rhizomes. To date, at least 43 triterpenoid glycosides were isolated from black cohosh, and they exhibited various biological activities including anticancer activity,[73,142,146] anti-HIV activity,[147] and inhibitory effect on catecholamine secretion.[148] With regard to polyphenolic derivatives, 13 compounds have been isolated from the rhizomes and roots of black cohosh, such as hydroxycinnamic acid derivatives (e.g. caffeic acid, ferulic acid, and isoferulic acid), fukiic acid ester derivatives (e.g. fukinolic acid, cimicifugic acid A, and cimicifugic acid B), and piscidic acid ester derivatives (e.g. cimicifugic acid E and F).[92,97] These compounds have been reported to have estrogenic,[97] anti-inflammatory,[133] and antioxidant activities,[75] and exhibit inhibitory effects on the enzymatic activities of α-amylase, carboxypeptidase A,[149] and collagenase.[150] Even though many triterpenoid glycosides and polyphenols have been isolated from black cohosh, none of them have proven to be the active compounds responsible for alleviating menopausal symptoms.

Because of the increasing use of black cohosh, it is important to better understand the complexity of phytochemical mixtures in black cohosh. In a preliminary phytochemical study of black cohosh, we detected many minor polyphenols by an HPLC-PDA method. A comparison of black cohosh with a well-studied related species, *A. simplex*, showed that the latter contains more than 79 triterpenoid glycosides, and 28

polyphenols.[91,151-158] This suggests that black cohosh may also contain other unidentified polyphenolic compounds.

As part of a continuing phytochemical study of black cohosh, we have investigated in greater detail the polyphenolic constituents of black cohosh. The standardized black cohosh alcoholic extract was fractionated. Two new polyphenolic compounds (**1** and **2**) were isolated, along with fifteen known polyphenolic compounds (**3-17**). Compounds **1** and **2** were evaluated for their antioxidant activity in the DPPH free-radical assay and for their growth effects on the estrogen sensitive breast cancer cell line MCF-7, using a cell proliferation assay. In addition, six polyphenolic compounds, protocatechuic acid, protocatechualdehyde, *p*-coumaric acid, 1-isoferuloyl-β-D-glucopyranoside, ferulate-1-methyl-ester and cimicifugic acid D are reported for the first time from black cohosh.

3.2. Experimental Section

3.2.1. General experimental procedures Melting points were determined on a Mel-Temp II melting point apparatus (Laboratory Devices Inc., Holliston, MA) and were uncorrected. Optical rotations were measured on a Jasco DIP-140 Digital Polarimeter (Rudolph Research Analytical, Flanders, NJ). UV spectra were obtained in a Lambda 2 UV/vis Spectrophotometer (Perkin-Elmer, Boston, MA). ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AMX-300 MHz NMR spectrometer, operating at 300 and 75 MHz, respectively. All NMR spectra were obtained in CD_3OD using trimethylsilane (TMS) as internal standard, with chemical shifts expressed in δ and coupling constant (J) in Hertz. Electrospray Ionization Mass Spectrometry (ESIMS) was performed with a ThermoQuest Finnigan LCQ instrument (San Jose, CA) equipped with Xcalibur software.

Samples were dissolved in MeOH and introduced by direct injection. The capillary voltage was 10 V, the spray voltage was 4.5 kV, and the tube lens offset was 0 V. The capillary temperature was 230°C. High Resolution ESIMS (HRESIMS) was performed on a 70-SE-4F mass spectrometer (Micromass). Samples were dissolved in MeOH. HPLC analyses were carried out on a Waters 2690 Separations Module equipped with a Waters 996 photodiode array detector and Waters Empower[32] software using a Phenomenex Aqua C_{18} column (4.6 × 250 mm, 5 μm) and a solvent system of 5:95 to 50:50 MeCN/1% aqueous acetic acid in a linear gradient. The flow rate was 1 mL/min and the column was used at room temperature and a 30-min run time for analysis of subfractions. Preparative HPLC was carried out using a Waters 600 controller with a Waters 486 tunable absorbance detector and a Phenomenex, Nucleosil 10 C_{18} column (21.1 × 250 mm, 10 μm), eluting with solvent system I [MeCN/0.1% aqueous acetic acid (9:1)], solvent system II [MeOH/0.1% aqueous acetic acid (1:3)], solvent system III [a linear gradient of MeOH (from 33% to 50%) in 0.1% aqueous acetic acid], solvent system IV [MeCN/0.1 % aqueous acetic acid (2:8)]. The flow rate was 10 mL/min. The column was used at room temperature, and a 60-min run time. TLC analyses were performed on silica gel 60 F_{254} plates (Merck, Darmstadt, Germany), with compounds visualized by spraying with a vanillin solution (1 g vanillin in 10 mL conc. H_2SO_4 and 90 mL EtOH). Sephadex LH-20 (25-100 μm; Pharmacia Fine Chemicals, Piscataway, NJ), C_{18} reversed-phase silica gel (40 μm; J. T. Baker, Phillipsburg, NJ), and Diaion HP-20 (Supelco, Bellefonte, PA) were used for column chromatography.

3.2.2. Plant material The standardized black cohosh (*A. racemosa*) extract (lot number 9-2044) was supplied by PureWorld Botanicals Inc., South Hackensack, NJ, USA.

3.2.3. Cell culture MCF-7, human breast cancer, estrogen sensitive cells were obtained from the ATCC (Manassas, VA). Cells were grown in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL) in a humidified incubator at 37°C, with a 5% CO_2 atmosphere.

3.2.4. DPPH free-radical scavenging assay The DPPH assay was performed on fractions and purified isolates as previously described.[159] Reaction mixtures containing test samples (dissolved in DMSO) and 400 μM DPPH ethanolic solution in 96-well microtiter plates were incubated at 37°C for 30 min, and absorbances were measured at 515 nm. Final concentrations of test materials were typically in a 1-50 μg/mL range. Percent inhibition by sample treatment was determined by comparison with a DMSO-treated control group. IC_{50} values denote the concentration of sample required to scavenge 50% DPPH free radicals.

3.2.5. Cell proliferation assay MCF-7 cells were transferred to phenol–red-free DMEM containing 2 mM of L-glutamine and 5% charcoal-stripped serum and passaged at least two times and washed two times with phosphate buffered saline prior to seed. Cells were seeded into a 24-well plate at density 2 x 10^4 cells and allowed to attach for 24 h in phenol red-free DMEM containing 2 mM of L-glutamine and 5% charcoal-stripped serum. The medium was then replaced with fresh medium with or without the indicated test compounds at a range of concentrations (1-40 μg/mL). After 4 days, the numbers of attached viable cells was counted using a Coulter Counter, model Z_F (Coulter

Electronics Inc., Hialeah, FL). Cell viability was calculated by comparing cell counts in treated samples relative to cell counts in untreated cells.[97,160]

3.2.6. Extraction and isolation A black cohosh powdered extract (0.5 kg) was re-extracted with 80% MeOH/water at room temperature overnight (12 h). After the MeOH was removed *in vacuo*, the resulting aqueous solution was sequentially partitioned with hexane and *n*-BuOH. The hexane, *n*-BuOH, and aqueous extracts were concentrated *in vacuo* at 40°C. A portion (30 g) of the residue from the *n*-BuOH extract was fractionated over Diaion HP-20 (600 g) column chromatography (CC), eluting with water/MeOH (1:1), MeOH, and acetone to give four fractions (B_{1-4}). Fraction B_2 (3.2 g) was chromatographed over C_{18} (120 g) and eluted with gradients of MeCN in 0.1% aqueous acetic acid (5% to 50% MeCN) to obtain 10 subfractions (B_{2a-j}). Fraction B_{2b} (270 mg) was further separated over C_{18} (12 g) CC with gradients of MeCN in 0.1% aqueous acetic acid (5% to 15% MeCN) to obtain 3 fractions (B_{2b1-3}). Fraction B_{2b2} (20.3 mg) was further purified by preparative HPLC eluting with solvent system I to yield **3** (4.4 mg) and **4** (2.3 mg). Fraction B_{2d} (390 mg) was chromatographed over Sephadex LH-20 (80 g) and eluted with MeOH/H_2O (9:1) to yield **6** (290 mg). Fraction B_{2e} (50 mg) was chromatographed over Sephadex LH-20 (10 g) and eluted with MeOH/H_2O (9:1) to obtain 6 subfractions (B_{2e1-6}). Fraction B_{2e4} (9.3 mg) was rechromatographed over Sephadex LH-20 (20 g) and eluted with MeOH/H_2O (9:1) to yield **11** (1.6 mg). Fraction B_{2f} (150 mg) was chromatographed over Sephadex LH-20 (30 g) and eluted with MeOH/H_2O (9:1) to obtain 8 subfractions (B_{2f1-8}). Fractions B_{2f3} (4 mg) and B_{2f8} (2 mg) were rechromatographed over Sephadex LH-20 (2 g) and eluted with MeOH/H_2O (9:1) to yield **5** (0.8 mg) and **1** (1.1 mg), respectively. Fraction B_{2h} (82 mg) was purified by

preparative HPLC eluting with solvent system II to give **8** (32.2 mg) and **10** (12 mg). Fraction B_{2i} (100 mg) was chromatographed over Sephadex LH-20 (20 g) and eluted with MeOH/H_2O (9:1) to yield **10** (84.2 mg).

A portion (30 g) of the residue from the water extract was fractionated over Diaion HP-20 (600 g) CC eluting with water, water/MeOH (1:1), MeOH, and acetone to give 7 combined fractions ($W_{1\text{-}7}$). Fraction W_3 (2.12 g) was chromatographed over C_{18} (100 g) CC, eluted with gradients of MeCN in 0.1% aqueous acetic acid (5% to 35% MeCN) to obtain 8 combined fractions ($W_{3a\text{-}h}$). Fractions W_{3c} (170 mg), W_{3d} (70.9 mg), W_{3e} (82.0 mg), and W_{3g} (94.9 mg) were subjected over preparative HPLC eluting with solvent system III to yield crude fukinolic acid (102.2 mg), crude cimicifugic acid D (6.4 mg), crude cimicifugic acid A (39.1 mg), and crude cimicifugic acid B (46.6 mg), respectively. The crude fukinolic acid, cimicifugic acid D, cimicifugic acid A, and cimicifugic acid B were further purified by preparative HPLC eluting with solvent system IV to yield **12** (54.4 mg), **15** (2.2 mg), **13** (19.4 mg), and **14** (30.1 mg), respectively.

Fraction W_4 (120.4 mg) was chromatographed over C_{18} (8 g) CC eluted with gradients of MeCN in 0.1% aqueous acetic acid (10 % to 40% MeCN) to yield 4 combined fractions ($W_{4a\text{-}d}$). Fraction W_{4d} (20.2 mg) was subjected over preparative HPLC eluting with the solvent system III to obtain crude cimicifugic acid E (6.1 mg), and crude cimicifugic acid F (11.4 mg). Crude cimicifugic acids E and F were further purified by preparative HPLC eluting with the solvent system IV to yield **16** (3.8 mg) and **17** (8.7 mg). Fraction W_5 (84.4 mg) was chromatographed over C_{18} (40 g) CC eluted with gradients of MeCN in 0.1% aqueous acetic acid (15 % to 50% MeCN) to obtain 8 combined fractions ($W_{5a\text{-}g}$). Fraction W_{5d} (32.8 mg) was chromatographed over Sephadex

LH-20 (5 g) CC eluted with MeOH/H_2O (9:1) to obtain 10 fractions (W_{5d1-10}). Fraction W_{5d6} (6 mg) was further purified by preparative HPLC eluting with solvent system IV to yield **2** (2.1 mg). Fraction W_6 (355 mg) was subjected over Sephadex LH-20 CC (80 g), eluting with MeOH/H_2O (9:1), to obtain 10 combined fractions (W_{6a-j}). Fractions W_{6b} (10.4 mg) and W_{6f} (15.1 mg) were further purified over preparative HPLC eluting with the solvent system II to give **7** (4.3 mg) and **9** (1.1 mg), respectively.

These are the descriptions of the compounds from the black cohosh extract.

Compound 1: yellow, amorphous powder; mp 149-150 °C; $[\alpha]^{25}_D$ –23.6° (*c* 0.0005, MeOH); UV (MeOH) λ_{max} (log ε) 339 (3.34), 295 (3.28), 237 (3.31) nm; ^{1}H NMR and ^{13}C NMR data, see Table1; positive HRESMS *m/z* 359.0774 $[M+H]^+$ (calculated for $C_{18}H_{15}O_8$, 359.0767)

Compound 2: yellow, amorphous powder; mp 112-113 °C; $[\alpha]^{25}_D$ + 22.4° (*c* 0.001, MeOH); UV (MeOH) λ_{max} (log ε) 328 (3.17), 290 sh (3.06), 225 (3.10) nm; ^{1}H NMR and ^{13}C NMR data, see Table1; positive HRESMS *m/z* 463.1252 $[M+H]^+$ (calculated for $C_{22}H_{23}O_{11}$, 463.1240)

Protocatechuic acid (3): white powder; Negative ESIMS m/z 153 $[M-H]^-$. ^{1}H and ^{13}C NMR data are consistent with previously published literature.[161]

Protocatechualdehyde (4): white powder; Negative ESIMS m/z 137 $[M-H]^-$. ^{1}H and ^{13}C NMR data are consistent with previously published literature.[162]

***p*-Coumaric acid (5):** yellowish-white powder; Negative ESIMS m/z 163 $[M-H]^-$. ^{1}H and ^{13}C NMR data are consistent with previously published literature.[163]

Caffeic acid (6): yellowish powder; Negative ESIMS m/z 179 $[M-H]^-$. ^{1}H and ^{13}C NMR data are consistent with previously published literature.[164]

Methyl caffeate (7): yellowish powder; Negative ESIMS m/z 193 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[165]

Ferulic acid (8): white powder; Negative ESIMS m/z 193 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[164]

Ferulate-1-methyl-ester (9): yellowish powder; Negative ESIMS m/z 207 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[165]

Isoferulic acid (10): white powder; Negative ESIMS m/z 179 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[166]

1-Isoferuloyl-β-D-glucopyranoside (11): yellowish powder; Negative ESIMS m/z 355 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[167]

Fukinolic acid (12): yellow, amorphous powder; Negative ESIMS m/z 433 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[158]

Cimicifugic acid A (13): yellow, amorphous powder; Negative ESIMS m/z 447 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[158]

Cimicifugic acid B (14): yellow, amorphous powder; Negative ESIMS m/z 447 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[158]

Cimicifugic acid D (15): yellow, amorphous powder; Negative ESIMS m/z 433 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[157]

Cimicifugic acid E (16): yellow, amorphous powder; Negative ESIMS m/z 431 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[157]

Cimicifugic acid F (17): yellow, amorphous powder; Negative ESIMS m/z 431 $[M-H]^-$. ^1H and ^{13}C NMR data are consistent with previously published literature.[157]

3.3. Results and Discussion

A black cohosh powdered extract was redissolved in 80% MeOH/water overnight. After the methanol was removed *in vacuo*, the resulting aqueous fraction was sequentially partitioned with hexane and *n*-butanol. The *n*-butanol-soluble fraction was separated by a combination of chromatographic procedures to obtain a new compound (**1**), along with seven known compounds, namely: protocatechuic acid (**3**),[161] protocatechualdehyde (**4**),[162] *p*-coumaric acid (**5**),[163] caffeic acid (**6**),[164] ferulic acid (**8**),[164,168] isoferulic acid (**10**),[166] and 1-isoferuloyl-β-D-glucopyranoside (**11**).[167] Chromatographic purification of the water-soluble fraction yielded a new compound (**2**) and other eight known compounds, namely: methyl caffeate (**7**),[165] ferulate-1-methyl-ester (**9**),[165] fukinolic acid (**12**),[158] cimicifugic acid A (**13**),[158] cimicifugic acid B (**14**),[158] cimicifugic acid D (**15**),[157] cimicifugic acid E (**16**),[157] and cimicifugic acid F (**17**).[157] Six known compounds (**3**, **4**, **5**, **9**, **11**, and **15**) were isolated for the first time from *A. racemosa*. The known compounds were identified by comparison of their spectral data (UV, MS, NMR) with published reports.

Compound **1** was isolated as a yellow amorphous powder and gave a molecular peak at *m/z* 359.0749 corresponding to $[M+1]^+$ in the positive HRESIMS, and the molecular formula $C_{18}H_{14}O_8$. The negative ESIMS of **1** exhibited significant fragment peaks at *m/z* 357 $[M-1]^-$ and 339 $[M-H_2O]^-$. Compound **1** exhibited UV (MeOH) [λ_{max} (log ε) 339 (3.34), 295 (3.28), 237 (3.31) nm] absorption characteristic of lignan containing a dibenzylbutyrolactone skeleton with a double bond at the 2,6-position of the γ-butyrolactone ring.[169-171] The ^{1}H NMR spectrum of **1** (Table 3.1) displayed two groups of typical AMX spin system signals for 1,2,4-trisubstituted phenyl ring protons at δ_H 7.30

(1H, *d*, *J* = 2.1 Hz, H-2′), 7.24 (1H, *dd*, *J* = 2.1, 8.4 Hz, H-6′), and 6.71 (1H, *d*, *J* = 8.4 Hz, H-5′), and at δ_H 7.10 (1H, *d*, *J* = 2.4 Hz, H-2″), 7.02 (1H, *dd*, *J* = 2.4, 8.4 Hz, H-6″), and 6.67 (1H, *d*, *J* = 8.4 Hz, H-5″). The ^{1}H NMR spectrum also showed one olefinic proton signal at δ_H 7.60 (1H, *s*, H-6) and a methylene signal at δ_H 4.69 and 4.43 (each 1H, *d*, *J* = 10.5 Hz, H-4). The ^{13}C NMR and DEPT spectrum of **1** showed a total of 18 signals, comprising two carbonyl carbons (δ_C 172.2 and 195.8), two quaternary carbons (δ_C 80.3 and 123.8), one methylene carbon (δ_C 77.7), one methine carbon (δ_C 144.4), and 12 aromatic signal carbons, corresponding to two groups for 1,2,4-trisubstituted phenyl ring carbons at δ_C 124.9 (C-1″), 118.1 (C-2″), 144.8 (C-3″), 149.0 (C-4″), 114.7 (C-5″), 125.3 (C-6″), 124.9 (C-1′), 115.9 (C-2′), 144.5 (C-3′), 151.5 (C-4′), 114.2 (C-5′), and 122.7 (C-6′), respectively. The cross peaks from the methine carbon C-6 (δ_C 144.4) to the olefinic proton at δ_H 7.60, and from the methylene carbon (C-4) to δ_H 4.69 and 4.43 were observed in the HSQC spectrum. The HMBC spectrum (Figure 3.2) showed the correlations from the olefinic proton (δ_H 7.60) to C-2″, C-6″, C-1, and C-3; and the correlations from the methylene protons (δ_H 4.69 and 4.43) to C-1, C-5, and C-3. In addition, the cross peaks from H-2′ to C-4′, C-6′, and C-5; H-6′ to C-2′, C-4′, and C-5 were also observed in the HMBC spectrum. The olefinic proton signal appeared at a low field (δ_H 7.60) due to the deshielding effect of β-carbonyl group (C-1),[170] indicating that compound **1** is in *E*-configuration. Due to the limited amount isolated, the absolute configuration of **1** has been tentatively assigned by analogy to (7*E*)-7,8-dehydro-7′-oxomatairesinal and 8′-hydroxyhinokinin.[172,173] Because the specific rotation of **1** is opposite in sign to that of (7*E*)-7,8-dehydro-7-oxomatairesinal, the stereochemistry of

C-8′ is suggested to be *S*-configuration. According to 1D and 2D NMR experiments, the structure of **1** was determined to be (3*S*)-2-(3″,4″-dihydroxyphenylmethylene)-3-hydroxy-3-(3′,4′-dihydroxybenzoyl)-γ-butyrolactone. The ^{1}H, ^{13}C, HSQC and HMBC spectra of compound **1** are shown in the Appendix.

Compound **2** was isolated as a yellow amorphous powder and gave a molecular peak at *m/z* 463.1252 corresponding to $[M+1]^{+}$ in the positive HRESIMS, and the molecular formula $C_{22}H_{22}O_{11}$. The negative ESIMS of **2** exhibited significant fragment peaks at *m/z* 461 $[M-1]^{-}$ and 253 $[M-208]^{-}$. The ^{1}H and ^{13}C NMR data (Table 3.2) of **2**, assigned by 1D and 2D NMR techniques that included HSQC, HMBC, and NOSEY, were similar to those of fukinolic acid,[158] except for two additional signals at δ_H 3.91 and 3.89 for methoxy groups, indicating that **2** is a fukiic acid ester derivative. The ^{1}H and ^{13}C NMR data of compound **2** showed the presence of fukiic acid and 3,4-dimethoxycinnamoyl moieties in its structure. The cross peaks from H-2′ (δ_H 7.30) to C-4′, C-6′, and C-7′; H-5′ (δ_H 7.02) to C-1′ and C-3′; H-6′ (δ_H 7.25) to C-2′ and C-4′; H-7′ (δ_H 7.82) to C-2′, C-6′, and C-9′; and H-8′ (δ_H 6.58) to C-1′ and C-9′ for the 3,4-dimethoxycinnamoyl moiety were observed in the HMBC spectrum. In addition, the fukiic acid moiety was confirmed by the correlation from H-2″ (δ_H 6.76) to C-4″, C-6″, and C-7″; H-5″ (δ_H 6.66) to C-1″ and C-3″; H-6″ (δ_H 6.61) to C-4″; H-7″ (δ_H 2.94 and 3.06) to C-2″, C-6″, and C-3; and H-2 (δ_H 5.66) to C-9′, C-7″, C-1, C-3 and C-4 in the HMBC experiment. Two methoxy groups were located by the analysis of the HMBC and NOESY spectra. In the HMBC spectrum, a methoxy proton (δ_H 3.91) showed a correlation with C-3′ (δ_C 149.5), while the another methoxy proton (δ_H 3.89) showed a correlation with C-4′ (δ_C 151.7). In the NOESY experiment, the correlation between a

methoxy protons (δ_H 3.91) with the carbon signal at δ_C 110.2 (C-2′) and between a methoxy protons (δ_H 3.89) with the carbon signal at δ_C 111.3 (C-5′), were observed. This suggests that two methoxy groups are from the 3,4-dimethoxycinnamoyl moiety (Figure 1). The site of esterification of fukiic acid by the (*E*)-3,4-dimethoxycinnamoyl moiety was confirmed by the correlation between the proton at δ_H 5.66 (H-2) with the carbon δ_C 166.6 (C-9′) in the HMBC spectrum. This correlation is commonly found in fukiic acid or piscidic acid ester derivatives from the *Actaea* species.[157,158] Because **2** has a positive optical rotation which is similar to fukinolic acid, cimicifugic acid A, and B,[158] the absolute configuration of **2** is deduced to be 2*R*, 3*S*-configurations.[157,158] Thus, compound **2** was determined to be (2*R*, 3*S*)-2-*O*-(3′,4′-dimethoxy-*E*-cinnamoyl)-3-hydroxy-3-[(3″,4″-dihydroxyphenyl)methyl]-butanedioic acid. The ^{1}H, ^{13}C, HSQC, HMBC, and NOESY spectra of compound **2** are shown in the Appendix.

The antioxidant activities of **1** and **2** were measured in the DPPH free radical assay. Both **1** and **2** showed high antioxidant activities with IC_{50} values of 26.94 and 37.40 μM, respectively. The known compounds **3-17** were also screened for their antioxidant activities in the DPPH assay and displayed antioxidant activities with IC_{50} values of 49.72, 49.62, 344.12, 58.26, 216.18, 121.41, 297.04, 289.14, 321.41, 12.91, 21.87, 23.05, 55.30, 65.46, and 151.47 μM, respectively, at a concentration comparable to ascorbic acid (105.46 μM). The radical scavenging activity on DPPH assay decreases in the following order: fukinolic acid (**12**) > compound **1** > cimicifugic acid A (**13**) > cimicifugic acid B (**14**) > compound **2** > protocatechuic acid (**3**) > protocatechualdehyde (**4**) > cimicifugic acid D (**15**) > caffeic acid (**6**) > cimicifugic acid E (**16**) > ferulic acid (**8**) > cimicifugic acid F (**17**) > isoferulic acid (**10**) > ferulate-1-methyl ester (**9**) >

1-isoferulate-β-D-glucopyranoside (**11**) > *p*-coumaric acid (**5**). These results are in agreement with previous reports of structure-activity relationship of antioxidant hydroxycinnamic acid derivatives. For example, the presence of the *O*-hydroxy group of 4-hydroxycinnamic acid (*p*-coumaric acid) increases its antioxidant activity by further stabilizing the phenoxyl radical; the presence of the *O*-electron donating methoxy group of *p*-hydroxycinnamic acid also increases the activity but less effectively than the *O*-hydroxy group.[38,46] Thus, the antioxidant activity is in the order caffeic acid (**6**) > ferulic acid (**8**) > isoferulic acid (**10**) > *p*-coumaric acid (**5**). The esterification of hydroxycinnamic acid derivative by alkyl group or sugar moiety decreases its antioxidant activity,[38,46] such as the antioxidant activity of methyl caffeate (**7**) < caffeic acid (**6**), ferulate-1-methyl ester (**9**) < ferulic acid (**8**), and 1-isoferulate-β-D-glucopyranoside (**11**) < isoferulic acid (**10**). Fukiic acid ester derivatives (**2**, **12-14**) and piscidic acid ester derivatives (**15-17**) display higher activity than hydroxycinnamic acid derivatives (**5-11**), probably due to the additional catechol ring. The antioxidant activity of both groups followed the pattern above, which showed the activity of fukinolic acid (**12**) > cimicifugic acid A (**13**) > cimicifugic acid B (**14**) > compound **2** and cimicifugic acid D (**15**) > cimicifugic acid E (**16**) > cimicifugic acid F (**17**).

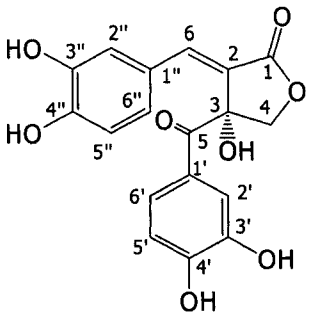
According to its chemical structure, compound **1** is identified as a member of the dibenzyl-γ-butyrolactones type of lignans.[174] Considering that black cohosh is rich in phenylpropanoids such as caffeic acid and isoferulic acid, compound **1** may be formed by two phenylpropanoid units via phenol oxidation coupling, as in the case of other lignans. This could be further verified by the isolation of several phenylpropanoid esters from black cohosh.[92] Compound **2**, a phenylpropanoid ester dimer, probably formed between

fukiic acid and (*E*)-3,4-dimethoxycinnamic acid via esterification. Fukiic acid and (*E*)-3,4-dimethoxycinnamic acid have been previously isolated from the Asian *Actaea* species, but no ester linked-dimer derivative of these two compounds had been reported.[158]

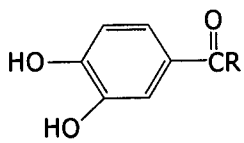
Because of their structural similarity to enterolactone and fukinolic acid, respectively, Compounds **1** and **2** were evaluated for their capacity to stimulate MCF-7 cell proliferation. Enterolactone and fukinolic acid have been reported to have a stimulating effect on MCF-7 cell proliferation.[97,160] The growth effect on MCF-7 cells of **1** and **2** were carried out as described in section 3.2.5. Estradiol and enterolactone were also tested as positive controls in this assay. It was found that the maximum cell proliferation of MCF-7 cells was obtained with 3.7 nM estradiol (3.6-fold). Both compounds **1** and **2** induced only a slight increase in cell proliferation of 1.24-fold (5 μg/mL, 14 μM) ($p = 0.003$) and 1.14-fold (10 μM) ($p = 0.82$), respectively, when compared with untreated cells. Enterolactone stimulated cell proliferation of 1.84-fold (5 μg/mL, 16.8 μM), when compared with untreated cells.

3.4. Conclusion

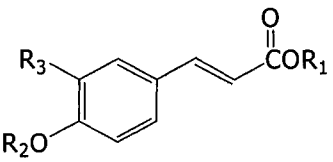
Two novel polyphenolic compounds (**1** and **2**), along with 15 known polyphenolic compounds (**3-17**) were isolated from the standardized black cohosh powdered extract. Two novel compounds displayed high activity in the DPPH assay and weak activity on growth of proliferation of MCF-7 cells.



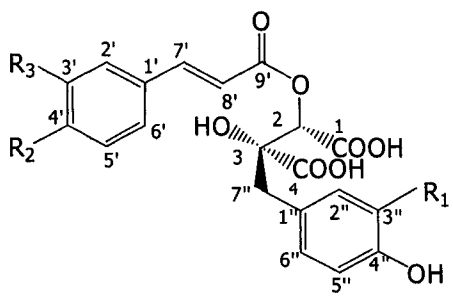
1



3 R =OH
4 R = H

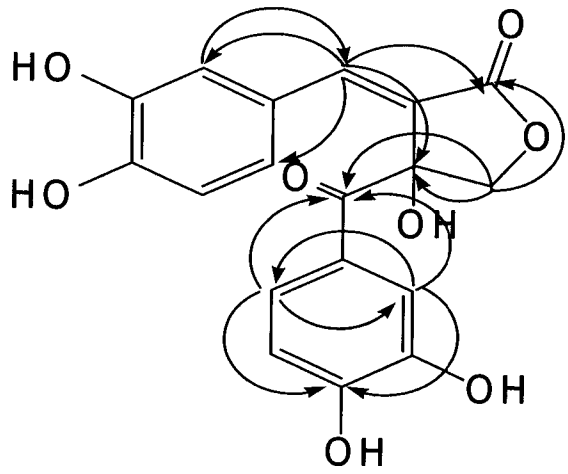


5 R_1 = H R_2 = H R_3 = H
6 R_1 = H R_2 = H R_3 = OH
7 R_1 = CH_3 R_2 = H R_3 = OH
8 R_1 = H R_2 = H R_3 = OCH_3
9 R_1 = CH_3 R_2 = H R_3 = OCH_3
10 R_1 = H R_2 = CH_3 R_3 = OH
11 R_1 = Glc R_2 = CH_3 R_3 = OH



2 R_1 = OH R_2 = OCH_3 R_3 = OCH_3
12 R_1 = OH R_2 = OH R_3 = OH
13 R_1 = OH R_2 = OH R_3 = OCH_3
14 R_1 = OH R_2 = OCH_3 R_3 = OH
15 R_1 = H R_2 = OH R_3 = OH
16 R_1 = H R_2 = OH R_3 = OCH_3
17 R_1 = H R_2 = OCH_3 R_3 = OH

Figure 3.1. Polyphenolic constituents isolated from *A. racemosa*



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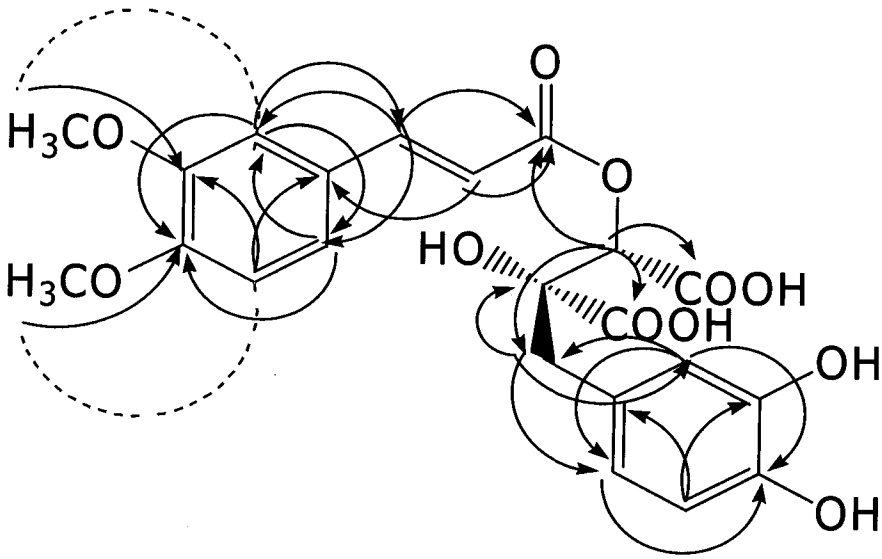

2

HMBC H⟶C

NOSEY H--------H

Figure 3.2. Key HMBC and NOESY correlations of compounds 1 and 2

Table 3.1. NMR Spectral data of compound 1 in CD_3OD

Position	δ_C	δ_H (int., mult., J in Hz)	HMBC (^{13}C No.)
1	172.2 s		
2	123.8 s		
3	80.3 s		
4	77.7 t	4.43 (1H, d, 10.5)	1, 3, 5
		4.69 (1H, d, 10.5)	
5	195.8 s		
6	144.4 d	7.60 (1H, s)	2″, 6″, 1, 3
1′	124.9 s		
2′	115.9 d	7.30 (1H, d, 2.1)	4′, 6′, 5
3′	144.5 s		
4′	151.5 s		
5′	114.2 d	6.71 (1H, d, 8.4)	1′, 3′
6′	122.7 d	7.24 (1H, dd, 8.4, 2.1)	2′, 4′, 5
1″	124.9 s		
2″	118.1 d	7.10 (1H, d, 2.4)	4″, 6″, 6
3″	144.8 s		
4″	149.0 d		
5″	114.7 d	6.67 (1H, d, 8.4)	1″, 3″
6″	125.3 d	7.02 (1H, dd, 8.4, 2.4)	2″, 4″

Table 3.2. NMR Spectral data of compound 2 in CD_3OD

Position	δ_C	δ_H (int., mult., J in Hz)	HMBC (^{13}C No.)	NOSEY (^{1}H No.)
1	169.5 t			
2	76.5 d	5.66 (1H, s)	1, 3, 4, 9′, 7″	7″
3	78.7 s			
4	173.5 s			
1′	127.4 s			
2′	110.2 d	7.30 (1H, d, 1.8)	4′, 6′, 7′	7′, 8′
3′	149.5 s			
4′	151.7 s			
5′	111.3 d	7.02 (1H, d, 8.4)	1′, 3′	6′
6′	122.9 d	7.25 (1H, dd, 8.4, 1.8)	2′, 4′	5′, 7′, 8′
7′	146.2 d	7.82 (1H, d, 15.9)	2′, 6′, 9′	2′, 6′
8′	114.3 d	6.58 (1H, d, 15.9)	1′, 9′	2′, 6′
9′	166.6 s			
1″	126.7 s			
2″	117.4 d	6.76 (1H, d, 1.8)	4″, 6″, 7″	
3″	114.3 s			
4″	143.9 s			
5″	114.5 d	6.66 (1H, d, 8.1)	1″, 3″	
6″	121.6 d	6.61 (1H, dd, 8.1,1.8)	4″	
7″	40.8 t	2.94 (1H, d, 13.8) 3.06 (1H, d, 13.8)	2″, 6″, 3	2
3′-OMe	55.1 q	3.91 (3H, s)	3′	2′
4′-OMe	55.0 q	3.89 (3H, s)	4′	5′

Table 3.3. DPPH free radical scavenging activity for isolated compounds[a]

Compound	IC_{50} (μM)
Compound **1**	26.94 ± 0.23
Compound **2**	37.40 ± 0.53
Protacatechuic acid (**3**)	49.72 ± 0.80
Protocatechualdehde (**4**)	49.62 ± 0.78
p-Coumaric acid (**5**)	344.12 ± 3.46
Caffeic acid (**6**)	58.26 ± 0.33
Methyl caffeate (**7**)	216.18 ± 0.26
Ferulic acid (**8**)	121.40 ± 1.33
Ferulate-1-methyl-ester (**9**)	297.04 ± 4.26
Isoferulic acid (**10**)	289.14 ± 4.21
1-Isoferuloyl-β-D-glucopyranoside (**11**)	321.44 ± 3.86
Fukinolic acid (**12**)	12.91 ± 0.29
Cimicifugic acid A (**13**)	21.88 ± 0.58
Cimicifugic acid B (**14**)	23.05 ± 0.07
Cimicifugic acid D (**15**)	55.30 ± 0.91
Cimicifugic acid E (**16**)	65.46 ± 0.45
Cimicifugic acid F (**17**)	151.47 ± 6.64
Ascorbic acid[b]	105.47 ± 2.71

[a]Values are the mean ± SD of triplicate determinations [b]Positive control

Chapter 4

Analysis of polyphenolic compounds of *Actaea* species

4.1. Intoduction

Despite the documented clinical efficacy of black cohosh for the treatment of menopausal symptoms,[69] little is known about its active compounds. Formononetin, an estrogenic isoflavone, has been reported from black cohosh[77,78] However, subsequent studies have verified that there is no detectable formononetin in black cohosh.[82-84] Even though triterpenoid glycosides are recognized as a major group of compounds in black cohosh, no studies have proven them to be the active compounds responsible for alleviating menopausal symptoms. Therefore, some recent studies have looked toward other compounds to explain the bioactivity of black cohosh. Due to the lack of estrogenic activity of black cohosh extract, and the contradictory evidence regarding the presence of formononetin,[79-81] the mechanism of action of black cohosh may not involve hormonal signaling through the estrogenic receptors. Some investigators have proposed that the mechanism of action of black cohosh may instead involve the serotonergic pathway, and have demonstrated the affinity of black cohosh extract to bind with serotonin receptors.[85] More research on the bioactive constituents of black cohosh is obviously needed.

Due to the increasing demand for black cohosh, mistakenly collecting the closely related species where their ranges overlap with black cohosh may occur. Although phytochemical investigations on this genus have been conducted and many triterpenoid glycosides and polyphenolic derivatives similar to those of black cohosh have been reported,[92,93,153,158,175] there are currently no reports of phytochemical variation among wild populations of native North American *Actaea* species. It is important to determine and compare the chemical profiles among them in order to distinguish black cohosh (*A. racemosa*) from its closely related species. Some investigators have used DNA

fingerprinting techniques to distinguish *A. racemosa* from three related species. However, Zerega *et al.* could not analyze all samples due to the instability of DNA and the absence of DNA in a typical alcoholic extract,[176] showing the difficulties inherent in these studies. Chemical techniques, such as HPLC are an alternative method that can be used to assist in the identification not only of the plant material, but also of botanical products made with black cohosh extracts.

Interest in black cohosh polyphenols has increased recently. Burdette *et al.* examined the antioxidant activity of the black cohosh methanolic extract, as well as that of nine polyphenolic compounds isolated from black cohosh using the DPPH assay.[75] They found that the extract and all the polyphenols exhibited antioxidant activity. Since the mechanism of action of black cohosh has not been determined, some authors propose that it may be the result of a complex synergistic action among its chemical constituents.[61] The clinical effect of black cohosh may, in part, be related to its antioxidant activity. Some investigators have found increased oxidative stress in menopausal women which can reduced after giving the antioxidant estradiol.[177,178] Due to the strong UV absorption of polyphenols, high-performance liquid chromatography with diode array detection method (HPLC-DAD) is an appropriate analytical method.[179] Numerous studies have been developed for the qualitative and quantitative analyses of polyphenolic compounds including flavonoids and caffeic acid derivatives.[180-187] In particular, two analytical methods have been reported for black cohosh. Jiang *et al.* reported an HPLC-DAD method to quantify six polyphenols.[186] Li *et al.* reported an HPLC method with mass spectrometry to identify polyphenols in black cohosh extracts.[187] However, neither method was validated for quality control purposes.

The current study was therefore conducted to qualitatively and quantitatively analize the eight major antioxidant polyphenols isolated from black cohosh, in four North American *Actaea* species. In this study, a RP-HPLC method with DAD was developed and validated. The antioxidant activities of the extract of black cohosh and three related species were also tested and compared.

4.2. Experimental Procedures

4.2.1. Chemicals

HPLC grade MeCN, and methanol (J. T. Baker, Phillipsburg, USA) were used for sample preparation and HPLC analysis. Reagent grade methanol, hexane, *n*-butanol, and acetic acid (VWR, Seattle, WA, USA) were used for the extraction and separation of reference compounds 1-8. DPPH (Sigma Chemical Co., St. Louis, MO, USA) and dimethyl sulfoxide (Aldrich, Milwaukee, USA) were used for the DPPH radical scavenging assay. The stationary phases for open column chromatography were octadecyl (C_{18} 40 μm) (J. T. Baker, Phillipsburg, USA), Sephadex LH-20 (25-100 μm; Pharmacia Fine Chemicals, Piscataway, NJ), and Diaion HP-20 (Supelco, Bellefonte, USA). All reference compounds were prepared by column chromatography and preparative HPLC following procedures described in section 3.2.6.

4.2.2. Equipment

HPLC analyses were carried out on a Waters 2695 Separations Module (Milford, MA, USA) equipped with a Waters 996 photodiode array detector and Waters Empower software using a Phenomenex Aqua C_{18} column (4.6 × 250 mm, 5 μm). The chromatographic elution was achieved using a gradient solvent system consisting of 5% aqueous acetic acid (A) and MeCN (B). The gradients were started with 5% B, and

reached 15% B at 8 min; B was kept at 15% B from min 8 to min 20, then increased until it reached 38% at 55 min, and 100% B at 56-59 min; the system was returned to initial conditions at 60 min and held for 5 min for the next injection. The flow rate was 1 mL/min. The UV-vis spectra were recorded from 200-400 nm, while absorbance at 320 nm was used for quantification.

Preparative HPLC was carried out using a Waters 600 controller with a Waters 486 tunable absorbance detector and Waters Empower software with a Phenomenex Nucleosil C_{18} column (21.1 × 250 mm, 10 μm) and an isocratic solvent system of 0.1% aqueous acetic acid/MeCN (80:20), a flow rate of 10 mL/min, column at room temperature, and 60 min run time.

UV spectra were obtained in a Lambda 2 UV-vis spectrophotometer (Perkin-Elmer, Boston, MA). ^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker AMX-300 MHz NMR spectrometer, operating at 300 and 75 MHz, respectively. All NMR spectra were obtained in CD_3OD, with chemical shifts expressed in δ and coupling constant (J) in Hertz. MS was performed on a Thermo Finnigan LCQ instrument (San Jose, CA) in the negative mode. The instrument was equipped with an electrospray ionization (ESI) source and controlled by Xcalibur software. Samples were dissolved in MeOH and introduced by direct injection. The capillary voltage was 10 V, the spray voltage was 4.5 kV, and the tube lens offset was 0 V. The capillary temperature was 230°C.

4.2.3. Plant material

The air-dried rhizomes and roots of *A. rubra* (lot number SK200301), *A. pachypoda* (lot number DB727), and *A. podocarpa* (lot number DB710303) were

purchased from Botanical Liaison, LLC, Boulder, CO, USA. The air-dried rhizomes and roots of *A. racemosa* (lot number 9-2677) and the standardized *A. racemosa* extract (lot number 9-2044) were prepared by PureWorld Botanicals Inc., South Hackensack, NJ, USA. Voucher specimens of *A. rubra, A. pachypoda, A. podocarpa,* and *A. racemosa* were deposited at the herbarium at The New York Botanical Garden.

4.2.4. Sample preparation

Ground rhizomes and roots (1.0 g) were extracted by sonication (30 min) in 15 mL 80% MeOH/water in 20 mL PTFE-capped vials. After cooling to room temperature, the supernatant solution was filtered into a 100 mL round bottom flask. The residue was reextracted two more times as described above. The combined solution was evaporated to dryness *in vacuo* at 40°C. The dried extract was kept at -20°C before HPLC analysis.

A portion of dried extract was dissolved in 80% MeOH/water to generate a solution with a concentration of 10 mg/mL, which was sebsequently filtered through a 0.45-μm membrane filter just prior to HPLC analysis.

4.2.5. Standard preparation

Stock solutions containing 1.06 mg/mL of caffeic acid, 1.00 mg/mL of ferulic acid, 1.04 mg/mL of isoferulic acid, 1.02 mg/mL of fukinolic acid, 1.00 mg/mL of cimicifugic acid A, 1.00 mg/mL of cimicifugic acid B, 1.04 mg/mL of cimicifugic acid E, and 1.03 mg/mL of cimicifugic acid F in 80% MeOH/water were prepared. Solutions were stored at 4°C in the dark.

4.2.6. Calibration curves

For calibration purposes, working solutions were freshly prepared by diluting each stock solution with 80% MeOH/water. Calibration curves were established on five

data points covering the following concentration ranges: 1.04-265 μg/mL for caffeic acid, 0.98-250 μg/mL for ferulic acid, 1.02-260 μg/mL for isoferulic acid, 1.00-255 μg/mL for fukinolic acid, 0.98-250 μg/mL for cimicifugic acid A, 0.98-250 μg/mL for cimicifugic acid B, 1.02-260 μg/mL for cimicifugic acid E, and 1.03-260 μg/mL for cimicifugic acid F. Samples of each standard solution (10 μL) were used for HPLC analysis. Each calibration curve was obtained by plotting the peak area of a standard at each level prepared versus the concentration of the standard.

4.2.7. Identification of constituents

Peaks were identified on the basis of their retention time (t_R) values and UV spectra by comparison with the standard solution. Ambiguous peaks were confirmed by spiking the extracts with pure standard.

4.2.8. Assay validation

Validation of this assay was in compliance with the Guidelines of the International Conference on Harmonization of Technical Requirements for the Registration of Pharmaceuticals for Human Use.[188]

4.2.8.1. Recovery

For the purpose of the recovery study, caffeic acid, fukinolic acid, and cimicifugic acid E were chosen to be representative of the three main classes of polyphenolic constituents of black cohosh: caffeic acid, fukiic acid ester, and piscidic acid ester derivatives. Recovery for the analytical method was evaluated by adding known amounts of the three representative standards (ca. 2 mg each) to ground rhizomes and roots of *A. racemosa* (1 g) prior to extraction. The sample was prepared as previously described in section 2.4. The recovery was determined by subtracting the values obtained for the

control matrix preparation from the sample added with standards, the resulting values were divided by the amount of standards, and expressed as percentage.

4.2.8.2. Precision and accuracy

Inter-day precision and accuracy were evaluated by performing six injections of a standard mixture solution at three concentration levels for three consecutive days. Intra-day precision and accuracy were evaluated by performing six injections of a standard mixture solution at three concentration levels over one day. The precision at each concentration was determined by the percent relative standard deviation (R.S.D., %) of the measured concentration of standards, while the accuracy was accessed for each standard and expressed as percent relative error (R.E., %) by comparing the nominal concentration with measured concentration.

4.2.8.3. Limits of detection and quantification

The limits of detection (LOD) and limits of quantification (LOQ) were calculated as signal-to-noise ratios with nominal values of 3:1 and 10:1, respectively. LOD and LOQ experiments were evaluated by performing three injections of individual standard solutions at the LOD and LOQ concentrations.

4.3. Results and Discussion

4.3.1. Optimization of separation conditions

The composition of several mobile phases has been published for the analysis of hydroxycinnamic acid derivatives such as caffeic acid and ferulic acid.[186,187,189-191] Jiang *et al.* used 10% aqueous formic acid-MeCN gradients as mobile phase on a HPLC-DAD method,[186] while others used gradients of 0.05% aqueous acetic acid and MeCN in a LC-MS method for the identification polyphenols in black cohosh.[187] Formic or acetic

acids were added in those two gradient systems because they can reduce the peak tailing of polyphenolic derivatives. To develop a suitable solvent system for HPLC separation of the eight major polyphenols isolated from black cohosh, a binary solvent mixture of formic acid (or acetic acid) and MeCN was examined after varying the concentration of acids. No significant difference between formic acid and acetic acid was found. The concentration of acid from 5-10% provided base line separation. A lower concentration of acid (5%) was chosen to protect column stability for long-term use. In this study, mixtures of 5% aqueous acetic acid and MeCN were used as mobile phase. To optimize the mobile phase for a binary gradient profile, different compositions of MeCN in 5% aqueous acetic acid were studied. The gradient conditions of MeCN and 5% aqueous acetic acid described in 4.2.2 gave baseline separation of all polyphenolic reference compounds and of all four *Actaea* species extracts.

To select a wavelength for quantification, the UV-vis spectra were recorded from 200-400 nm due to their UV-vis spectra. The 320 nm wavelength gave an optimized absorbance for all quantified polyphenols. A typical HPLC-DAD chromatogram at 320 nm of a standard mixture is shown in Figure 4.2. The retention time and UV spectral data of the standards are reported in Table 4.1.

4.3.2. Assay validation

The method was validated with respect to linearity, precision, accuracy, recovery, and sensitivity. Linear regression analysis for each standard was performed by the external standard method. Good linearity of five-point calibration curves were obtained for all standards between peaks area and concentration ($r^2 > 0.99$) over the range test (ca.

1-250 μg/mL). The parameters of each calibration curve (slope, intercept, and correlation coefficient) are reported in Table 4.2.

The average recovery (%) and percent relative standard deviation (R.S.D., %) for caffeic acid, fukinolic acid, and cimicifugic acid E were 99.92 (0.08), 101.18 (0.22), and 101.33 (0.13), respectively (Table 4.3). Similar recoveries for closely related compounds would be expected.

Intra- and inter-day analyses of the same solution containing all polyphenols in three different concentrations (1, 30, and 250 μg/mL) were used to validate the precision and accuracy of the method (Table 4.4). The precision was calculated as R.S.D. (%) at the different concentrations of the standard mixture solution; the R.S.D. of all standards varied between 0.15 and 1.97% (n=6) on the same day, but it was from 0.17 to 1.99% (n=18) on different days. The accuracy was calculated as R.E. (%). The observed concentrations were in good agreement with the actual concentration. The R.E. (%) ranged from –1.91 to 1.83% (n=6) on the same day and from –1.82 to 1.96 % (n=18) between days. These data demonstrated that the method established for the measurement of the polyphenols is accurate.

The limits of detections (LOD) and limits of quantification (LOQ) of all eight polyphenols were established by means of the baseline noise method. The LOD and LOQ for these compounds were found to be in the range of 24.8-78.3 ng/mL and 82.7-260.9 ng/mL (Table 4.5).

4.3.3. Quantification of polyphenols from *Actaea* species

HPLC chromatograms of the four *Actaea* extracts are shown in Figure 4.3, and the content of the eight polyphenolic compounds in these extracts are found in Table 4.6.

The total weight of the eight main polyphenolic compounds is 3.29% in the extract for *A. pachypoda*; 5.97% for *A. podocarpa*; 4.03% for *A. racemosa*; and 8.33% for *A. rubra*. *A. racemosa*, and the related species *A. rubra* have a similar chemical profile, differing in the ratio of polyphenols. The other two species have a polyphenolic profile that is distinct from *A. racemosa*. *A. pachypoda* lacks cimicifugic acid F and *A. podocarpa* lacks isoferulic acid. Among the eight polyphenolic compounds, fukinolic acid is the most abundant polyphenol in *A. pachypoda* and *A. racemosa*, while the most abundant polyphenols in *A. podocarpa* and *A. rubra* are cimicifugic acid A and cimicifugic acid B, respectively. The *A. pachypoda* extract contains only two main polyphenols, fukinolic acid and cimicifugic acid A, and these compounds account for 90.29% of the total polyphenols found in the extract. The *A. podocarpa* extract also contains 2 main polyphenols, cimicifugic acid A and cimicifugic acid E, and these compounds account for 97.24% of the total polyphenols found in the extract.

This HPLC method can be used to clearly distinguish *A. racemosa* from *A. pachypoda* and *A. podocarpa*. The HPLC chromatograms show that the *A. racemosa* extracts contain all eight major polyphenols, while *A. pachypoda* contains only two major polyphenols, fukinolic acid and cimicifugic acid A; cimicifugic acid F was not found in the extract. In the same manner, *A. podocarpa* contains two major polyphenols, cimicifugic acid A and cimicifugic acid E, but isoferulic acid was not found in the extract. Other authors have developed an HPLC method to identify black cohosh based on its triterpenoid constituents.[96] *A. racemosa* can be distinguished from the related Asian species *A. cimicifuga* by the presence of the triterpenoid glycoside cimicifugoside M. However, it is difficult to interpret triterpenoid chromatograms due to overlapping peaks.

The analysis of polyphenolic compounds provides a simpler spectrum, which still allows us to distinguish black cohosh from its closely related species.

4.3.4. DPPH radical scavenging activity of *Actaea* extracts

The DPPH radical scavenging activities of the extract of four *Actaea* species are reported in Table 4.7. The average IC_{50} values of radical scavenging activity for *A. pachypoda, A. podocarpa*, *A. racemosa*, and *A. rubra* are 191.59, 111.09, 144.64, and 79.31 μg/mL, respectively. The antioxidant activity of the extracts correlates to their polyphenolic content. Of the four species, *A. rubra* is the richest in total polyphenols (Table 4.6) and its extracts displayed the highest DPPH radical scavenging activity (Table 4.7), whereas *A. pachypoda* has the lowest content of total polyphenols and its extracts displayed the lowest for DPPH radical scavenging activity.

4.4. Conclusion

A new RP-HPLC method with DAD was developed and validated to quantify eight major polyphenol compounds in four North American *Actaea* species. A baseline separation of all eight polyphenols has been achieved in the extracts of the four species. This study provides initial phytochemical profiles of polyphenols for four North American *Actaea* species. However, more studies are needed to examine the differences in polyphenolic profiles among various wild populations of these four species.

Table 4.1. Retention time and UV band of quantified polyphenol compounds

Compound	$t_R \pm$ SD (min)	UV band (nm)
Caffeic acid (**1**)	12.26 ± 0.13	240, 295 sh, 324 max
Ferulic acid (**2**)	21.18 ± 0.31	240, 295 sh, 323 max
Isoferulic acid (**3**)	23.89 ± 0.38	240, 295 sh, 323 max
Fukinolic acid (**4**)	25.77 ± 0.55	236, 288 sh, 330.5 max
Cimicifugic acid A (**5**)	35.86 ± 0.27	236, 288 sh, 329 max
Cimicifugic acid B (**6**)	37.12 ± 0.25	236, 288 sh, 329 max
Cimicifugic acid E (**7**)	40.86 ± 0.19	236, 288 sh, 329 max
Cimicifugic acid F (**8**)	41.81 ± 0.19	236, 288 sh, 329 max

Table 4.2. Calibration curves of quantified polyphenol compounds[a]

Compound	Slope (*m*)	Intercept (*b*)	Correlation coefficient (r^2)	Linear range (μg/mL)
Caffeic acid	56806	+5350	0.9999	1.04-265
Ferulic acid	51345	-35580	0.9970	0.98-250
Isoferulic acid	45991	-63864	0.9984	1.02-260
Fukinolic acid	25868	-5786	0.9999	1.00-255
Cimicifugic acid A	23099	-74804	0.9998	0.98-250
Cimicifugic acid B	25258	-2672	0.9999	0.98-250
Cimicifugic acid E	21513	-41574	0.9999	1.02-260
Cimicifugic acid F	20194	+21490	0.9991	1.03-260

[a]For each curve the equation is $y = mx+b$, where y is the peak area, x is the concentration of the analyte (μg/mL), m is the slope, b is the intercept; r^2 is the correlation coefficient.

Table 4.3. Recovery studies for caffeic acid, fukinolic acid, and cimicifugic acid E

Compound	Added amount (mg)	Recovery (%)	Mean (n =3)	R.S.D. (%)
Caffeic acid	2.12	99.83-99.97	99.92	0.08
Fukinolic acid	2.10	101.03-101.44	101.18	0.22
Cimicifugic acid E	2.38	101.24-101.47	101.33	0.13

Table 4.4. Precision and accuracy of the method

Compounds	Standard concentration (μg/mL)	Intra-day			Inter-day		
		Measured amount[a] (μg/mL)	R.S.D. (%)	R.E. (%)	Measured amount[b] (μg/mL)	R.S.D. (%)	R.E. (%)
Caffeic acid	1.03	1.01 ± 0.01	0.78	-1.60	0.99 ± 0.05	0.86	-1.82
	30.65	30.51 ± 0.06	0.19	-0.45	30.83 ± 0.29	0.93	0.58
	265.50	264.45 ± 0.82	0.31	-0.38	267.94 ± 3.33	1.24	0.92
Ferulic acid	1.54	1.52 ± 0.05	0.63	-1.91	1.51 ± 0.06	1.59	-1.49
	30.55	30.34 ± 0.05	0.17	-0.67	30.73 ± 0.34	1.15	0.58
	322.42	321.08 ± 1.04	0.33	-0.42	325.83 ± 4.51	1.39	1.06
Isoferulic acid	2.36	2.40 ± 0.04	0.63	1.77	2.39 ± 0.06	1.61	1.71
	30.55	30.45 ± 0.05	0.15	-0.32	30.79 ± 0.31	1.06	0.78
	273.58	271.53 ± 0.83	0.36	-0.75	275.46 ± 3.72	1.36	0.69
Fukinolic acid	1.09	1.07 ± 0.04	1.89	-1.28	1.06 ± 0.07	1.97	-1.73
	28.96	29.31 ± 0.11	0.38	1.19	29.24 ± 0.09	0.31	0.96
	256.80	257.42 ± 0.74	0.29	-0.24	259.55 ± 2.12	0.82	1.07
Cimicifugic acid A	2.24	2.25 ± 0.02	1.92	1.60	2.26 ± 0.07	1.99	1.45
	31.88	31.94 ± 0.06	0.17	0.20	31.92 ± 0.05	0.17	0.13
	268.42	267.59 ± 0.69	0.26	-0.31	269.74 ± 2.06	0.78	0.49
Cimicifugic acid B	1.07	1.08 ± 0.01	0.17	0.20	1.08 ± 0.05	1.87	0.39
	22.80	23.02 ± 0.12	0.52	0.99	22.98 ± 0.08	0.36	0.80
	216.86	217.54 ± 0.50	0.23	0.34	219.23 ± 1.64	0.75	1.12
Cimicifugic acid E	2.69	2.71 ± 0.01	1.65	1.83	2.73 ± 0.09	1.91	1.96
	32.55	32.60 ± 0.06	0.18	0.16	32.71 ± 0.12	0.39	0.51
	252.40	251.88 ± 0.67	0.26	-0.20	254.18 ± 2.17	0.86	0.70
Cimicifugic acid F	1.09	1.07 ± 0.03	1.97	-1.02	1.10 ± 0.07	1.63	0.26
	27.28	27.34 ± 0.07	0.26	0.24	27.34 ± 0.07	0.26	0.22
	170.65	171.01 ± 0.30	0.17	0.20	172.53 ± 1.30	0.75	1.10

[a]Data are the mean ± SD of six injections, [b]Data are the mean ± SD of eighteen injections

Table 4.5. Limits of detection (LOD) and limits of quantification (LOQ)

Compound	LOD (ng/mL)[a]	LOQ (ng/mL)
Caffeic acid	24.8 ± 0.15	82.7 ± 0.51
Ferulic acid	51.6 ± 1.17	172.0 ± 3.89
Isoferulic acid	52.9 ± 1.15	176.3 ± 3.82
Fukinolic acid	66.9 ± 0.59	222.9 ± 1.96
Cimicifugic acid A	66.3 ± 1.01	220.9 ± 3.37
Cimicifugic acid B	69.9 ± 0.52	233.2 ± 1.73
Cimicifugic acid E	72.8 ± 0.94	242.6 ± 3.08
Cimicifugic acid F	78.3 ± 0.94	260.9 ± 3.13

[a]Data are the mean ± SD of triplicate determinations

Table 4.6. Content of the eight major polyphenols in *Actaea* species

Compound	*A. pachypoda* (%w/ext.)[a]	*A. podocarpa* (%w/ext.)	*A. racemosa* (%w/ext.)	*A. rubra* (%w/ext.)
Caffeic acid	$0.0636 \pm 5.5\times10^{-4}$	$0.0022 \pm 2.9\times10^{-5}$	$0.1188 \pm 2.3\times10^{-4}$	$0.0158 \pm 4.7\times10^{-4}$
Ferulic acid	$0.0712 \pm 3.0\times10^{-4}$	$0.1008 \pm 1.4\times10^{-4}$	$0.1486 \pm 2.5\times10^{-4}$	$0.0309 \pm 2.3\times10^{-3}$
Isoferulic acid	$0.0177 \pm 1.1\times10^{-4}$	<LOQ	$0.5737 \pm 6.2\times10^{-4}$	$0.0759 \pm 7.4\times10^{-4}$
Fukinolic acid	$1.8887 \pm 8.7\times10^{-3}$	$0.0442 \pm 3.0\times10^{-4}$	$1.1598 \pm 1.1\times10^{-3}$	$2.1887 \pm 2.9\times10^{-3}$
Cimicifugic acid A	$1.0825 \pm 5.0\times10^{-3}$	$2.9577 \pm 4.4\times10^{-3}$	$0.5883 \pm 1.9\times10^{-3}$	$1.9632 \pm 1.0\times10^{-3}$
Cimicifugic acid B	$0.0532 \pm 1.4\times10^{-3}$	$0.0089 \pm 8.9\times10^{-3}$	$1.0389 \pm 7.3\times10^{-3}$	$3.1688 \pm 6.1\times10^{-3}$
Cimicifugic acid E	$0.1149 \pm 9.0\times10^{-3}$	$2.8446 \pm 5.1\times10^{-3}$	$0.1850 \pm 7.4\times10^{-4}$	$0.3851 \pm 3.7\times10^{-4}$
Cimicifugic acid F	<LOQ	$0.0087 \pm 5.1\times10^{-4}$	$0.2201 \pm 3.3\times10^{-4}$	$0.5000 \pm 8.1\times10^{-4}$
Total phenolics	3.2908	5.9671	4.0332	8.3284

[a] Data are the mean ± SD of triplicate determinations

Table 4.7. DPPH free radical scavenging activity of the *Actaea* extracts[a]

Extract or compound	IC_{50} (μg/mL)
A. pachypoda extract	191.59 ± 3.66
A. podocarpa extract	111.09 ± 1.06
A. racemosa extract	144.64 ± 1.77
A. rubra extract	79.31 ± 2.67
Ascorbic acid[b]	18.56 ± 0.48

[a]Values are the mean ± SD of triplicate determinations. [b]Positive control

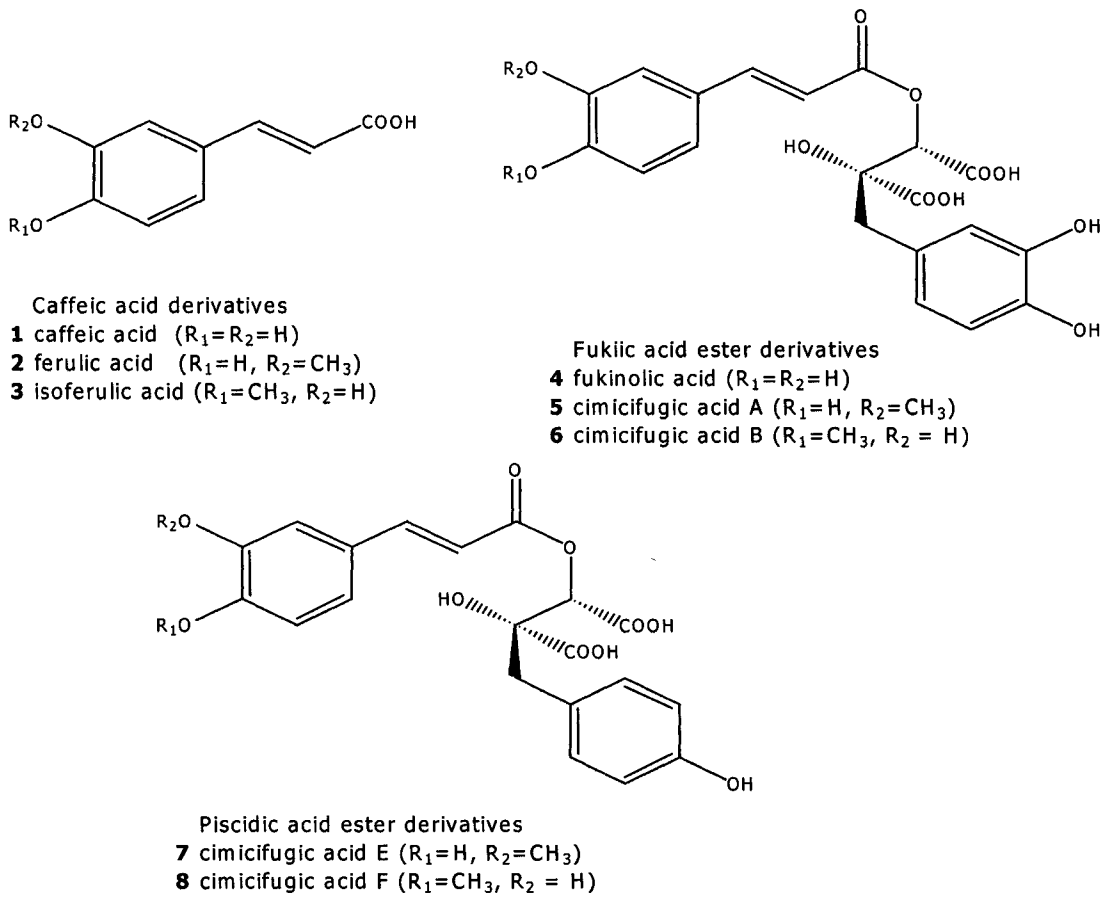

Figure 4.1. Structures of the quantified polyphenolic compounds, caffeic acid (1), ferulic acid (2), isoferulic acid (3), fukinolic acid (4), cimicifugic acid A (5), cimicifugic acid B (6), cimicifugic acid E (7), and cimicifugic acid F (8)

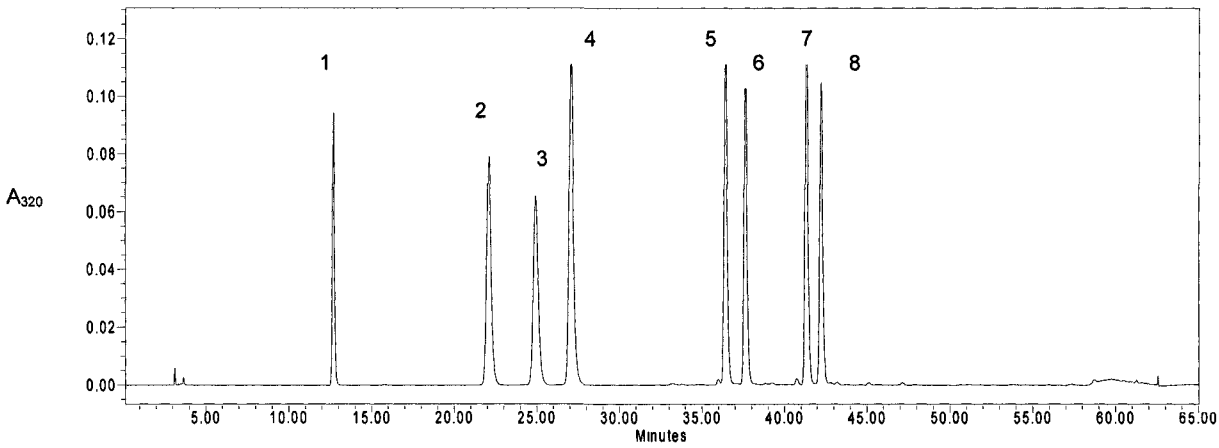

Figure 4.2. HPLC-DAD chromatogram of the eight polyphenols at 320 nm: caffeic acid (1), ferulic acid (2), isoferulic acid (3), fukinolic acid (4), cimicifugic acid A (5), cimicifugic acid B (6), cimicifugic acid E (7), and cimicifugic acid F (8)

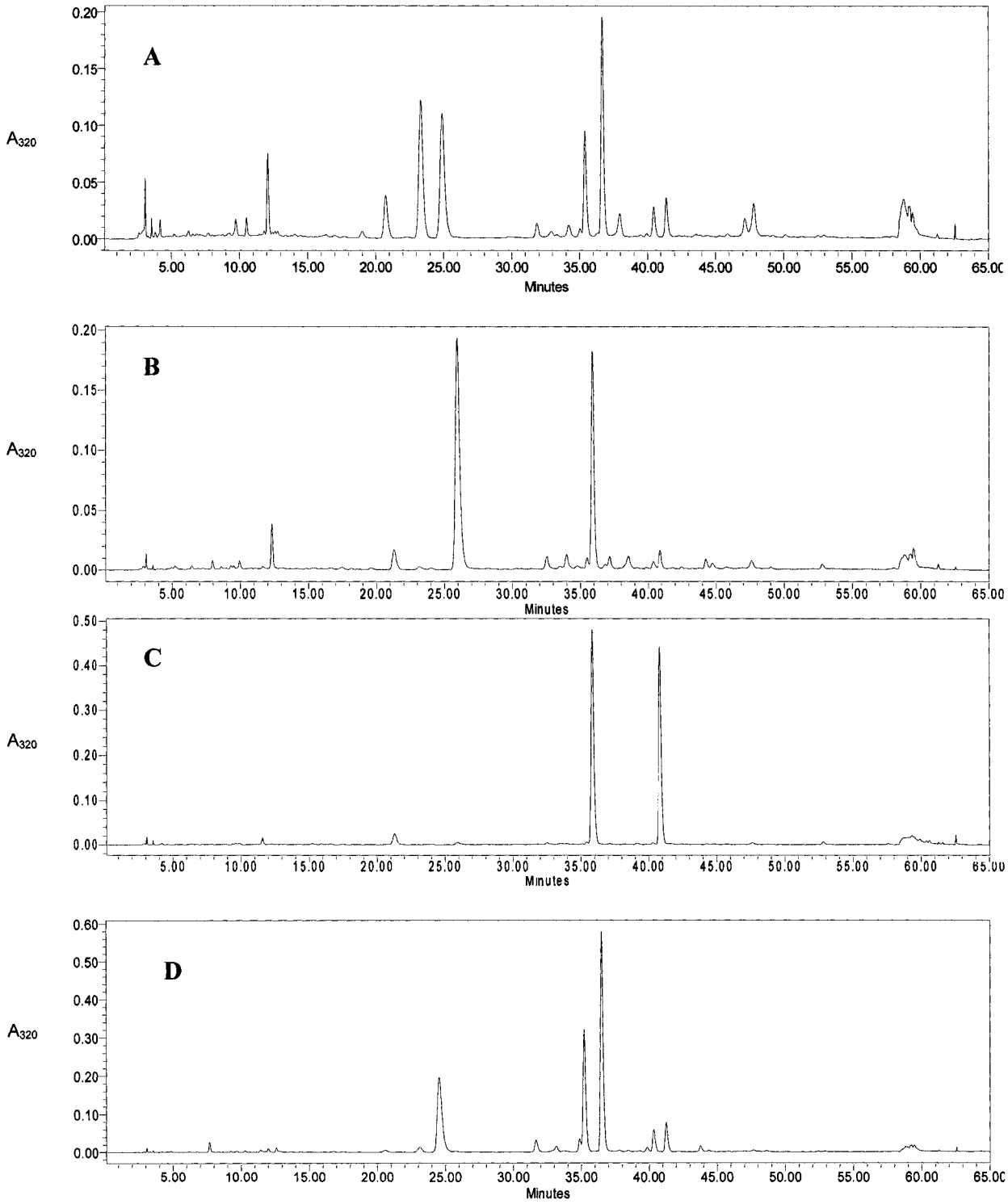

Figure 4.3. Comparison of HPLC-DAD chromatograms at 320 nm for the extract of *A. racemosa* (A), *A. pachypoda* (B), *A. podocarpa* (C), and *A. rubra* (D)

Chapter 5

Conclusions

Activity-guided isolation and identification of the standardized black cohosh powdered extract resulted in the isolation of two novel polyphenolic compounds, (3*S*)-2-(3″,4″-dihydroxyphenylmethylene)-3-hydroxy-3-(3′,4′-dihydroxybenzoyl)-γ–butyrolactone (**1**) and (2*R*,3*S*)-2-*O*-(3′,4′-dimethoxy-*E*-cinnamoyl)-3-hydroxy-3-[(3″,4″-dihydroxyphenyl)methyl]-butanedioic acid (**2**), together with fifteen known polyphenols described above. The structures of the novel compounds were determined on the basis of the NMR spectroscopic analysis.

Compound **1** displayed high antioxidant activity in the DPPH assay with an IC_{50} value of 26.94 μM, and stimulated MCF-7 cell proliferation with a 1.24-fold (14 μM), as compared to untreated cells. The structure of compound **1**, identified as a member of the dibenzyl-γ-butyrolactones type of lignans, was deduced to be formed by two phenylpropanoid units via phenol oxidation coupling as in the case of lignans.

Compound **2** displayed high antioxidant activity in the DPPH assay with an IC_{50} value of 37.40 μM, and stimulated the growth of MCF-7 cell proliferation at 1.14-fold (10 μM), when compared to untreated cells. Compound **2** is classified as a phenylpropanoid ester dimer, a kind of compound commonly found in *Actaea*, in addition to fukinolic acid, and cimicifugic acids A, B, C, D, E, and F. Compound **2** is likely formed via esterification of fukiic acid and (*E*)-3,4-dimethoxycinnamic acid. The latter two compounds have been previously isolated from the Asian species, but their ester-linked dimer had not been reported.

Fifteen polyphenolic compounds were also isolated from black cohosh: protocatechuic acid, protocatechualdehyde, *p*-coumaric acid, caffeic acid, methyl caffeate, ferulic acid, ferulate-1-methyl-ester, isoferulic acid, 1-isoferuloyl-β-D-

glucopyranoside, fukinoilic acid, cimicifugic acids A, B, D, E, and F. Their structures were determined by comparing their spectral data (MS, ^{1}H, ^{13}C) with reported values. Six of these compounds, namely: protocatechuic acid, protocatechualdehyde, *p*-coumaric acid, ferulate-1-methyl-ester, 1-isoferuloyl-β-D-glucopyranoside, and cimicifugic acid D, are reported for the first time from black cohosh. The fifteen polyphenolic compounds were tested in the DPPH assay and displayed IC_{50} values ranging from 12.91 to 321.41 μM. Fukinolic acid had the highest antioxidant activity, while *p*-coumaric acid had the lowest activity.

Due to the increasing use of black cohosh, over-harvesting of black cohosh from wild populations may occur. Sometimes, closely related species may be collected where their ranges overlap with that of black cohosh. Since there are currently no reports of phytochemical variations among wild populations of native North American *Actaea* species, it is important to determine and compare the chemical profiles among them in order to distinguish black cohosh from closely related species. Thus, an HPLC analytical method was conducted in this project.

A reversed-phase high-performance liquid chromatography (RP-HPLC) with diode array detection (DAD) method was developed and validated to quantify polyphenols in the four North American *Actaea* species. This method allowed the identification and quantification of caffeic acid, ferulic acid, isoferulic acid, fukinolic acid, cimicifugic acids A, B, E and F in the rhizomes and roots of *Actaea* species. The highest levels of the eight measured polyphenols were found in *A. rubra*, the second highest in *A. podacarpa*, the third highest in *A. racemosa,* and the lowest in

A. pachypoda. The polyphenol levels corresponded with the antioxidant activities determined by the DPPH assay.

The validation of the method includes tests for sensitivity, linearity, precision, accuracy, and recovery. The limits of detection were found to be in the range of 24.8-78.3 ng/mL for the eight major polyphenols. Linear calibration curves were obtained for the eight compounds tested, and had a correlation coefficient (r^2) > 0.99. The precision and accuracy of the method were evaluated by analyzing three sets of a standards mixture solution on three consecutive days with R.S.D. (%) and relative error (%) < 2.00. The recovery rates were 99.83-101.44 % for tested standards.

This project provides a more complete understanding of polyphenolic constituents of black cohosh, which may lead to find the bioactive compounds responsible for its clinical efficacy, and also provides the primary chemical profiles among the North American *Actaea* species.

Appendix

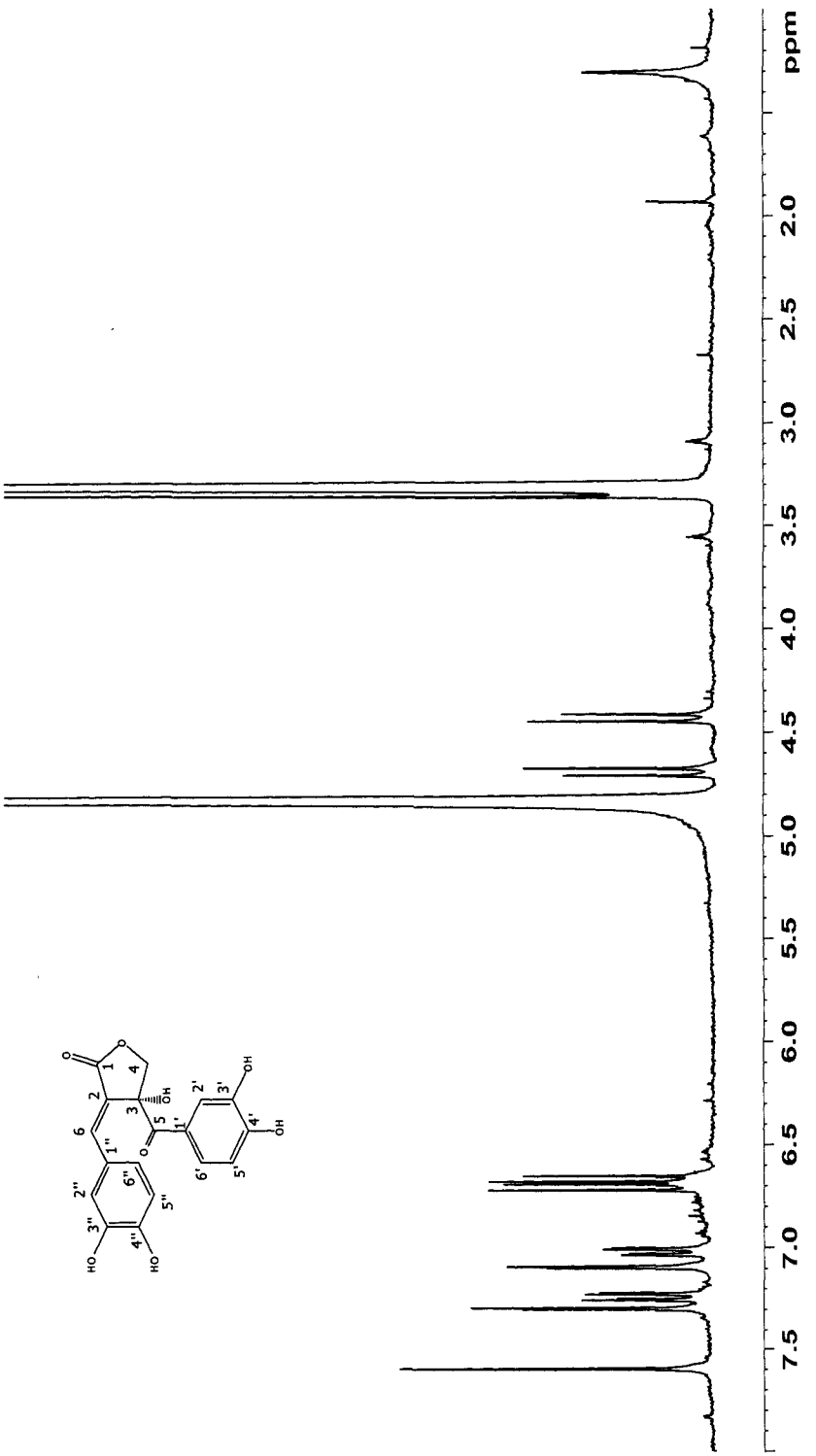

Figure A.1. ^{1}H NMR spectrum of compound 1 recorded at 300 MHz in CD_3OD

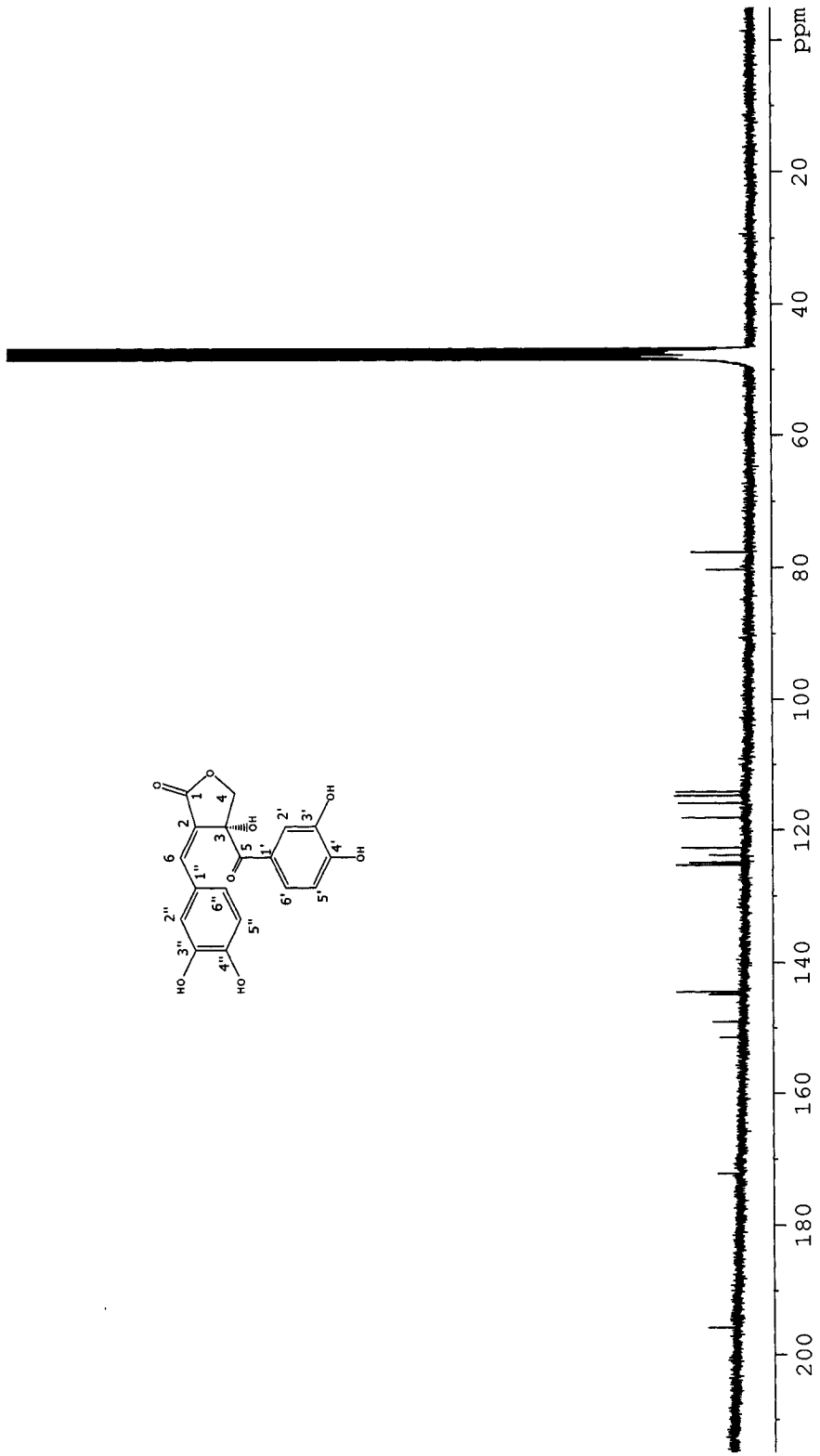

Figure A.2. ^{13}C NMR spectrum of compound 1 recorded at 75 MHz in CD_3OD

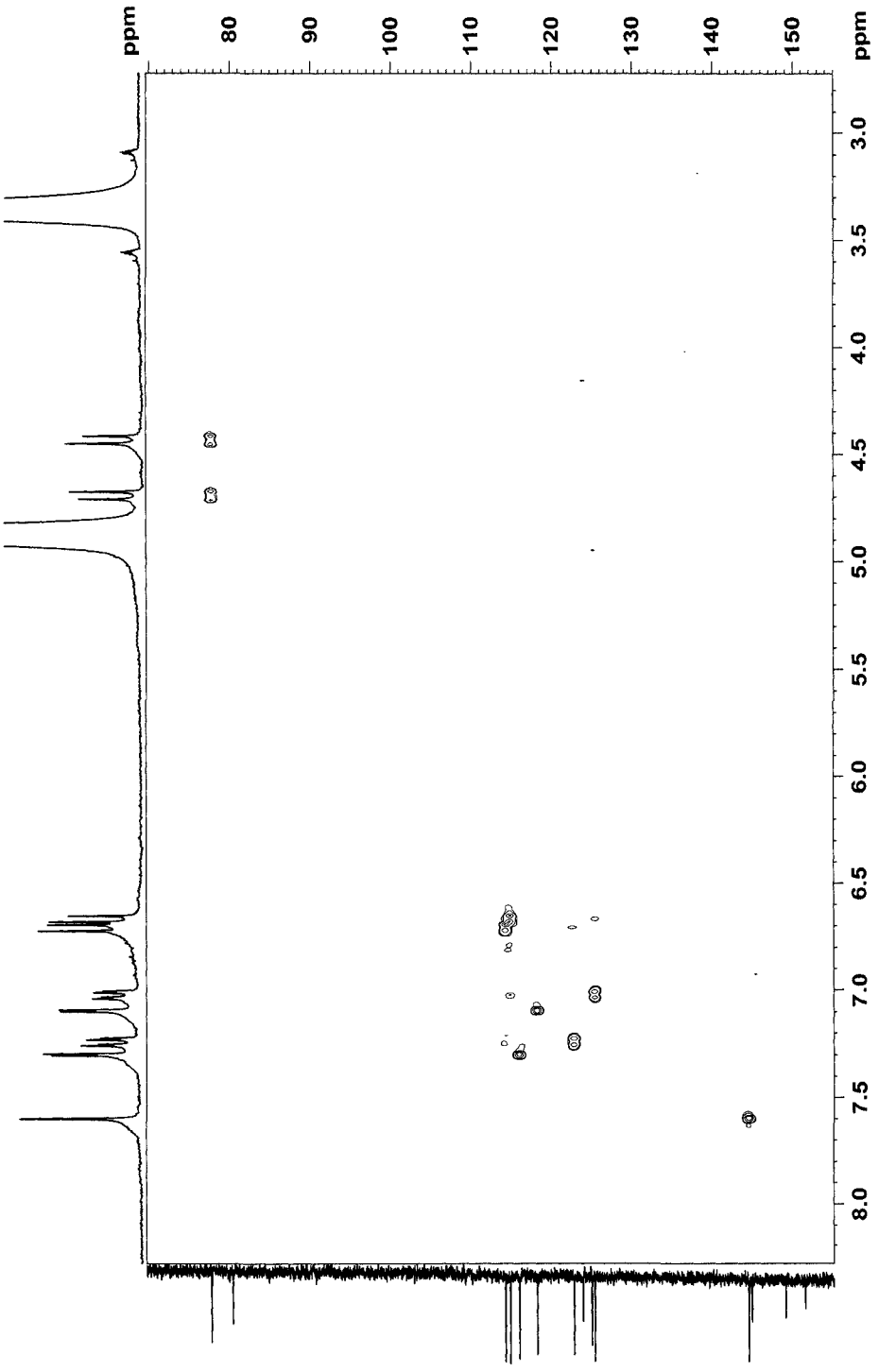

Figure A.3. HSQC spectrum of compound 1 recorded at 300 MHz in CD_3OD

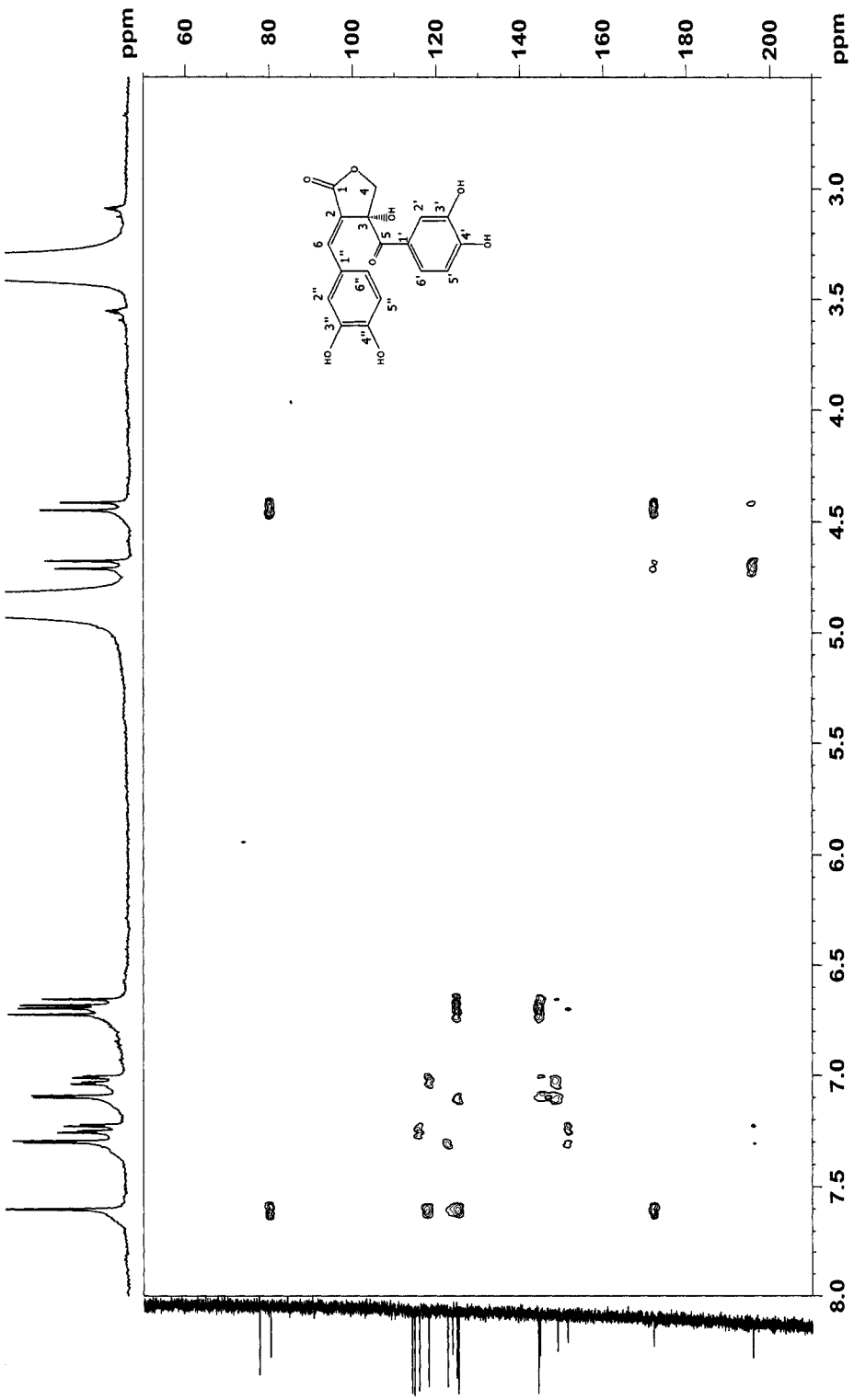

Figure A.4. HMBC spectrum of compound 1 recorded at 300 MHz in CD_3OD

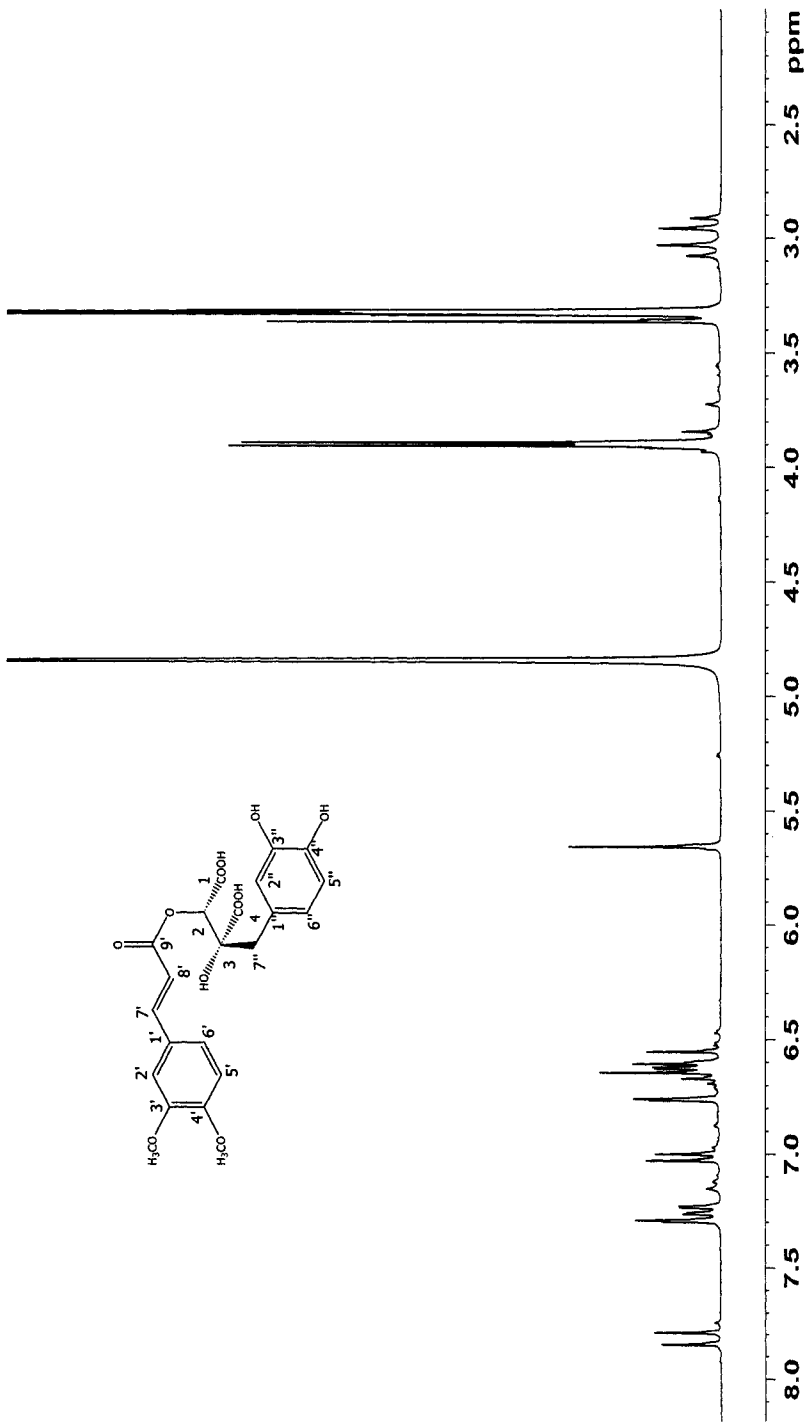

Figure A.5. ^{1}H NMR Spectrum of compound 2 recorded at 300 MHz in CD_3OD

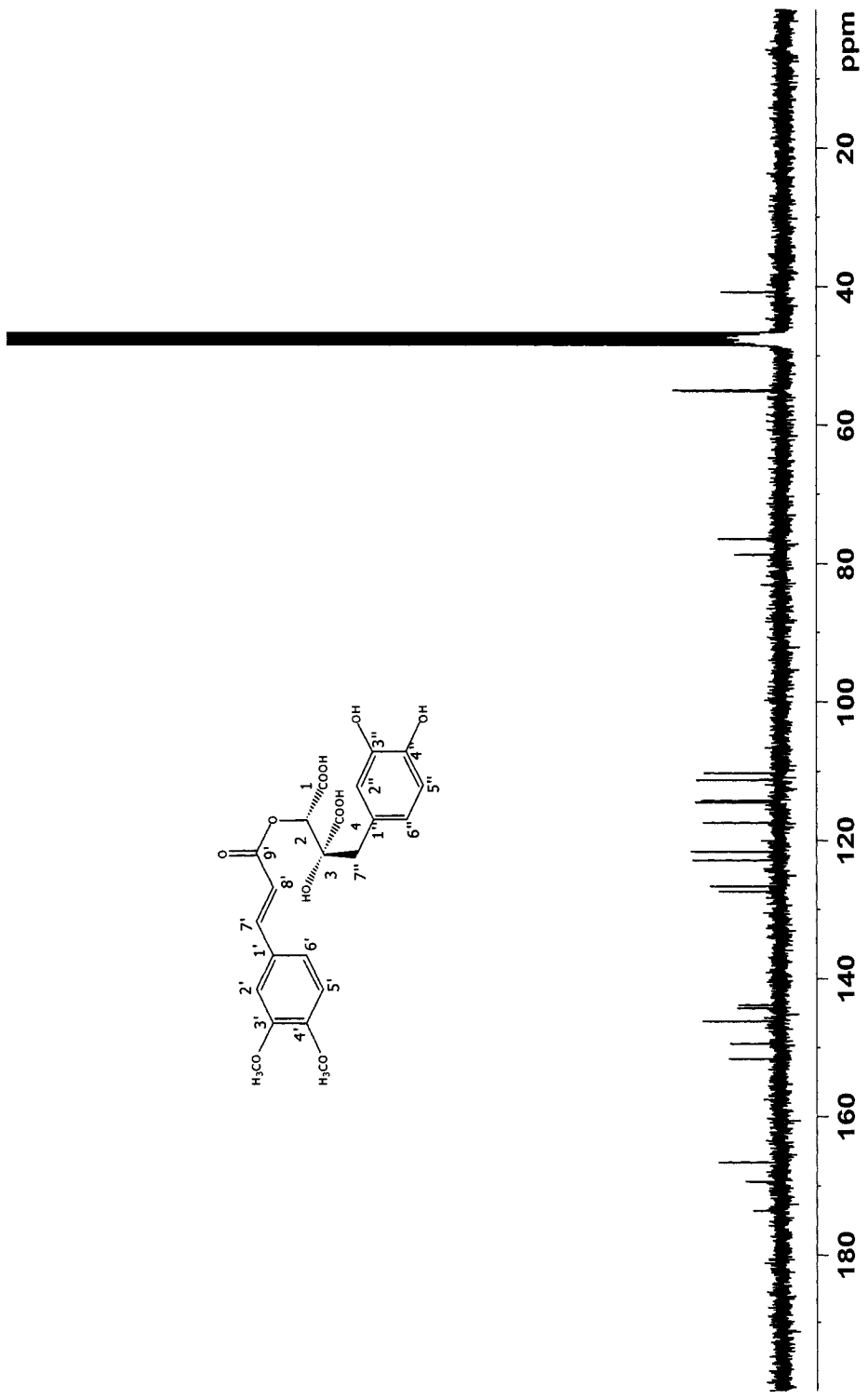

Figure A.6. ^{13}C NMR spectrum of compound 2 recorded at 75 MHz in CD_3OD

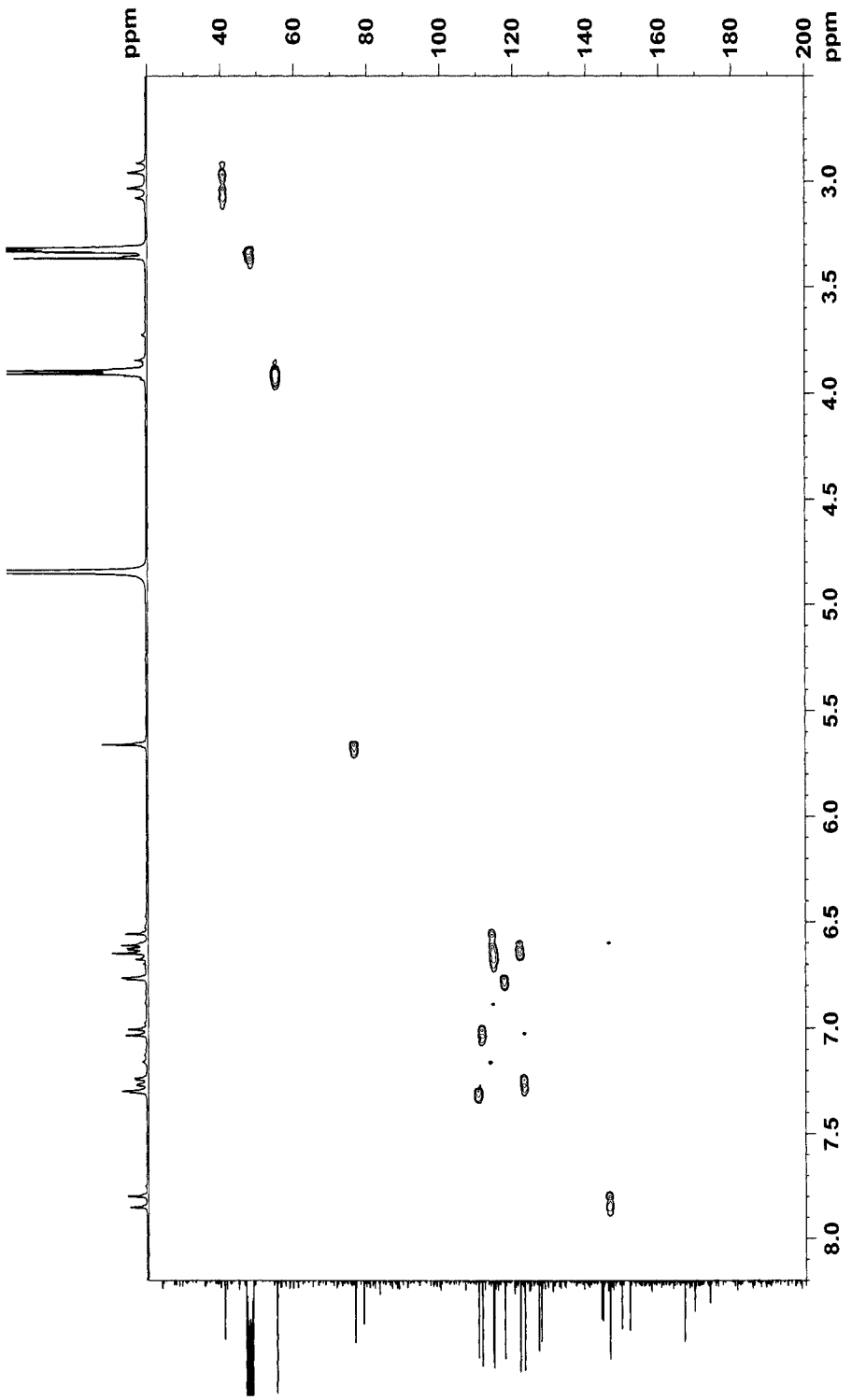

Figure A.7. HSQC spectrum of compound 2 recorded at 300 MHz in CD_3OD

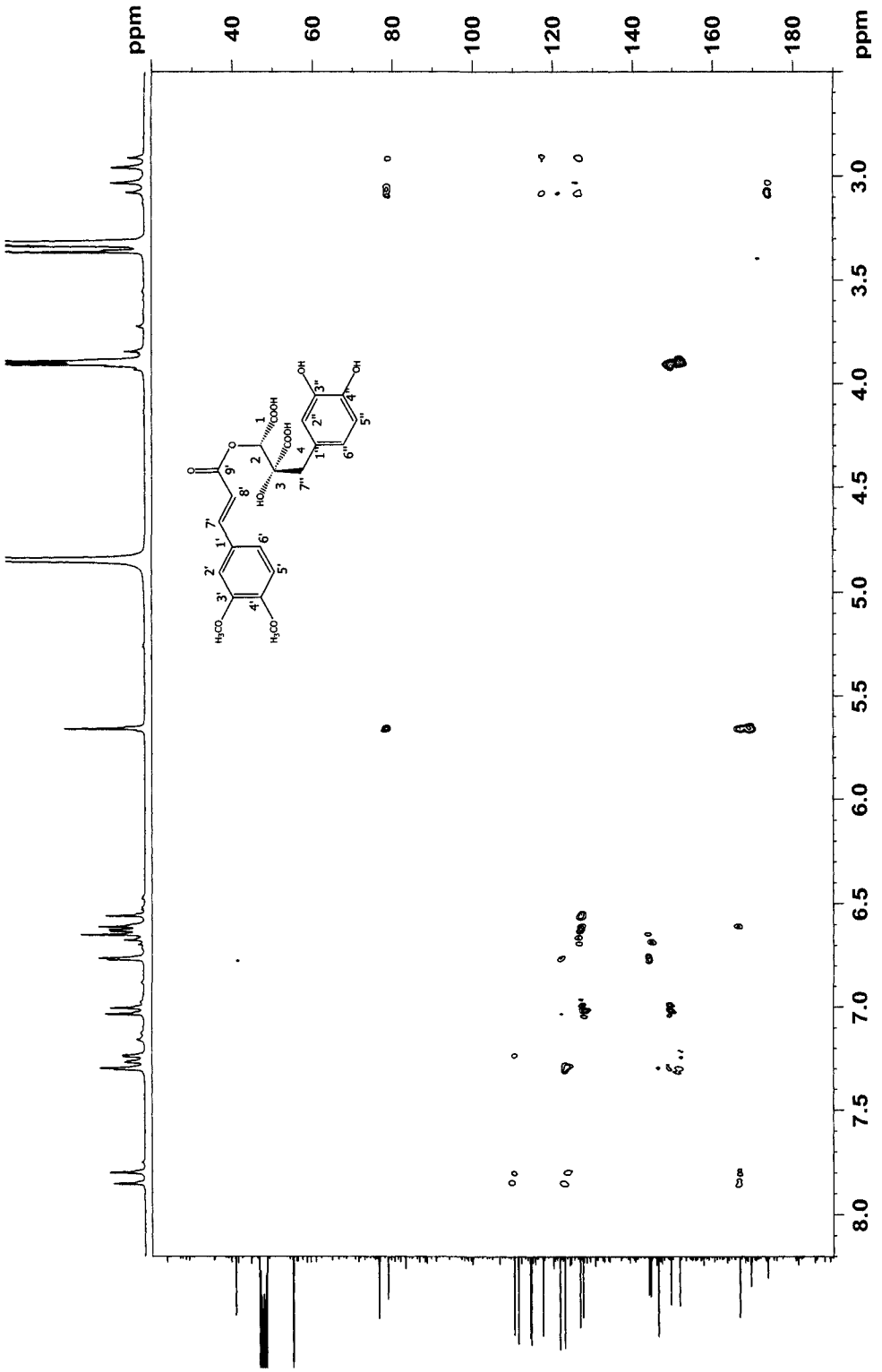

Figure A.8. HMBC spectrum of compound 2 recorded at 300 MHz in CD_3OD

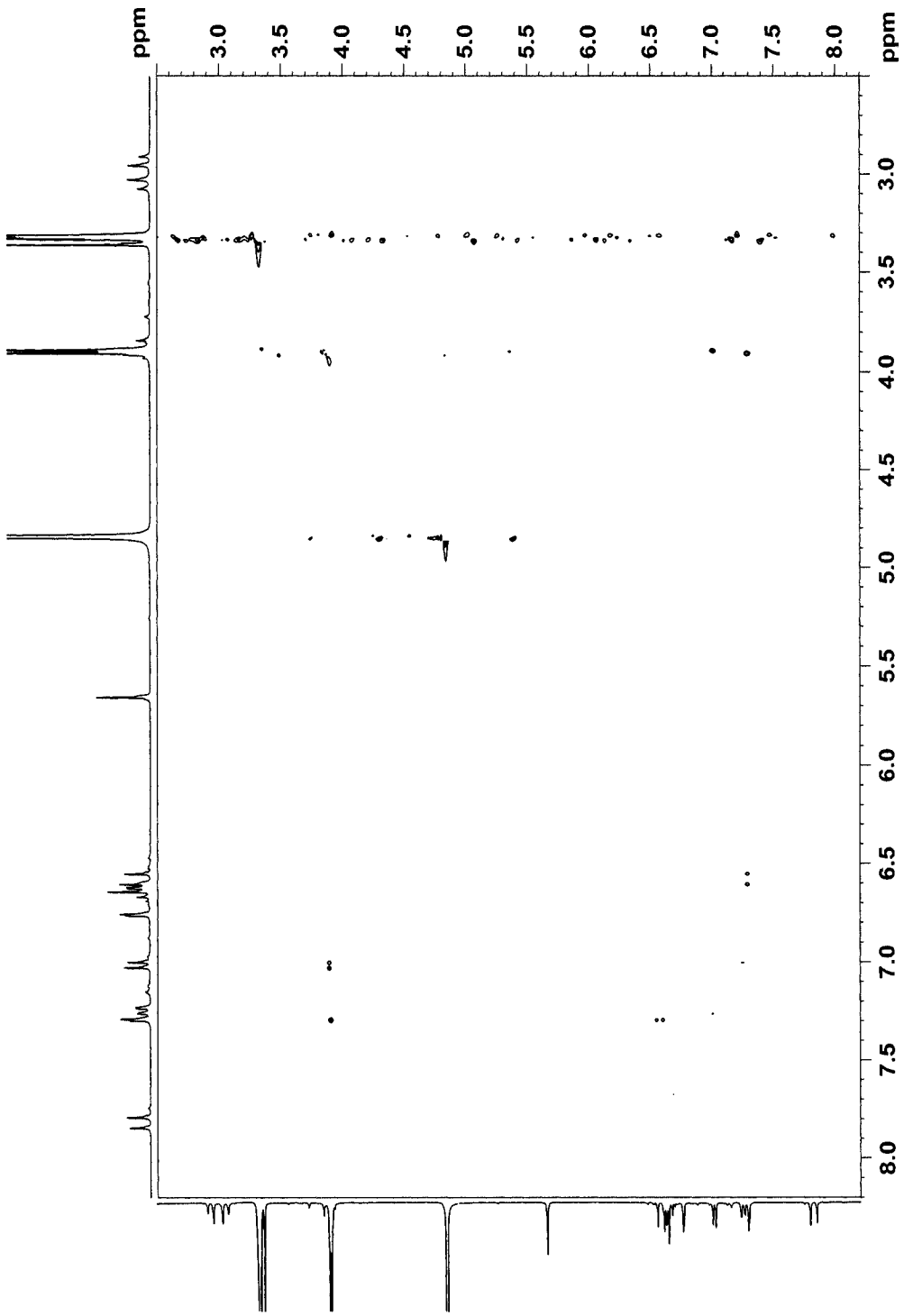

Figure A.9. NOSEY spectrum of compound 2 recorded at 300 MHz in CD_3OD

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