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## Materials and methods for reduction of protein tau and treatment of neurodegenerative diseases

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(12) **United States Patent**  
**Dickey et al.**

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(45) **Date of Patent:** **Jan. 27, 2015**

(54) **MATERIALS AND METHODS FOR  
REDUCTION OF PROTEIN TAU AND  
TREATMENT OF NEURODEGENERATIVE  
DISEASES**

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(US)

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patent is extended or adjusted under 35  
U.S.C. 154(b) by 0 days.

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§ 371 (c)(1),

(2), (4) Date: **Mar. 28, 2013**

(87) PCT Pub. No.: **WO2012/012798**

PCT Pub. Date: **Jan. 26, 2012**

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**Related U.S. Application Data**

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23, 2010.

(51) **Int. Cl.**  
**C07C 43/20** (2006.01)

(52) **U.S. Cl.**  
USPC ..... **568/633**

(58) **Field of Classification Search**  
USPC ..... **568/633**  
See application file for complete search history.

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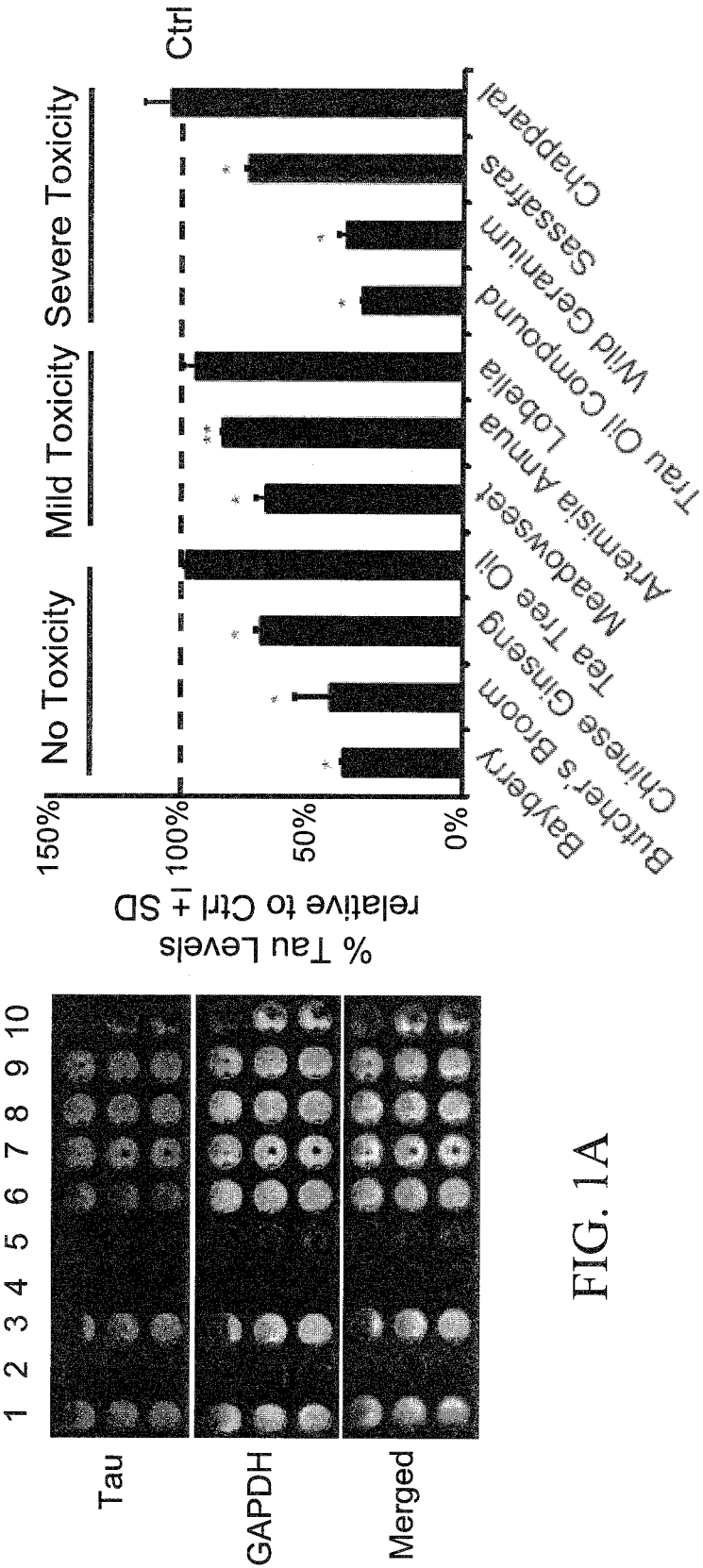
*Primary Examiner* — Rei-tsang Shiao

(74) *Attorney, Agent, or Firm* — Saliwanchik, Lloyd & Eisenschenk

(57) **ABSTRACT**

The subject invention provides a myricanol compound that is in predominant form of (+)- $\alpha$ R,11S-myricanol as compared to (−)- $\alpha$ S,11R-myricanol. In one embodiment, the (+)- $\alpha$ R,11S-myricanol is isolated from *Myrica cerifera*, and is in about 86% enantiomeric excess of (−)- $\alpha$ S,11R-myricanol. The subject invention also pertains to therapeutic compositions and methods for treatment of neurodegenerative diseases, in particular, neurodegenerative diseases associated with abnormal accumulation of protein tau. Specifically exemplified herein is the therapeutic use of myricanol and myricanone isolated from root barks of *Myrica* species. Also provided are methods for preparing extracts of the subject invention from *Myrica* species.

**3 Claims, 16 Drawing Sheets**



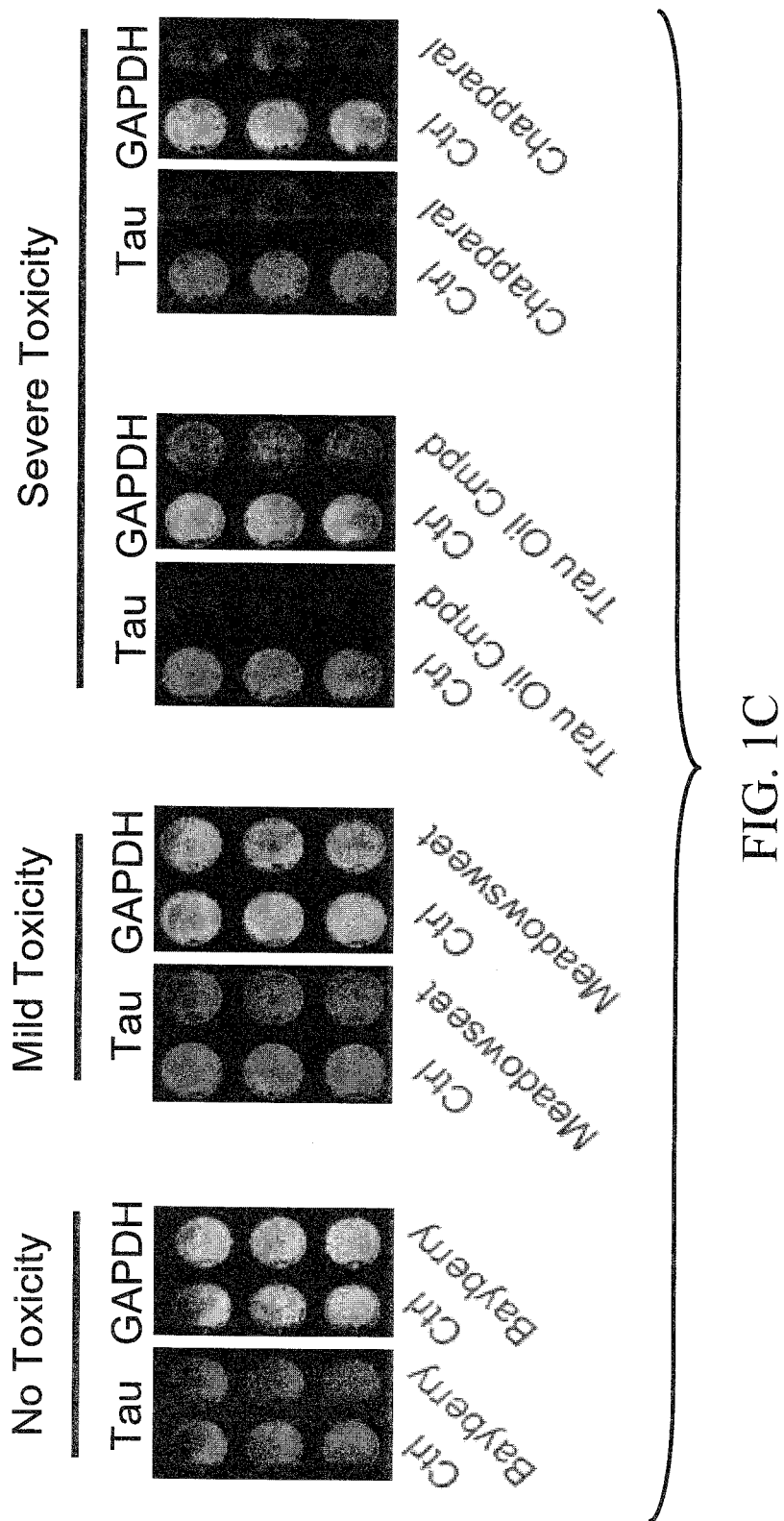


FIG. 2A

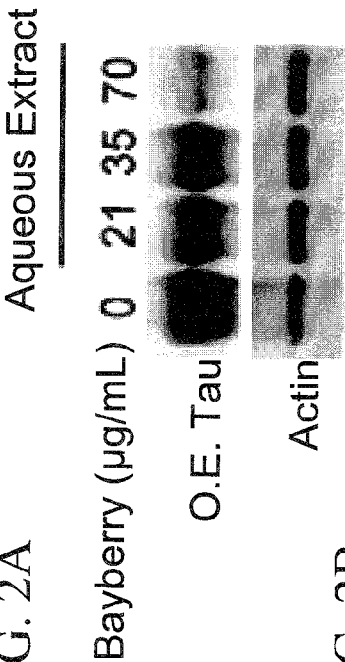


FIG. 2B

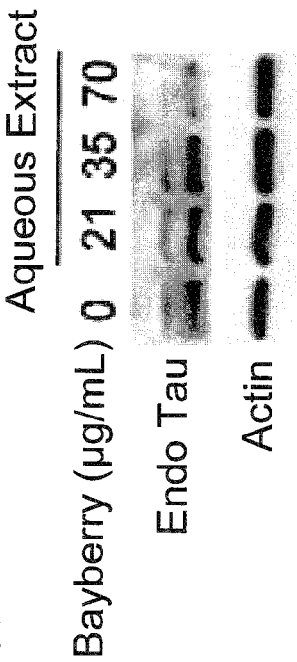
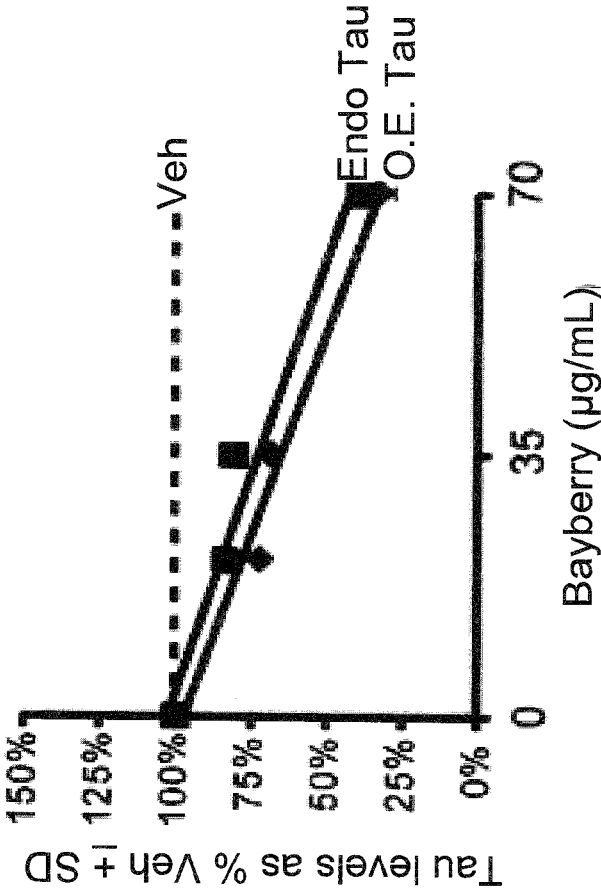


FIG. 2C



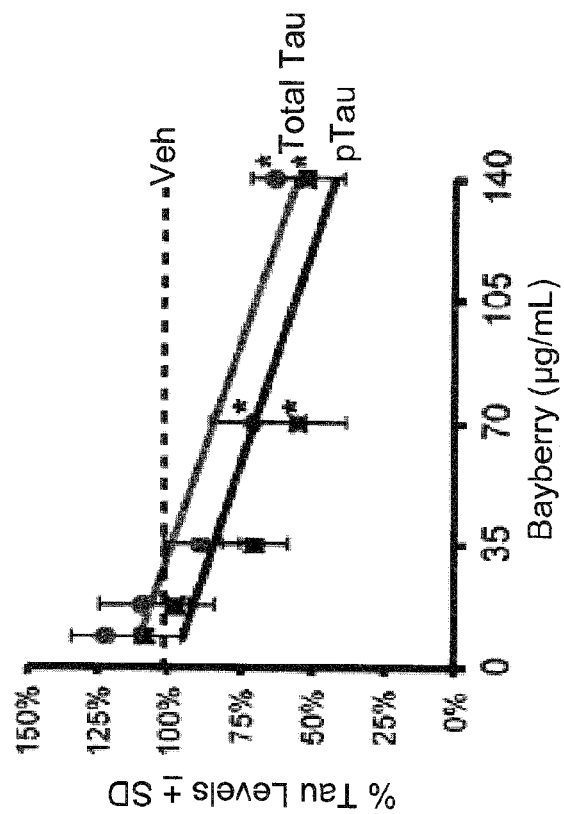


FIG. 2E

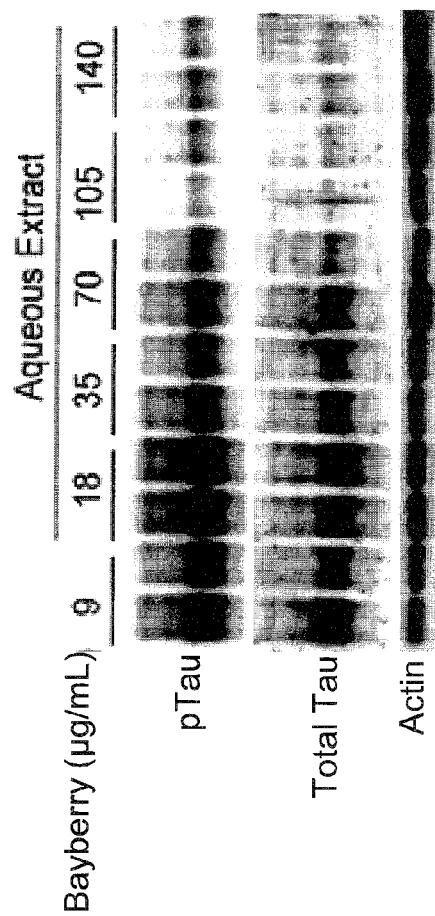
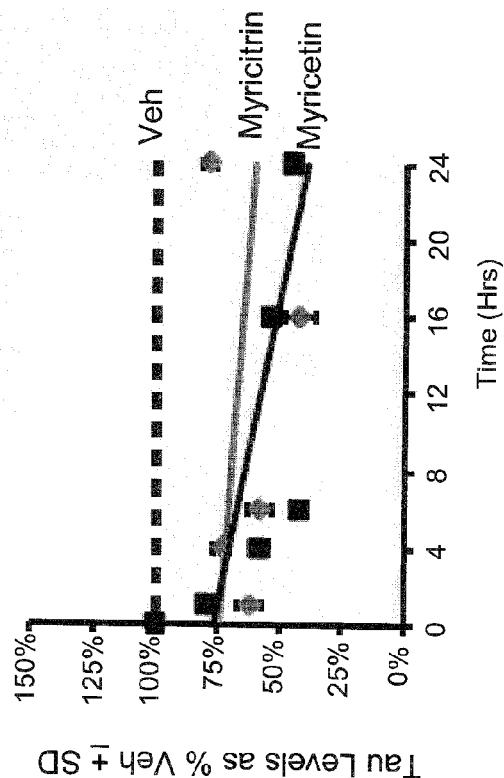
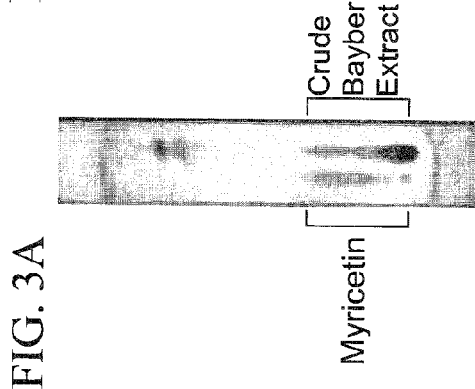
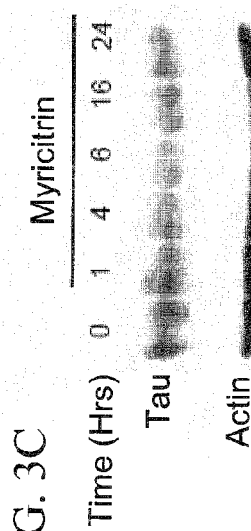
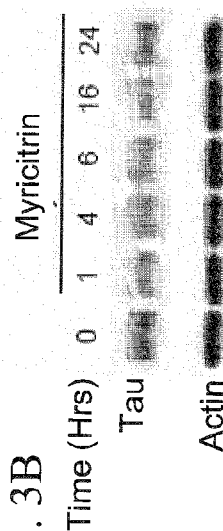
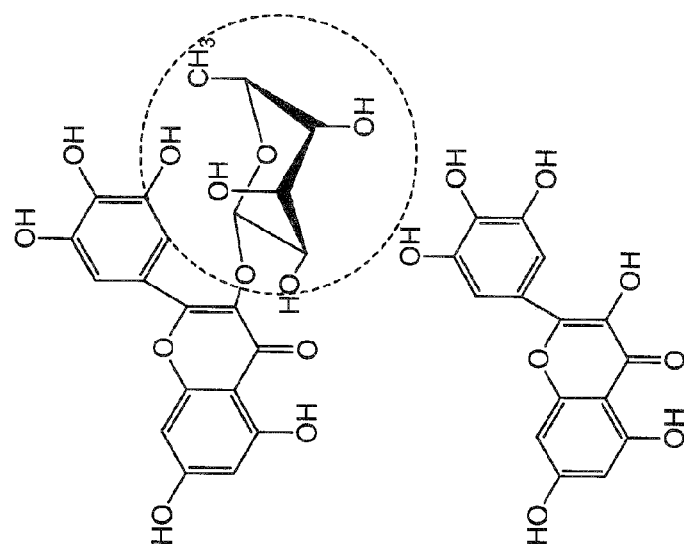


FIG. 2D



Bayberry Extract

M  
P  
L  
C

8 UV Active Fractions

FIG. 4A

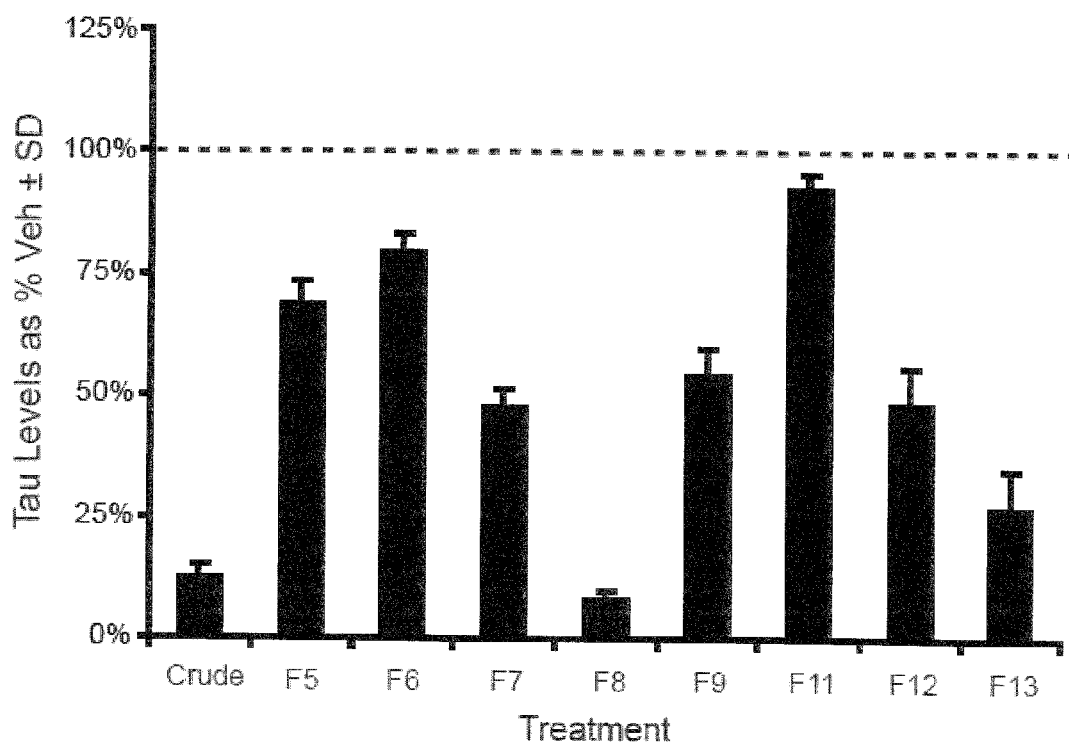


FIG. 4D



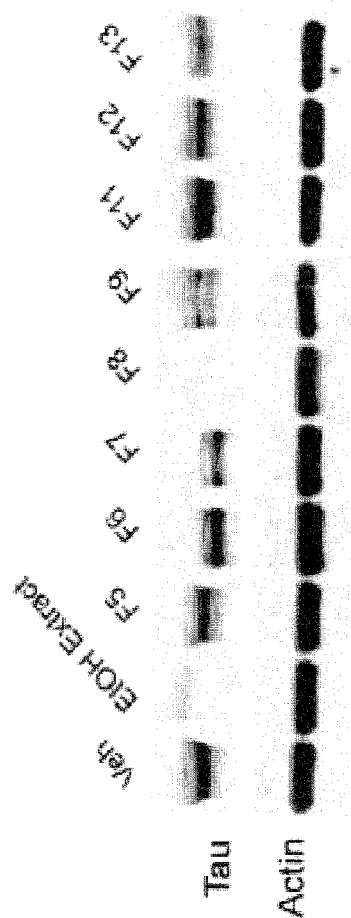


FIG. 4C

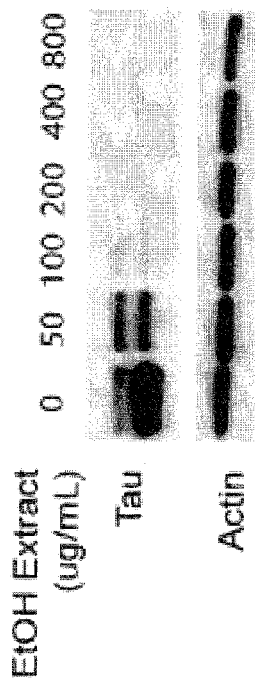


FIG. 4B

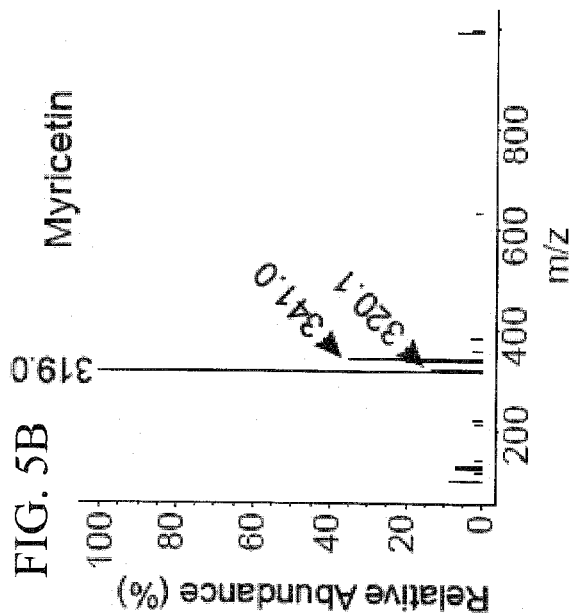


FIG. 5B

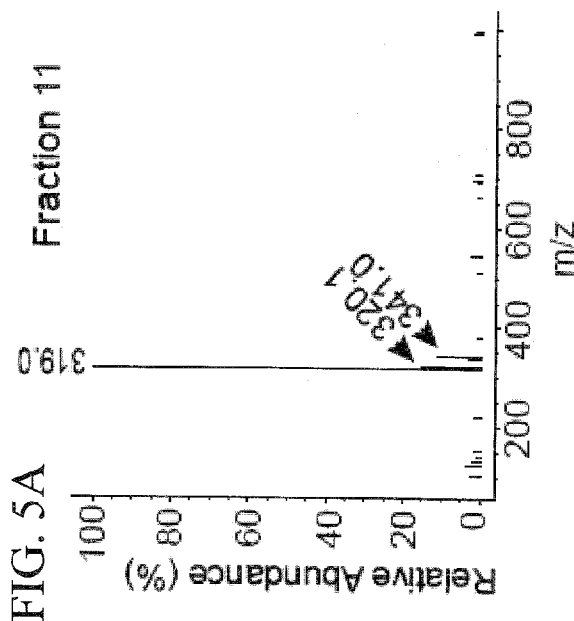


FIG. 5A

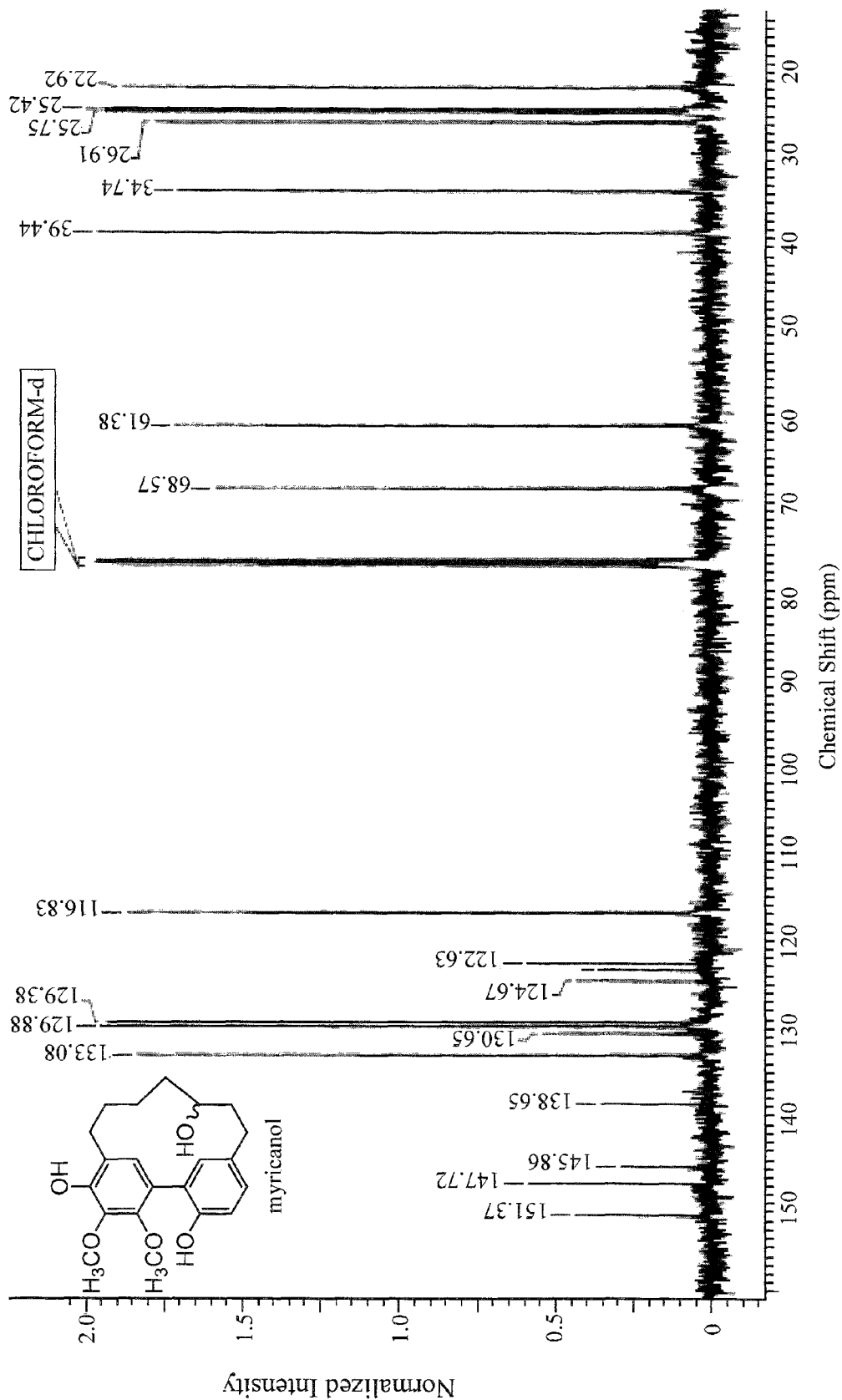


FIG. 6

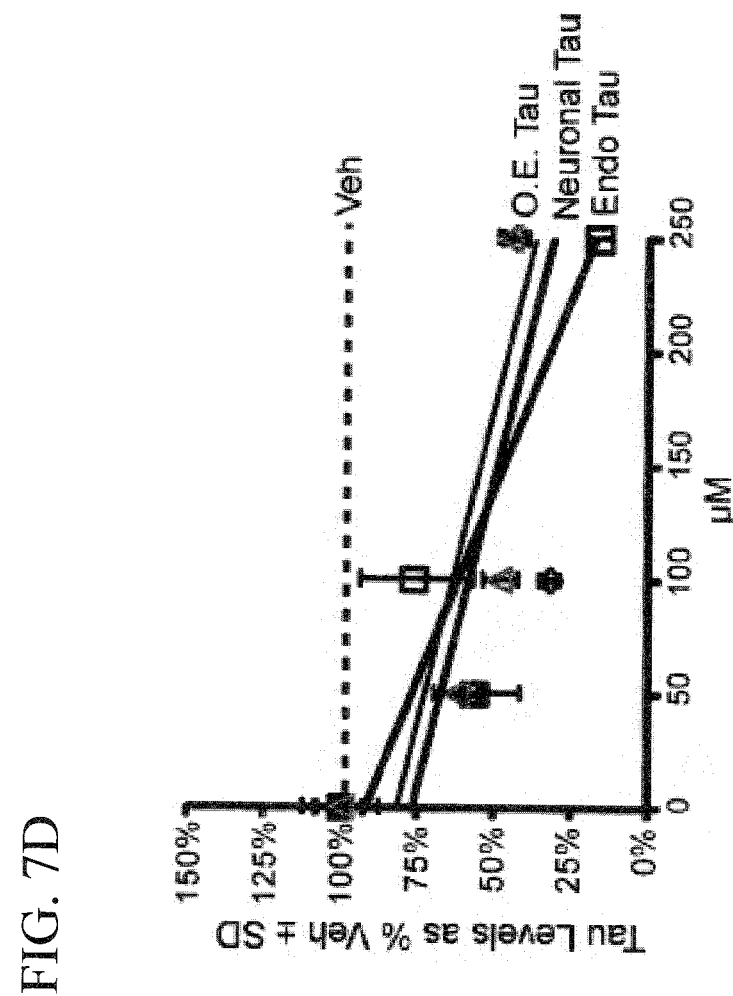
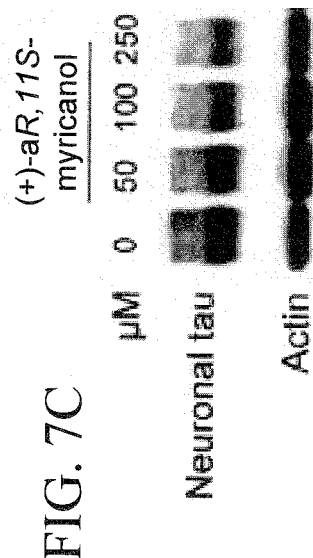
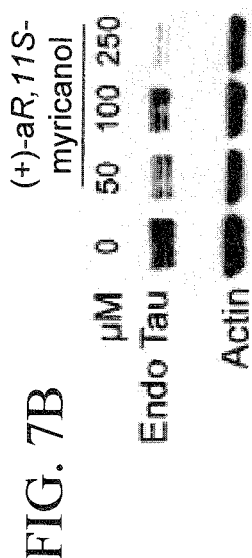
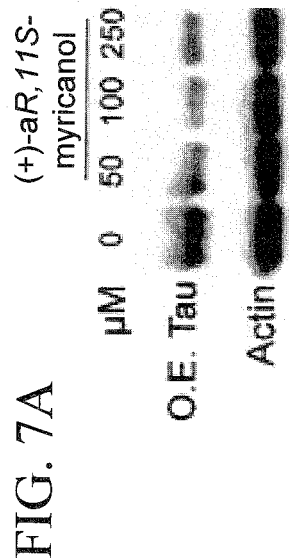


FIG. 7E

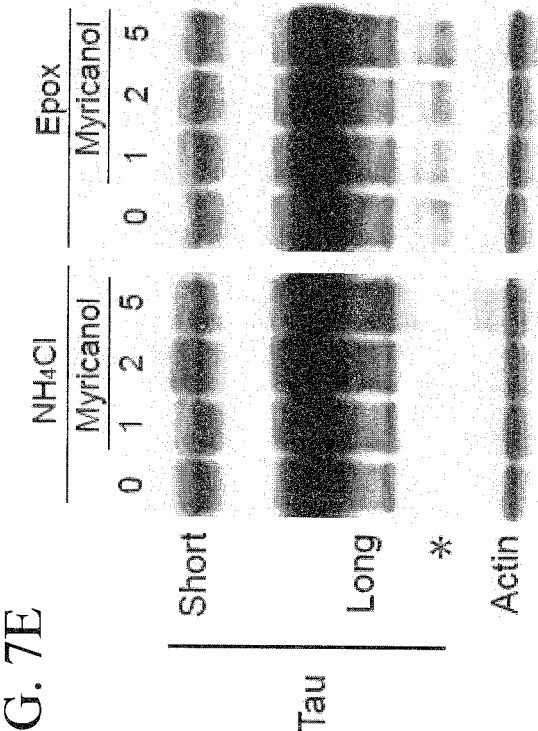
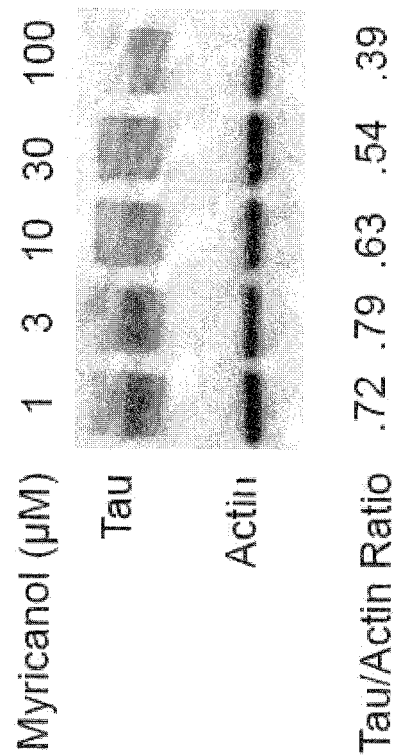


FIG. 7F



MYRICANOL(+-)-Whelk-5%  
 C:\EZStart\Projects\Default\Method\CS-Whelk-5%-90min.met  
 C:\EZStart\Projects\Default\Data\MYRICANOL(+-)-Whelk-5%.dat

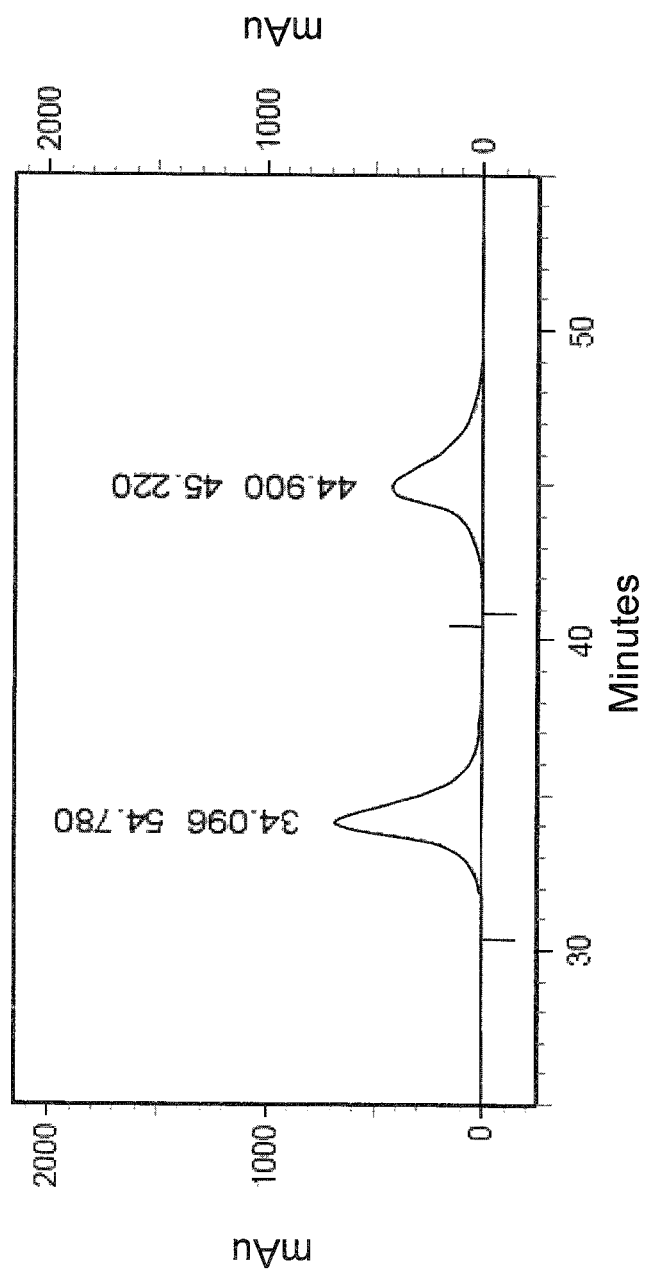
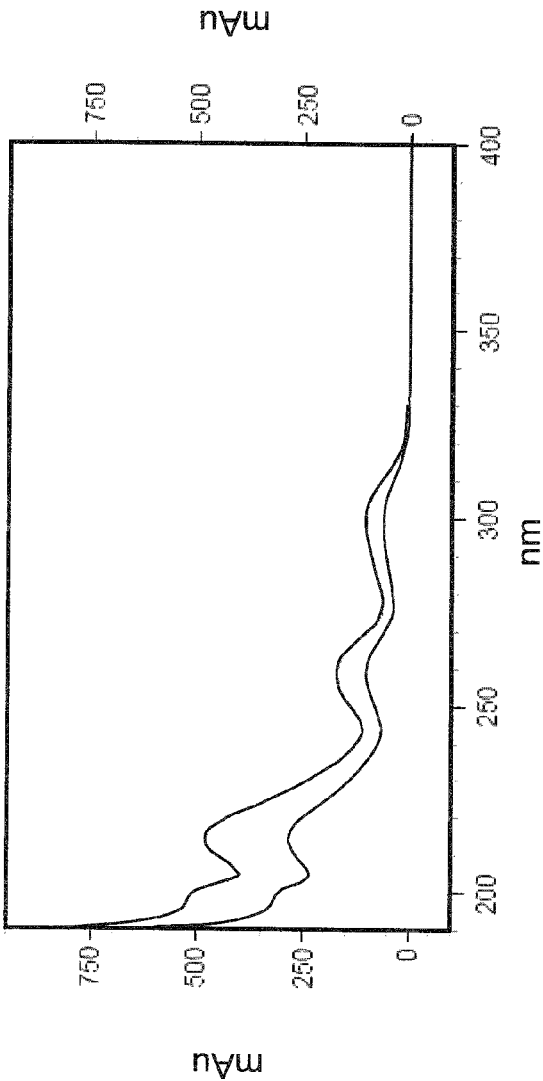


FIG. 8A



3: 214 nm, 4nm  
Results

Pk # Name	Retention Time	Area Percent
1	34.096	54.780
2	44.900	45.220
Totals		100.000

FIG. 8B

BB8-10-Whelk5%  
C:\EZStart\Projects\Default\Data\BB8-10-Whelk5%.dat  
C:\EZStart\Projects\Default\Method\CS-ADH-1.0%-0.8ml.met  
OD-H column 20%IPA @ 0.8ml/min

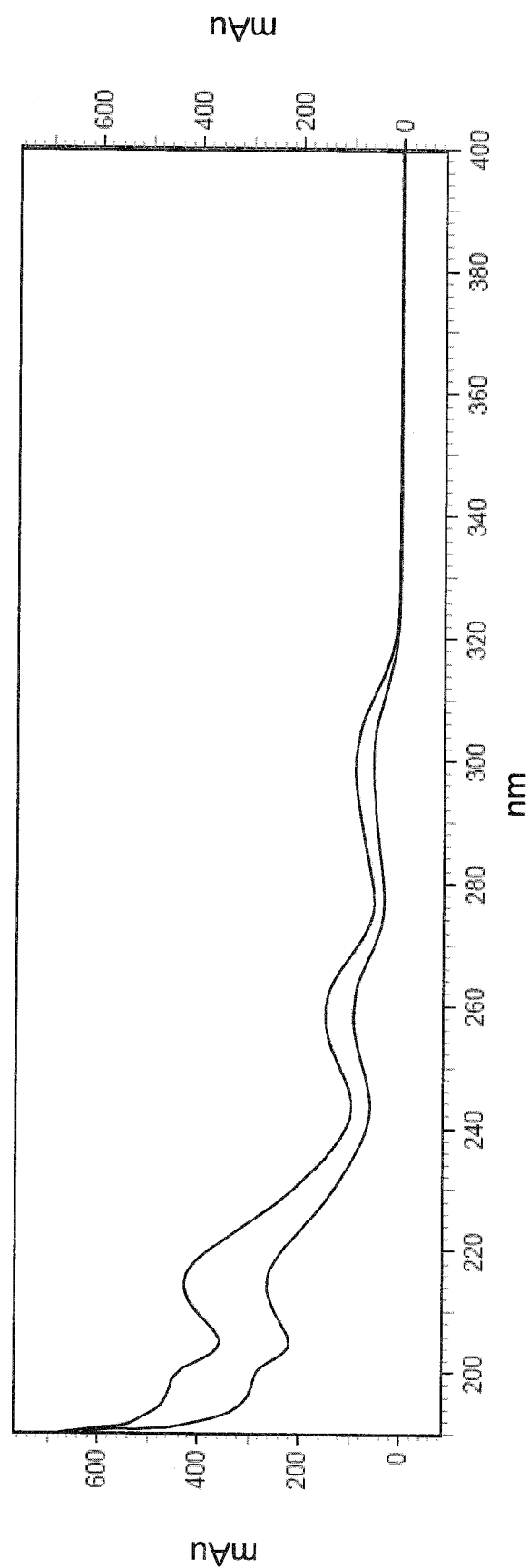
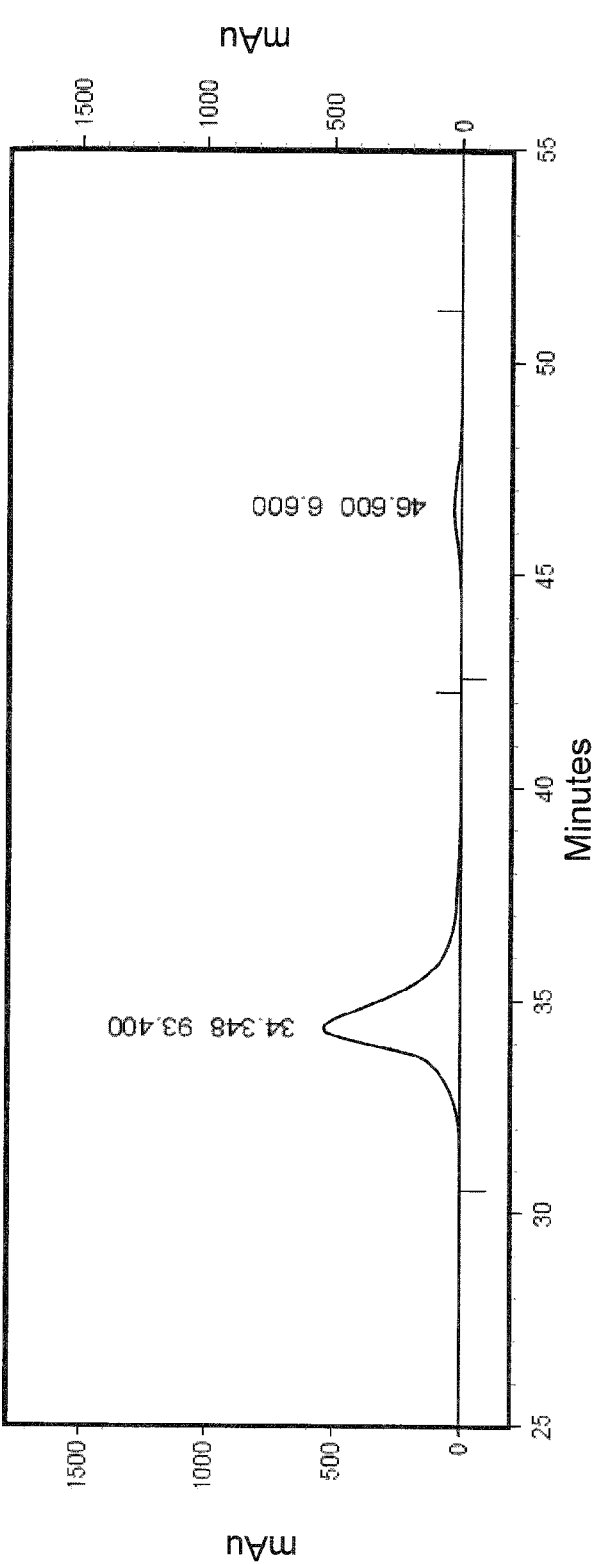


FIG. 9A



2: 219 nm, 4nm Results

Pk #	Retention Time	Area Percent
1	34.348	93.400
2	46.600	6.600
Totals		100.000

FIG. 9B



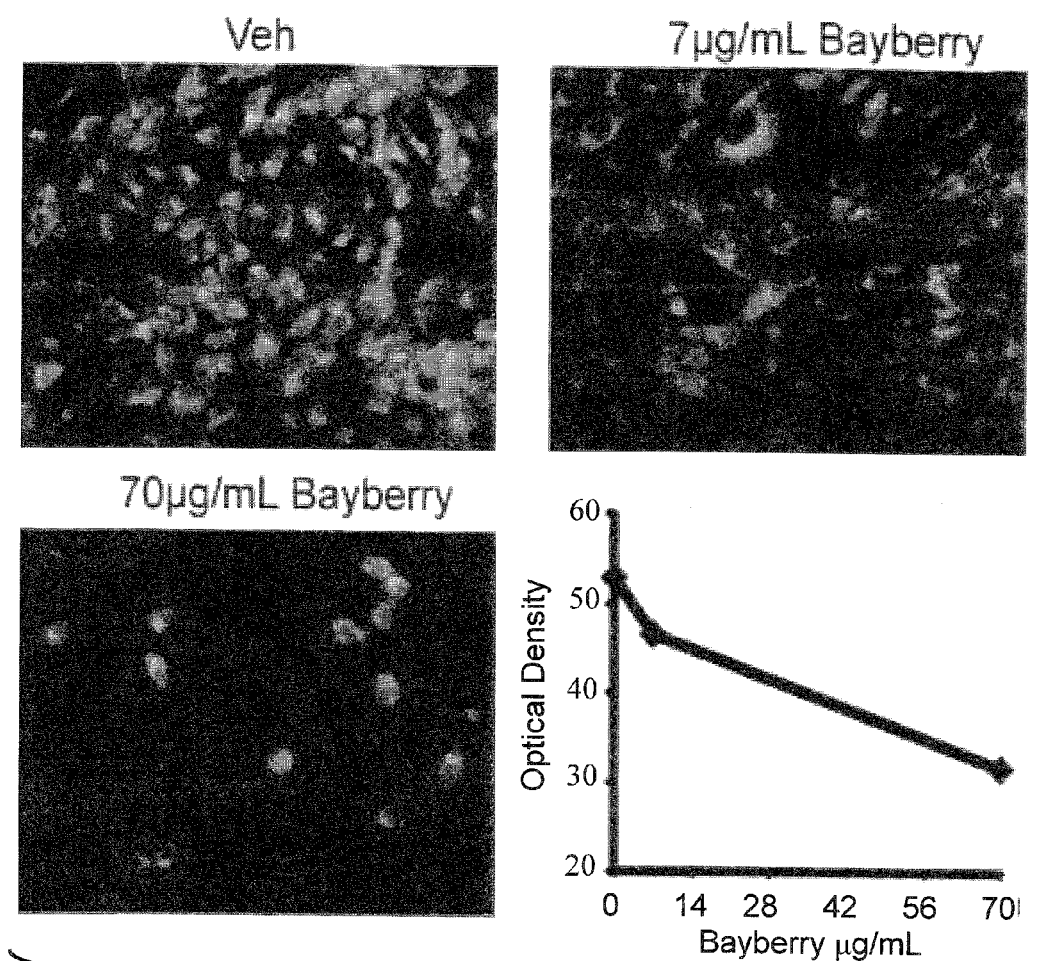
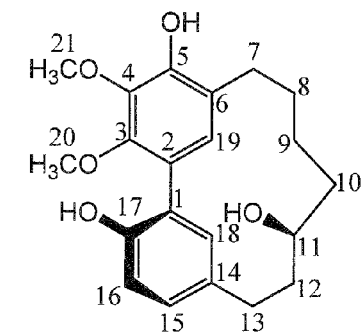
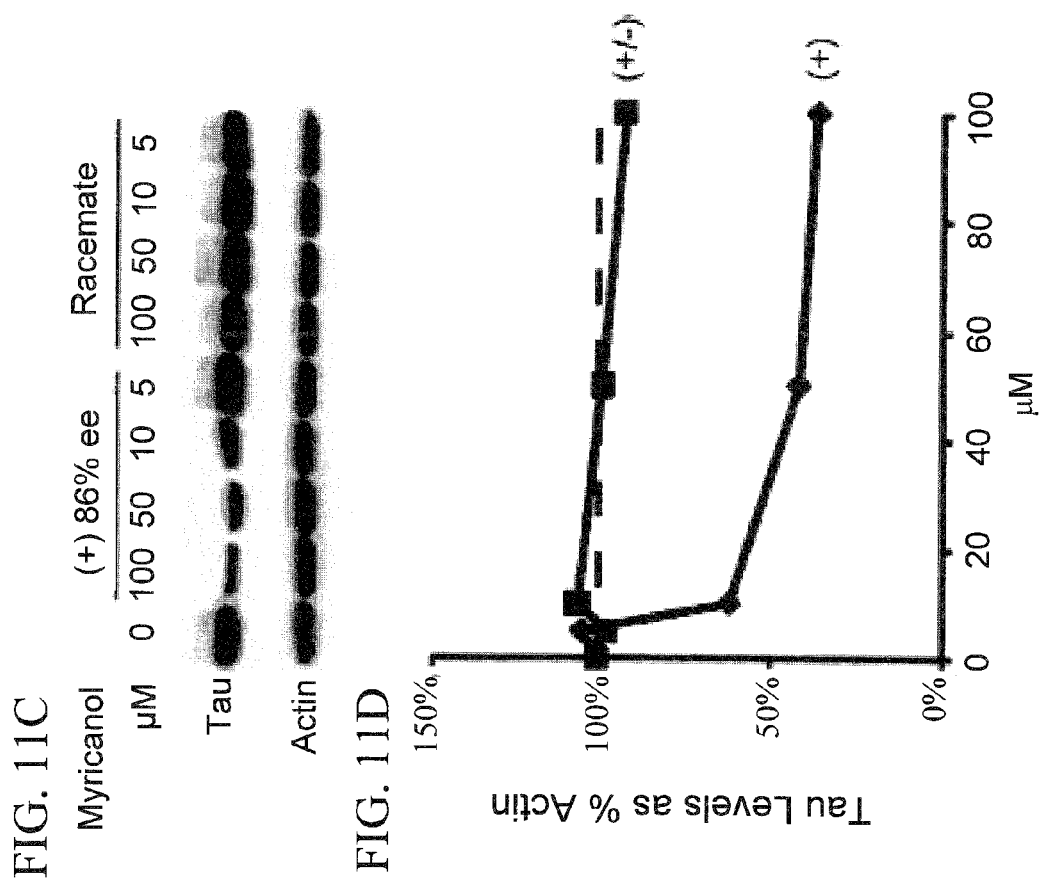
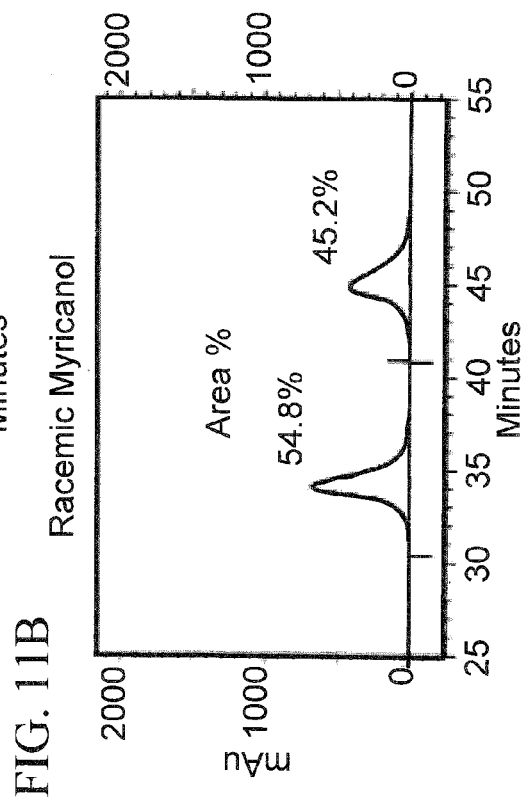
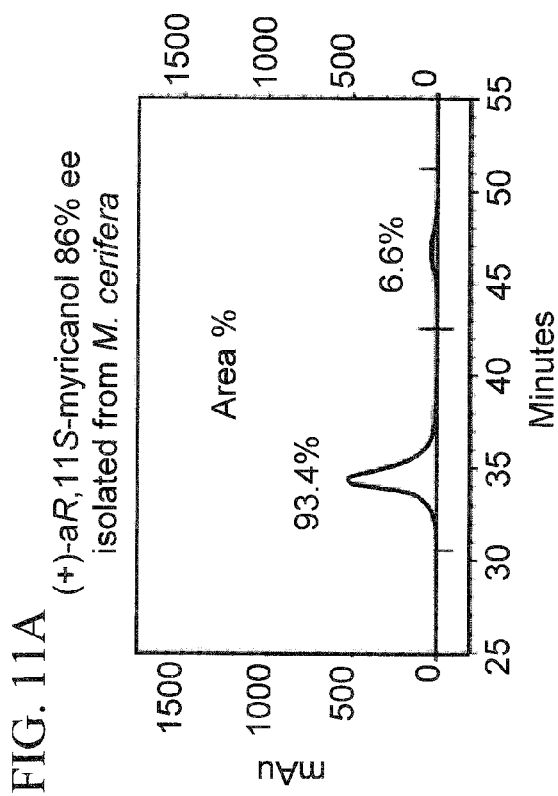


FIG. 10



(+)-3: (+)-aR, 11S-myricanol

FIG. 12



1

# MATERIALS AND METHODS FOR REDUCTION OF PROTEIN TAU AND TREATMENT OF NEURODEGENERATIVE DISEASES

## GOVERNMENTAL SUPPORT

This invention was made with government support under Grant #AG031291 awarded by the National Institutes of Health. The government has certain rights in the invention.

## CROSS-REFERENCE TO RELATED APPLICATIONS

This application is the U.S. national stage application of International Patent Application No. PCT/US2011/045213, filed Jul. 25, 2011, which claims the benefit of U.S. Provisional Patent Application No. 61/367,141, filed Jul. 23, 2010, the disclosures of each of which are hereby incorporated by reference in their entirety.

## BACKGROUND OF THE INVENTION

Intracellular aggregation of abnormal species of phosphorylated tau (protein tau) is a major pathologic feature of a family of neurodegenerative diseases collectively referred to as the tauopathies. Tau normally functions to stabilize microtubules in neurons; however, it pathologically aggregates more than 15 neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease. The most common tauopathy is Alzheimer's disease, in which paired helical filaments (PHFs) of mis-folded protein tau aggregates in neurofibrillary tangles, in dystrophic neuritis of senile plaques, and in cell processes in the neuropil. Abnormal accumulation of protein tau is closely linked with postsymptomatic progression in Alzheimer's disease. Abnormal accumulation of protein tau in the cytoplasm of neuronal and glial cells also represents major structural hallmarks in the pathology of Pick's disease, corticobasal degeneration and progressive supranuclear palsy.

At present, researchers on the development of therapeutics for tauopathies focus primarily on agents that prevent abnormal phosphorylation or aggregation of tau proteins. However, it has been discovered that while aggregation of hyperphosphorylated protein tau is visible evidence of tauopathies, these neurofibrillary tangles appear to be less toxic than soluble intermediates of protein tau. High levels of tau intermediates, particularly aberrant tau species failed to be cleared from cells, cause cognitive dysfunction in AD and tauopathies. Therefore, agents that degrade or destabilize tau intermediates, clear aberrant tau species from cells, or otherwise reduce intracellular tau levels, are promising therapeutics for AD and tauopathies.

Natural products chemistry has produced several effective AD therapy leads, including the amyloid aggregation inhibitor curcumin, isolated from tumeric, the microtubule stabilizer paclitaxel from the Pacific yew tree, and the *Streptomyces*-derived Hsp90 inhibitor geldanamycin.

The *Myrica* plant belongs to the family of Myricaceae and is widely distributed throughout the world. Many *Myrica* species are edible and have been used in folk medicines. *Myrica cerifera* (also known as bayberry) is abundantly present in the Southeast and Central United States, and has been used for treatment of fevers, jaundice, ulcers, diarrhea, and dysentery. *Myrica cerifera* and its chemical constituent

2

myricanol, however, have not been previously reported to play any role in reduction of levels of protein tau.

## BRIEF SUMMARY OF THE INVENTION

The subject invention provides novel and advantageous therapeutic compositions and methods for treatment of neurodegenerative diseases, in particular, neurodegenerative diseases associated with abnormal accumulation of protein tau. Specifically exemplified herein is the therapeutic use of myricanol and myricanone isolated from root barks of *Myrica* species.

In one aspect, the subject invention provides a myricanol compound that is in predominant form of (+)- $\alpha$ R,11S-myricanol as compared to (-)- $\alpha$ S,11R-myricanol. In one embodiment, the (+)- $\alpha$ R,11S-myricanol is isolated from *Myrica cerifera*, and is in about 86% enantiomeric excess of (-)- $\alpha$ S,11R-myricanol.

The subject invention also provides therapeutic compositions comprising *Myrica* extracts for treatment of neurodegenerative diseases. Also provided are methods for preparing extracts of the subject invention from *Myrica* species.

The subject invention also provides pharmaceutical compositions, comprising an effective amount of the compounds and *Myrica* extracts of the subject invention as an active ingredient and a pharmaceutically-acceptable carrier.

The therapeutic compositions and methods of the subject invention are useful for treating neurodegenerative diseases, such as for example, Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis (ALS), Huntington's disease, multiple sclerosis, Pick's disease, fronto temporal dementia, cortico-basal degeneration, progressive supranuclear palsy, Creutzfeldt-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, and Kuru.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows reduction of tau levels by various natural product extracts with varying degrees of toxicity. M17 cells were plated in 96-well plates and treated with 190 extracts for 24 hours. An In-Cell Western screen assay was performed to identify extracts capable of reducing tau levels relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Ten extracts were analyzed in triplicate per half-plate (A). Tau levels are shown as a percent of vehicle-treated cells after normalization to GAPDH (B). Toxicity was determined by calculating GAPDH levels relative to vehicle. Extracts causing no change, <25% change, or >25% change in GAPDH levels are shown as "No toxicity", "Mild toxicity" or "Severe toxicity", respectively. (C) shows efficacy of extracts in each toxicity category in reduction for tau levels.

FIGS. 2A-E show reduction of tau levels by aqueous bayberry extract in multiple cell models as well as ex-vivo slice cultures. HeLa cells stably overexpressing tau (HeLa-C3; A) and IMR32 cells with endogenous tau levels (B) were treated for 4 hrs with aqueous bayberry extract at 0, 21, 35, 70  $\mu$ g/ml (0, 0.3, 0.5, and 1.0% by volume). Quantification of the Western blots after actin normalization displays a downward trend in tau levels (C). Tau levels are shown as a percentage of vehicle-treated cells $\pm$ SD. A representative Western blot (D) is used to demonstrate the dose-dependent reductions in tau levels from whole brain murine slice cultures obtained from four wild type mice (2 males, 2 females) at 5 months of age. Treatments on whole brain murine slice cultures were performed in duplicate at doses of 0, 9, 18, 35, 70, 105, 140  $\mu$ g/ml (0, 0.125, 0.25, 0.5, 1.0, and 2.0% by volume) of the bayberry extract. Each point in the graph (E) to the right represents the

3

average tau reductions (n=8 slices) for all four mice at the given doses relative to the average vehicle $\pm$ SD. \*p<0.05 by student's t test.

FIG. 3 shows reduction of tau levels by myricitrin and myricetin. Raw bayberry root-bark powder was extracted in toluene followed by ethanol and then concentrated in vacuo. The extract was tested against standard myricetin via thin layer chromatography. The results indicated the presence of myricetin in the crude extract (A). Commercial myricitrin (50  $\mu$ M) and myricetin (50  $\mu$ M) were tested over time on HeLa—C3 cells (B & C). The results are displayed as a trend line of tau levels as a percent of vehicle $\pm$ SD (D).

FIG. 4 shows that bayberry contains a variety of compounds effective for reduction of tau levels. Bayberry extract was fractionated by MPLC (A). Bayberry ethanol extract displays anti-tau activity (B). Eight UV-active chromatographic fractions from the hydrolyzed bayberry extract were tested on HeLa—C3 cells. The cells were treated with 20  $\mu$ L of each UV active bayberry fraction (40  $\mu$ g/ml) for 4 hrs and analyzed by Western blot (C). Reduction of tau proteins is presented in the graph (D) as a percentage of vehicle-treated cells (dashed line) $\pm$ SD.

FIG. 5 shows liquid chromatography/mass spectrophotometry (LC/MS) results, confirming the presence of myricetin in bayberry extract. The resulting fractions from MPLC were subjected to LC/MS for identification of chemical constituents. Fraction 11 (A) displayed similar signals to that of myricetin (B), suggesting that myricetin was present in relatively high concentrations in Fraction 11.

FIG. 6 shows  $^{13}$ C NMR spectrum of Fraction 8, revealing that myricanol is the dominant chemical constituent. Fraction 8 was analyzed by  $^{13}$ C NMR and myricanol was identified to be the dominant compound in this fraction. These  $^{13}$ C NMR data are in agreement with published data of myricanol. The chemical structure of myricanol is shown as an insert in the spectrum.

FIG. 7 shows that myricanol extracted from bayberry reduces tau levels through the facilitation of proteasomal- and lysosomal-mediated degradation of protein tau. Extracted myricanol was used to treat HeLa—C3 cells that over-express tau (O.E.) (A), IMR32 cells that express endogenous (endo) tau (B), and murine brain slices (C), and Western blots were performed. The Western blot results were quantified and plotted as a percent of vehicle $\pm$ SD (D). Tau over-expressing HeLa cells were treated with either  $\text{NH}_4\text{Cl}$  or epoxomicin and then treated at 0, 1, 2, 5  $\mu$ L of extracted myricanol (E). “\*” indicates a cleavage product of tau that is absent in cells treated with  $\text{NH}_4\text{Cl}$ , revealing that  $\text{NH}_4\text{Cl}$  inhibits lysosomal-mediated degradation of protein tau. (F) shows the molar  $\text{EC}_{50}$  of myricanol for reduction of protein tau in HeLa—C3, as determined by Western blot.

FIG. 8 shows results of chiral HPLC, showing enantiomeric compositions of synthetic myricanol.

FIG. 9 shows results of chiral HPLC, showing enantiomeric compositions of myricanol isolated from *Myrica cerifera* (BB8-10).

FIG. 10 shows the anti-tau efficacy of the bayberry extract. Briefly, H4 neuroglioma cells in four-well chamber slides were treated with the indicated amounts of bayberry extract from Herb Pharm (mixed suspension with ethanol, glycerol, and water) for 24 hours. The treated cells were fixed, and tau immunoreactivity was assessed using standard (as opposed to near-infrared) immunofluorescent microscopy. Quantitation was derived using optical density with ImageJ software.

FIG. 11 shows that chirality plays an important role to anti-tau efficacy of myricanol. Using a chiral stationary phase, HPLC traces of (+)- $\alpha$ R,11S-myricanol from *M. cer-*

4

*ifera* (A) and commercially available (+/-)-myricanol (B) reveal 86% and 9% ee, respectively. Tau levels in HeLa—C3 cells are reduced significantly by enantiomerically enriched (+)- $\alpha$ R,11S-myricanol, but the racemic mixture showed no activity at similar concentrations (C and D).

FIG. 12 shows the chemical structure of (+)- $\alpha$ R,11S-myricanol isolated from *Myrica cerifera*.

#### BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is an amino acid sequence of a tau protein isoform (tau 352) useful according to the subject invention.

SEQ ID NO:2 is an amino acid sequence of a tau protein isoform (tau 441) useful according to the subject invention.

SEQ ID NO:3 is an amino acid sequence of a tau protein isoform (tau 383) useful according to the subject invention.

SEQ ID NO:4 is an amino acid sequence of a tau protein isoform (tau 758) useful according to the subject invention.

SEQ ID NO:5 is an amino acid sequence of a tau protein isoform (tau 776) useful according to the subject invention.

SEQ ID NO:6 is an amino acid sequence of a tau protein isoform (tau 412) useful according to the subject invention.

#### DETAILED DISCLOSURE OF THE INVENTION

The subject invention provides novel and advantageous materials and methods for treating neurodegenerative diseases, in particular, neurodegenerative diseases associated with abnormal accumulation of protein tau. Specifically exemplified herein is the therapeutic use of myricanol, a compound isolated from root barks of *Myrica* species.

The subject invention is based, at least in part, on the surprising discovery that *Myrica cerifera* and its chemical constituent myricanol potentially reduce endogenous and over-expressed tau levels in vitro and ex vivo. It is also discovered that myricanol reduces intracellular protein tau by facilitating clearance of protein tau via proteasomal- and lysosomal-mediated degradation. Myricanol is structurally similar to curcumin, a diarylheptanoid shown to modulate tau phosphorylation in cultured hippocampal neurons and possess anti-inflammatory and anti-amyloidogenic effects. In addition, it is discovered that myricanol more effectively reduces intracellular tau levels than myricetin, a chemical constituent abundantly present in *Myrica cerifera*. Myricitrin, a glycoside of myricetin, is also present in bayberry. Myricitrin is more chemically-stable and lipid soluble than myricetin. It is postulated that myricitrin may function as a pro-drug of myricetin in vivo, in which its glycoside group is cleaved by glycoside hydrolases in the intestinal tract to generate myricetin.

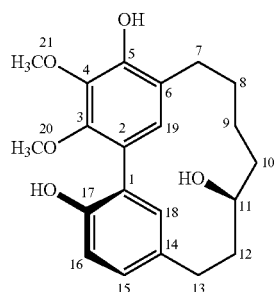
In one aspect, the subject invention provides a myricanol compound that is in predominant form of (+)- $\alpha$ R,11S-myricanol as compared to (–)- $\alpha$ S,11R-myricanol. In one embodiment, the (+)- $\alpha$ R,11S-myricanol is isolated from *Myrica cerifera*, and is in about 86% enantiomeric excess of (–)- $\alpha$ S,11R-myricanol.

The subject invention also provides therapeutic uses of myricanol and related compounds for treatment of neurodegenerative diseases, in particular, neurodegenerative diseases associated with abnormal accumulation of protein tau. The method comprises administering an effective amount of a composition comprising myricanol, or an alcohol, ester, or ether derivative of myricanol, or any salt thereof, to a subject. Myricanol and Related Compounds

In one aspect, the subject invention provides a myricanol compound that is in predominant form of (+)- $\alpha$ R,11S-myricanol as compared to (–)- $\alpha$ S,11R-myricanol, an alcohol,

5

ester, or ether derivative of the myricanol, or any salt thereof. The (+)- $\alpha$ R,11S-myricanol has the following structure:



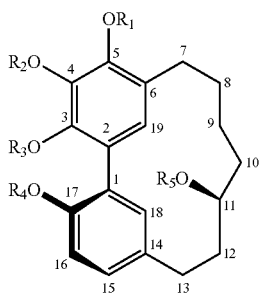
Before the current invention, (+)-11S-myricanol had always existed in a racemic mixture, and cannot be substantially separated from its enantiomer (−)- $\alpha$ S,11R-myricanol. The (+)-11S-enantiomer of myricanol had not previously been found to occur naturally.

In certain embodiments, the myricanol compound of the subject invention is at least 55%, 60%, 70%, 75%, 80%, 85%, 90%, 93%, 95%, 97%, and 100% of (+)- $\alpha$ R,11S-myricanol. In one embodiment, the myricanol compound of the subject invention is in about 93% (i.e., in about 86% enantiomeric excess) of (+)- $\alpha$ R,11S-myricanol.

In another embodiment, the subject invention provides that is substantially free from (−)- $\alpha$ S,11R-myricanol. In certain embodiments, the subject invention provides (+)- $\alpha$ R,11S-myricanol in at least 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or 100% enantiomeric excess.

The (+)- $\alpha$ R,11S-myricanol and the myricanol compound that is predominantly in the (+)- $\alpha$ R,11S form can be isolated from plants or can be chemically synthesized. In one embodiment, the (+)- $\alpha$ R,11S-myricanol and the myricanol compound that is predominantly in the (+)- $\alpha$ R,11S form are isolated from a *Myrica* species such as *Myrica cerifera*.

In certain embodiments, the subject invention also provides alcohol, ester, or ether derivatives of the myricanol compound, wherein the myricanol derivative is predominantly in the (+)- $\alpha$ R,11S form. The (+)- $\alpha$ R,11S form of the alcohol, ester, or ether derivatives of the myricanol compound have the following structure:



wherein  $R_1$ - $R_5$  are, independently, —H or any group that forms an ester or ether bond.

In one embodiment,  $R_2$  and  $R_3$  of formula I are identical.

In certain embodiments, one or more of  $R_1$ - $R_5$  of formula I can be, —H, unsubstituted or substituted alkyl, —COOH, —COOR, benzyl, or cyclic alkyl. In certain embodiments, any or all of  $R_1$ - $R_5$  have fewer than 6 carbon atoms.

6

In certain embodiments, one or more of  $R_1$ - $R_5$  of formula I can be an organic or inorganic acid group including, but not limited to, acetic acid, carboxylic acid, aspartic acid, formic acid, citric acid, benzoic acid, hippuric acid, malic acid, mucic acid, phosphoric acid, sulfuric acid, gluconic acid, maleic acid, succinic acid, tartaric, and lactic acid.

In certain embodiments, one or more of  $R_1$ - $R_5$  of formula I can be a carbohydrate moiety, in which a monosaccharide, disaccharide, oligosaccharide, or its derivative loses an —H in its hydroxyl group and thereby forms a radical. Suitable carbohydrate moieties can be derived, for example, from glucose, fructose, and sucrose.

“Alkyl” means a linear saturated monovalent radical of one to sixteen carbon atoms or a branched saturated monovalent of three to sixteen carbon atoms. It may include hydrocarbon radicals of one to four or one to three carbon atoms, which may be linear. Examples include methyl, ethyl, propyl, 2-propyl, n-butyl, iso-butyl, tert-butyl, pentyl, and the like.

“Alkenyl” means a linear or branched  $C_2$ - $C_{16}$  hydrocarbon radical that comprises one or more carbon-carbon double bonds. Examples include propenyl, buten-1-yl, isobutenyl, penten-1-yl, 2,2-methylbuten-1-yl, 3-methylbuten-1-yl, hexan-1-yl, hepten-1-yl, octen-1-yl, and the like.

“Acyl” means a radical —C(O)R where R is hydrogen, alkyl or cycloalkyl, or heterocycloalkyl. Examples include formyl, acetyl, ethylcarbonyl, and the like.

“Carboxyl” means the radical —C(O)OH.

“Carboalkoxy” means a radical —C(O)R where R is, for example, hydrogen, alkyl or cycloalkyl, heterocycloalkyl, halo, or alkyl halo.

“Halo” means fluoro, chloro, bromo or iodo.

“Haloalkyl” means alkyl substituted with one or more same or different halo atoms, e.g., —CH<sub>2</sub>Cl, —CH<sub>2</sub>Br, —CF<sub>3</sub>, —CH<sub>2</sub>CH<sub>2</sub>Cl, —CH<sub>2</sub>CCl<sub>3</sub>, and the like.

“Hydroxy” means the radical —OH.

“Hydroxyalkyl” means an alkyl radical as defined herein, substituted with one or more, preferably one, two or three hydroxy groups. Representative examples include, but are not limited to, hydroxymethyl, 2-hydroxyethyl, 2-hydroxypropyl, 3-hydroxypropyl, 1-(hydroxymethyl)-2-methylpropyl, 2-hydroxybutyl, 3-hydroxybutyl, 4-hydroxybutyl, 2,3-dihydroxypropyl, 2-hydroxy-1-hydroxymethylethyl, 2,3-dihydroxybutyl, 3,4-dihydroxybutyl and 2-(hydroxymethyl)-3-hydroxy-propyl, preferably 2-hydroxyethyl, 2,3-dihydroxypropyl and 1-(hydroxymethyl) 2-hydroxyethyl.

“Substituted,” as used herein, refers to a compound or chemical moiety in which at least one hydrogen atom of that compound or chemical moiety is replaced with a second chemical moiety. In certain embodiments, substituents include, but are not limited to, halogen; alkyl; heteroalkyl; alkenyl; alkynyl; hydroxyl, aryl, hydroxyalkyl, heteroaryl, hydroxy; alkoxy; amino; nitro; thiol; carbocyclic cycloalkyl, amino (primary, secondary or tertiary); o-lower alkyl; o-aryl, aryl; aryl-lower alkyl.

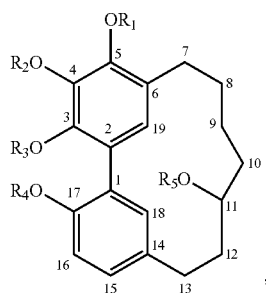
The enantiomeric forms of the compounds (e.g., isolated or chemically synthesized) of the invention are substantially free from one another (i.e., in enantiomeric excess). In other words, the “+” forms of the compounds are substantially free from the “−” forms of the compounds. Conversely, “−” forms of the compounds are substantially free of “+” forms of the compounds. In one embodiment of the invention, the enantiomeric compounds are in at least 80% of the “+” forms. In a preferred embodiment, the compounds are in at least about 90% of the “+” forms. In a more preferred embodiment, the compounds are in at least about 95% of the “+” forms. In an even more preferred embodiment, the com-

7

pounds are in at least about 97.5% of the “+” forms. In a most preferred embodiment, the compounds are in at least about 99% of the “+” forms.

The subject invention also provides therapeutic uses of myricanol and related compounds for treatment of neurodegenerative diseases, in particular, neurodegenerative diseases associated with abnormal accumulation of protein tau. The method comprises administering an effective amount of a composition comprising myricanol, or an alcohol, ester, or other derivative of myricanol, or any salt thereof, to a subject.

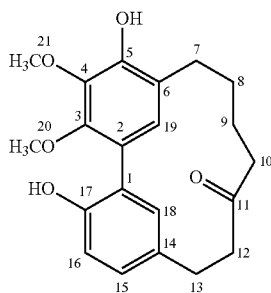
In certain embodiment, an alcohol, ester, or other derivative of myricanol has the structure of formula II:



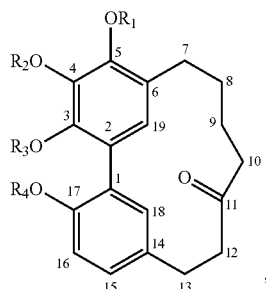
wherein R<sub>1</sub>-R<sub>5</sub> are, independently, —H or any group that forms an ester or ether bond.

Compounds useful for treatment of neurodegenerative diseases also include myricanone, an alcohol, ester, or other derivative of myricanone, or any salt thereof.

Myricanone has the following structure:



In one embodiment, the subject invention also provides therapeutic uses of an alcohol, ester, or other derivative of myricanone, having the structure of formula III:



wherein R<sub>1</sub>-R<sub>4</sub> are, independently, —H or any group that forms an ester or ether bond.

8

In one embodiment, R<sub>2</sub> and R<sub>3</sub> of formula II are identical.

In certain embodiments, one or more of R groups of the myricanol or myricanone derivatives can be —H, unsubstituted or substituted alkyl, —COOH, —COOR, benzyl, or cyclic alkyl. In certain embodiments, one or more of R groups have fewer than 6 carbon atoms.

In certain embodiments, one or more of R groups of the myricanol or myricanone derivatives can be an organic or inorganic acid group including, but not limited to, acetic acid, carboxylic acid, aspartic acid, formic acid, citric acid, benzoic acid, hippuric acid, malic acid, mucic acid, phosphoric acid, sulfuric acid, gluconic acid, maleic acid, succinic acid, tartaric, and lactic acid.

In certain embodiments, one or more of R groups of the myricanol or myricanone derivatives can be a carbohydrate moiety, in which a monosaccharide, disaccharide, oligosaccharide, or its derivative loses an —H in its hydroxyl group and thereby forms a radical. Suitable carbohydrate moieties can be derived, for example, from glucose, fructose, and sucrose.

In a specific embodiment, the subject invention provides methods for treating neurodegenerative diseases, particularly neurodegenerative diseases associated with abnormal accumulation of protein tau, by administering a composition comprising myricanol, myricanone, or an alcohol, ether, or ester of myricanol, or any salt thereof. In one embodiment, the composition comprises myricanol that is in a form of predominantly (+)-αR,11S-myricanol with respect to (–)-αS,11R-myricanol, or an alcohol, ether, or ester of (+)-αR,11S-myricanol, or any salt thereof.

In another embodiment, the subject method comprises administration, in addition to myricanol and/or myricanone, myricitrin and/or myricetin.

The subject invention further provides methods for treating or ameliorating neurodegenerative diseases by administering a composition comprising isolated enantiomeric compounds.

The subject invention further provides methods for treating or ameliorating neurodegenerative diseases by administering a composition comprising isolated compounds in a form, including but not limited to, a salt, crystalline, polymorph, amorphous, solvate, hydrate, ester, prodrug, metabolite, and any combination thereof.

The subject invention also contemplates administration of a prodrug or metabolite of Compound A. The term “prodrug,” as used herein, refers to a metabolic precursor of a compound of the subject invention or pharmaceutically acceptable form thereof. In general, a prodrug comprises a functional derivative of a compound, which may be inactive when administered to a subject, but is readily convertible in vivo into an active metabolite compound.

Conventional procedures for the selection and preparation of suitable prodrug derivatives are described, for example, in “Design of Prodrugs”, ed. H. Bundgaard, Elsevier, 1985. Preferably, a prodrug of the subject invention enhances desirable qualities of the compound of the subject invention, including but not limited to, solubility, bioavailability, and stability. Hence, the compounds employed in the present methods may, if desired, be delivered in a prodrug form. Prodrugs of the compounds employed in the subject invention may be prepared by modifying functional groups present in the compound such that the modifications are cleaved, either in routine manipulation or in vivo, to the parent compound.

The term “metabolite,” as used herein, refers to a pharmacologically active product, including for example, an active intermediate or an ultimate product, produced through in vivo metabolism of a compound of the subject invention in a subject. A metabolite may result, for example, from the ana-

bolic and/or catabolic processes of the administered compound in a subject, including but not limited to, the oxidation, reduction, hydrolysis, amidation, deamidation, esterification, deesterification, enzymatic cleavage, and the like.

Metabolites are typically identified by preparing a radiolabelled (e.g.,  $^{14}\text{C}$  or  $^3\text{H}$ ) isotope of a compound of the subject invention, administering it parenterally in a detectable dose (e.g., greater than about 0.5 mg/kg) to an animal such as rat, mouse, guinea pig, monkey, or to a human, allowing sufficient time for metabolism to occur (typically about 30 seconds to about 30 hours), and isolating its conversion products from the urine, blood or other biological samples. These products are easily isolated since they are labeled (others are isolated by the use of antibodies capable of binding epitopes surviving in the metabolite). The structure of metabolites can be determined in conventional fashion, e.g., by MS, LC/MS or NMR analysis. In general, analysis of metabolites is performed according to techniques well known to those skilled in the art of drug metabolism studies.

In an embodiment, the composition comprises myricanol and/or myricanol isolated from a *Myrica* species, such as for example, *Myrica cerifera*, *Myrica rubra*, *Myrica esculenta*, *Myrica faya*, *Myrica gale*, *Myrica hartwegii*, *Myrica heterophylla*, *Myrica holdridgeana*, *Myrica inodora*, *Myrica integra*, *Myrica nana*, *Myrica parvifolia*, *Myrica pennsylvanica*, *Myrica pilulifera*, *Myrica adenophora*, *Myrica californica*, *Myrica pubescens*, and *Myrica serrata*.

In a specific embodiment, the composition comprises myricanol and/or myricanol isolated from *Myrica cerifera*. In another specific embodiment, the composition comprises myricanol and/or myricanol isolated from root barks of *Myrica* species using the toluene-ethanol sequential extraction procedures of the subject invention.

#### *Myrica* Extracts

Another aspect of the subject invention provides therapeutic compositions comprising *Myrica* extracts comprising myricanol and/or myricanol for treatment of neurodegenerative diseases, in particular, neurodegenerative diseases associated with abnormal accumulation of protein tau. Also provided are methods for preparing extracts of the subject invention from *Myrica* species. In an embodiment, the subject invention provides a method for preparing *Myrica* extract, comprising:

- a) providing a sufficient quantity of raw material of a *Myrica* species;
- b) mixing the raw material of a *Myrica* species with toluene to yield a *Myrica* extract.

In a specific embodiment, the method for preparing *Myrica* extract comprises:

- a) providing a sufficient quantity of raw material of a *Myrica* species;
- b) mixing the raw material of a *Myrica* species with toluene to yield a toluene fraction and a first residue;
- c) mixing the first residue with ethanol to yield an ethanol fraction and a second residue;
- d) mixing the second residue with CMT buffer comprising about 30% chloroform, 10% methanol and 0.002% trifluoroacetic acid (TFA) to obtain a CMT extract; and
- e) fractionating the CMT extract using MPLC and recovering the fraction that comprises myricanol.

In specific embodiments, the *Myrica* species is selected from *Myrica cerifera*, *Myrica rubra*, *Myrica esculenta*, *Myrica faya*, *Myrica gale*, *Myrica hartwegii*, *Myrica heterophylla*, *Myrica holdridgeana*, *Myrica inodora*, *Myrica integra*, *Myrica nana*, *Myrica parvifolia*, *Myrica pennsylvanica*, *Myrica pilulifera*, *Myrica adenophora*, *Myrica californica*, *Myrica pubescens*, and/or *Myrica serrata*. In a preferred

embodiment, the *Myrica* species is *Myrica cerifera*. In another preferred embodiment, raw materials contain root barks of *Myrica* species, which are preferably ground into powders.

In an embodiment, the extraction procedure of the subject invention utilizes ethanol as a solvent for extraction. The solvent preferably comprises more than 70% ethanol, more than 80% ethanol, more than 90% ethanol, or even more than 95% ethanol.

In addition, it is preferred that the extraction procedure is carried out at room temperature. This temperature may be, for example, from about 20° C. to about 30° C., from about 22° C. to about 28° C., or from about 24° C. to about 26° C. In a specific embodiment, the extraction procedure is carried out at about 25° C.

In a specific embodiment, the *Myrica* extract obtained using the subject sequential extraction procedure is further separated using techniques known in the art, such as medium pressure liquid chromatography (MPLC) with a linear gradient of 0-20% methanol:chloroform (0.1% TFA). In an embodiment, the subject *Myrica* extract comprises myricanol. The subject *Myrica* extract may also comprise myricitrin and/or myricetin.

The subject invention further provides therapeutic compositions for treatment of neurodegenerative diseases, comprising one or more of fractions of the *Myrica cerifera* extract obtained using the subject sequential extraction procedure. In specific embodiments, the therapeutic composition comprises one or more of Fractions 5, 7, 8, 9, 11, 12, and 13 of the *Myrica cerifera* extract. In a further specific embodiment, the subject therapeutic composition comprises Fraction 8 of the *Myrica cerifera* extract.

In one embodiment, the *Myrica* extract comprises a myricanol compound that is in predominant form of (+)- $\alpha\text{R}$ ,11S-myricanol as compared to (–)- $\alpha\text{S}$ ,11R-myricanol.

In one embodiment, the extraction process of the present invention can be used to produce a myricanol compound that is in predominant form of (+)- $\alpha\text{R}$ ,11S-myricanol as compared to (–)- $\alpha\text{S}$ ,11R-myricanol.

#### Treatment of Neurodegenerative Diseases

The compounds and compositions of the subject invention, through administration to a subject, are useful for treating or ameliorating neurodegenerative diseases or conditions, in particular, neurodegenerative diseases or conditions associated with abnormally high levels of protein tau and/or abnormal accumulation of protein tau in neurons. In a preferred embodiment, the compounds and compositions of the subject invention are useful to treat or ameliorate Alzheimer's disease or Parkinson's disease.

The term "subject," as used herein, describes an organism, including mammals such as primates, to which treatment with the compositions according to the subject invention can be administered. Mammalian species that can benefit from the disclosed methods of treatment include, but are not limited to, apes, chimpanzees, orangutans, humans, monkeys; and other animals such as dogs, cats, horses, cattle, pigs, sheep, goats, chickens, mice, rats, guinea pigs, and hamsters. Typically, the subject is a human.

The term "tau protein" or any grammatical variation thereof (e.g., protein tau and tau etc.), as used herein, refers generally to any protein of the microtubule-associated tau protein family. Members of the tau family share the common features of a characteristic N-terminal segment, sequences of approximately 50 amino acids inserted in the N-terminal segment, a characteristic tandem repeat region consisting of 3 or 4 tandem repeats of 31-32 amino acids, and a C-terminal tail. Tau proteins of the subject invention may be in a form of

soluble tau intermediates, functional, aberrant, abnormally-truncated, mis-folded or mis-processed tau, and phosphorylated tau.

Preferably, tau protein of the subject invention is of mammalian origin, more preferably, of human origin. Specifically, tau proteins of the subject invention include microtubule-associated protein translated from the human chromosomal sequence of GenBank Accession No. AH005895 and naturally-occurring mammalian variants or isoforms thereof. Six human brain tau isoforms are currently known, including tau352 (GenBank Accession No. NP\_058525) (SEQ ID NO:1), tau441 (GenBank Accession No. NP\_005901) (SEQ ID NO:2), tau383 (GenBank Accession No. NP\_058518) (SEQ ID NO:3), tau758 (GenBank Accession No. NP\_058519) (SEQ ID NO:4), tau776 (GenBank Accession No. NP\_001116538) (SEQ ID NO:5), and tau412 (GenBank Accession No. NP\_001116539) (SEQ ID NO:6).

The term "treatment" or any grammatical variation thereof (e.g., treat, treating and treatment etc.), as used herein, includes but is not limited to, ameliorating or alleviating a symptom of a disease or condition, reducing, suppressing, inhibiting, lessening, or affecting the progression, severity, and/or scope of a condition, chance of re-occurrence or returning of a disease after a remission. For instance, the term "treatment" includes (i) ameliorating a symptom associated with a neurodegenerative disease in a patient diagnosed with the neurodegenerative disease; and/or (ii) relieving (such as attenuate the progress of) or remedying a neurodegenerative disease in a patient diagnosed with the neurodegenerative disease.

In one embodiment, the treatment method of the subject invention reduces tau levels and/or improves tau clearance. Normal, functional tau is less affected by clearance pathways in the cell than aberrant tau. In one embodiment, the treatment method of the subject invention modulates tau clearance by selectively targeting abnormal tau.

In an embodiment, the subject invention provides a method for treating or ameliorating a neurodegenerative disease or condition. The method comprises administering, to a subject in need of such treatment, an effective amount of compounds and compositions of the subject invention.

In an embodiment, the therapeutic composition is administered to a human subject who has symptoms of, or is diagnosed with, a neurodegenerative disease. In preferred embodiments, the therapeutic composition is administered to a human subject who has symptoms of, or is diagnosed with, a neurodegenerative disease associated with abnormal accumulation of protein tau. For instance, the therapeutic composition is administered to a human subject who has elevated levels of soluble protein tau and/or hyperphosphorylated protein tau in the nervous system, such as in the brain or cytoplasm of neuronal and glial cells. In addition, the therapeutic composition is administered to a human subject who exhibits pathologic features such as neurofibrillary tangles or senile plaques in neuronal cells and/or cell processes. In a specific embodiment, the therapeutic composition is administered to a human subject who has symptoms of, or is diagnosed with, Alzheimer's disease.

The identification of subjects who are in need of treatment for a neurodegenerative disease is well within the knowledge and ability of one skilled in the art. By way of example, a clinician skilled in the art can readily identify, by the use of clinical tests, neurologic and physical examination, and medical/family history, those patients who are suffering from a neurodegenerative disease as well as those who are predisposed to developing a neurodegenerative disease and thus readily determine if an individual is in need of treatment of

the subject invention. For instance, neurofibrillary tangles or senile plaques present in neuronal cells and/or cell processes can be determined using electron microscopy (EM) or other clinical techniques known in the art. In addition, spinal fluid or cerebral fluid samples or tissues samples from hippocampal tissue or frontal cortex tissue samples may be obtained from a subject and levels of protein tau present in the samples can be determined using routine techniques such as enzyme-linked immunosorbant assay (ELISA), western blot, and immunological assays.

The term "effective amount" or "therapeutically effective amount," as used herein, refers to an amount that is capable of preventing, treating or ameliorating a disease or condition or otherwise capable of producing an intended therapeutic effect. For instance, the effective amount of the compounds and compositions of the subject invention is an amount capable of reducing levels of protein tau in a subject. In certain embodiments, the effective amount enables a 5%, 25%, 50%, 75%, 90%, 95%, 99% and 100% reduction of levels of protein tau (e.g. soluble protein tau intermediates and/or aberrant protein tau) in a subject.

The compounds and compositions of the subject invention can be used to treat or ameliorate neurodegenerative diseases including, but not limited to, Alzheimer's disease, Parkinson's disease, Amyotrophic lateral sclerosis (ALS), Huntington's disease, multiple sclerosis, Pick's disease, fronto temporal dementia, cortico-basal degeneration, progressive supranuclear palsy, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, and Kuru.

The compounds and compositions of the subject invention can also be used to treat or ameliorate neurodegenerative diseases including, but not limited to, Down's syndrome, Argyrophilic grain disease, parkinsonism dementia complex of Guam, non-Guamanian motor neurone disease with NFT, Niemann-Pick disease type C, subacute sclerosing panencephalitis, postencephalitic parkinsonism, dementia pugilistica, myotonic dystrophy, prion protein amyloid antipathy, and Hallervorden-Spatz disease.

The compounds and compositions of the subject invention are particularly useful to treat or ameliorate a neurodegenerative disease involving tau pathologies (i.e., tauopathies) including, but not limited to, Alzheimer's disease, Parkinson's disease, frontotemporal dementia, frontotemporal dementia with Parkinsonism, frontotemporal lobe dementia, pal lidopontonigral degeneration, progressive supranuclear palsy, multiple system tauopathy, multiple system tauopathy with presenile dementia, Wilhelmsen-Lynch disease, Pick's disease, and Pick's disease-like dementia.

Specifically, the compounds and compositions of the subject invention are particularly useful to treat or ameliorate a disease or condition arising, at least in part, from abnormally high levels of protein tau in the nervous system, such as in cytoplasm of neuronal and glial cells and in neuronal and glial cell processes. Thus, the subject invention is particularly useful for treatment of neurodegenerative diseases and disorders, in which reduction of levels of protein tau in the nervous system would be beneficial.

In addition, the compounds and compositions of the subject invention are useful for alleviating or attenuating symptoms arising from or associated with neurodegenerative diseases, including cognitive dysfunction, impaired memory, impaired mental capacities, emotional disturbances, speech dysfunction, incontinence, tremor, postural instability, rigidity or stiff movement, muscle paralysis, and pain.



## Therapeutic Compositions and Formulations

The subject invention further provides therapeutic compositions that contain a therapeutically effective amount of the compounds and compositions and a pharmaceutically acceptable carrier or adjuvant.

The terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as used herein, include compositions, carriers, diluents and reagents, are used interchangeably, and represent that the materials are capable of administration to or upon a subject such as mammal.

The term "carrier" refers to an adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum oil such as mineral oil, vegetable oil such as peanut oil, soybean oil, and sesame oil, animal oil, or oil of synthetic origin. Particularly preferred pharmaceutical carriers for treatment of or amelioration of a neurodegenerative disease are carriers that can penetrate the blood/brain barrier.

Suitable carriers also include ethanol, dimethyl sulfoxide, glycerol, silica, alumina, starch, sorbitol, inositol, xylitol, D-xylose, mannitol, powdered cellulose, microcrystalline cellulose, talc, colloidal silicon dioxide, calcium carbonate, calcium phosphate, calcium aluminium silicate, aluminium hydroxide, sodium starch phosphate, lecithin, and equivalent carriers and diluents. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions.

Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The therapeutic composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary, depending on the type of the condition and the subject to be treated. In general, a therapeutic composition contains from about 5% to about 95% active ingredient (w/w). More specifically, a therapeutic composition contains from about 20% (w/w) to about 80% or about 30% to about 70% active ingredient (w/w).

The compounds and compositions of the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in detail in a number of sources which are well known and readily available to those skilled in the art. For example, Remington's Pharmaceutical Science by E. W. Martin describes formulations which can be used in connection with the subject invention. In general, the compositions of the subject invention will be formulated such that an effective amount of the bioactive compound(s) is combined with a suitable carrier in order to facilitate effective administration of the composition.

The therapeutic or pharmaceutical compositions of the subject invention can also be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is

well understood in the art and need not be limited based on formulation. Typically such compositions are prepared as injectables either as liquid solutions or suspensions; however, solid forms suitable for solution, or suspensions, in liquid prior to use also can be prepared. The preparation also can be emulsified, such as oil-in-water emulsion.

The compounds and compositions of the subject invention in prescription amounts can be readily made into any form of drug, suitable for administering to humans or animals. Suitable forms include, for example, tinctures, decoctions, and dry extracts. These can be taken orally, applied through venous injection mucous membranes or inhalation. The active ingredient can also be formulated into capsules, powder, pellets, pastille, suppositories, oral solutions, pasteurized gastroenteric suspension injections, small or large amounts of injection, frozen powder injections, pasteurized powder injections and the like. All of the above-mentioned methods are known to people skilled in the art, described in books and commonly used by practitioners of herbal medicine.

A tincture is prepared by suspending herbs in a solution of alcohol, such as, for example, wine or liquor. After a period of suspension, the liquid (the alcohol solution) may be administered for example, two or three times a day, one teaspoon each time.

A decoction is a common form of herbal preparation. It is traditionally prepared in a clay pot, but can also be prepared in glass, enamel or stainless steel containers. The formulation can be soaked for a period of time in water and then brought to a boil and simmered until the amount of water is reduced by, for example, half.

An extract is a concentrated preparation of the essential constituents of a medicinal herb. Typically, the essential constituents are extracted from the herbs by suspending the herbs in an appropriate choice of solvent, typically, water, ethanol/water mixture, methanol, butanol, iso-butanol, acetone, hexane, petroleum ether or other organic solvents.

The extracting process may be further facilitated by means of maceration, percolation, repercolation, counter-current extraction, turbo-extraction, or by carbon-dioxide hypercritical (temperature/pressure) extraction. After filtration to rid of herb debris, the extracting solution may be further evaporated and thus concentrated to yield a soft extract (extractum spissum) and/or eventually a dried extract (extractum siccum), by means of spray drying, vacuum oven drying, fluid-bed drying or freeze-drying. The soft extract or dried extract may be further dissolved in a suitable liquid to a desired concentration for administering or processed into a form such as pills, capsules, injections, etc.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients, e.g., compound, carrier suitable for administration.

## Routes of Administration

The compounds and compositions of the subject invention can be administered to the subject being treated by standard routes, including oral, inhalation, or parenteral administration including intravenous, subcutaneous, topical, transdermal, intradermal, transmucosal, intraperitoneal, intramuscular, intracapsular, intraorbital, intracardiac, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, sub-arachnoid, intraspinal, epidural and intrasternal injection, infusion, and electroporation, as well as co-administration as a component of any medical device or object to be inserted (temporarily or permanently) into a subject. In a preferred embodiment, the compounds and compositions of the subject invention are administered orally.

The amount of the therapeutic or pharmaceutical composition of the subject invention which is effective in the treatment of a neurodegenerative disease will depend on the nature of the disease, condition or disorder and can be determined by standard clinical techniques. In general, the dosage ranges from about 0.01 to about 2000 mg, about 0.01 to about 1000 mg, about 0.01 to about 500 mg, about 0.01 to about 300 mg, about 0.01 to about 200 mg, or about 0.01 to about 100 mg. Such a unit dose may be administered once to several times (e.g. two, three and four times) every two weeks, every week, twice a week, or every day, according to the judgment of the practitioner and each patient's circumstances.

Illustratively, dosage levels of the administered active ingredients can be: intravenous, 0.01 to about 20 mg/kg; intraperitoneal, 0.01 to about 100 mg/kg; subcutaneous, 0.01 to about 100 mg/kg; intramuscular, 0.01 to about 100 mg/kg; orally 0.01 to about 200 mg/kg and preferably about 1 to 100 mg/kg; intranasal instillation, 0.01 to about 20 mg/kg; and aerosol, 0.01 to about 20 mg/kg of animal (body) weight.

Once improvement of the patient's condition has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced as a function of the symptoms to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment should cease. Patients may however require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

In addition, in vitro assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the seriousness of the disease, condition or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from in vitro or animal model test systems.

## Materials and Methods

### Analytical Instrumentation

Specific rotations were measured on an Autopol IV automatic polarimeter using a Na lamp corrected to 20° C. UV data were obtained on a Varian Cary 50-Bio UV-visible spectrophotometer using a xenon lamp. Circular dichroism (CD) spectra were obtained with an Aviv Instruments model 215 CD spectrometer using a xenon lamp. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Varian 400 MHz spectrometer using residual protonated solvent as <sup>1</sup>H internal standard or <sup>13</sup>C absorption lines of solvents for <sup>13</sup>C internal standard. NMR data were obtained in CDCl<sub>3</sub> (purchased from Cambridge Isotope Laboratories). LC/MS data were obtained on an Agilent 1100 LC/MS TOF ESI mass spectrometer. Separations were conducted on a Teledyne Isco Combiflash Companion MPLC instrument using an appropriately sized normal-phase silica gel cartridge purchased from Teledyne Isco. Enantiomeric excess was measured via HPLC (Shimadzu Prominence LC-20AT HPLC system with a SPD-N20A diode array detector using a Whelk-O 1 column, solvent system: 5% IPA/hexanes, 0.8 mL/min.).

### In-Cell Western (ICW) Screen

Natural compounds tested in the screen were supplied by Herb Pharm, (Williams, Oreg.). In-Cell Western screen assay was performed on M17 cells as previously described (Dickey et al. (2005) *Curr Alzheimer Res* 2, 231-238). Briefly, M17 cells were plated in a 96-well plate with 200 µL complete media and treated with Herb Pharm compounds in triplicate (10% by volume).

Protein levels were illuminated with rabbit antihuman tau (1:500; DAKO) and mouse anti-human phosphoglycerate-3-phosphate dehydrogenase (GAPDH; 1:1500; BioDesign). Secondary infrared fluorescent antibodies were used for detection: one absorbing at 680 nm (AlexaFluor 680; Molecular Probes) and the other absorbing at 800 nm (IRDye 800 CW; Rockland, Md.). Toxicity was assessed based on GAPDH immunoreactivity.

### Cell Culture and Western Blot Analysis

HeLa cells stably expressing VS-tagged 4R0N tau were maintained under G418 selection in Opti-Mem plus 10% FBS (complete media; Invitrogen) as previously described (Jinwal et al. (2009) *J Neurosci* 29, 12079-12088). IMR32 cells were maintained in Opti-mem plus 10% FBS and 2% 200 mM L-glutamine (Cellgro, Mediatech, Inc, Herndon, Va.). IMR32, M17, and H4 cells were maintained in Opti-mem plus 10% FBS and 2% 200 mM L-glutamine (Cellgro, Mediatech, Inc., Herndon, Va.). All cells were treated as indicated and harvested as previously described (Jinwal et al. (2009) *J Neurosci* 29, 12079-12088).

Protein levels were determined by using the Peirce BCA kit. Western blotting was performed by running SDS-PAGE, followed by transfer onto Immobilon-P (Millipore), as previously described (Dickey et al. (2005) *Curr Alzheimer Res* 2, 231-23; Koren et al. (2010) *J. Bio. Chem.* 285, 2498-2505). Ex-Vivo Slice Cultures

4 non-transgenic 5-month-old mice were decapitated and their brains were quickly removed. 400 µm coronal sections were made using a vibratome as previously described (Jinwal et al. *J. Neurosci.* 2009, 29, 12079-12088; Qiu et al. *J. Neurosci.* 2006, 26, 12943-12955; Mirnikjoo et al. *J. Biol. Chem.* 2001, 276, 10888-10896. NP100572Z).

Briefly, slices were allowed to recover for 1 hour at RT in a 50/50 mixture of cutting solution (110 mM sucrose, 60 mM NaCl, 3 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 5 mM glucose, 0.6 mM ascorbate, 0.5 mM CaCl<sub>2</sub>, and 7 mM MgCl<sub>2</sub>) and artificial cerebral spinal fluid (ACSF; 125 mM NaCl, 2.5 mM KCl, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>, 25 mM glucose, 2 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub>) and then moved into ACSF at 30-32° C. for 1 hour. Slices were then moved in duplicate into an oxygenated multi-well plate at 30-32° C. containing ACSF and treated with aqueous bayberry extract at the following concentrations: 9, 18, 35, 70, 105, and 140 µg/ml. Sections were kept alive under the previously described conditions for 4 hrs and then snap frozen by liquid nitrogen. Sections were homogenized mechanically as previously described (Jinwal et al. *J. Neurosci.* 2009, 29, 12079-12088).

### Antibodies and Reagents

Anti-tau antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.) and Dako (Carpinteria, Calif.). Anti-actin and anti-GAPDH antibodies were purchased from Sigma Aldrich and Biodesign (Birmingham, Ala.). All of the aforementioned antibodies were used at a dilution of 1:1,000. Anti-S396/S404 phosphorylated tau antibody (pS396/404), provided by P. Davies of Albert Einstein College of Medicine (New York, N.Y.), was used at a dilution of 1:200. Secondary antibodies were purchased from Southern Biotech (Birmingham, Ala.) and used at a 1:1,000 dilution. Fluorescent secondary antibodies were purchased from Molecular Probes and Rockland Laboratories. All antibodies were diluted into 7% nonfat dry milk (Bio-Rad Laboratories) in TBS.

Myricitrin and (+/-)-myricanol were purchased from Indofine Chemical Co., Inc. (Hillsborough, N.J.). Myricetin was purchased from Sigma Aldrich. These compounds were all solubilized in DMSO to 50 mM.

## Bayberry Aqueous Extract

Bayberry (*M. cerifera*) root-bark powder was purchased from Frontier Natural Products Co-op (Norway, Iowa). An aqueous extract was prepared fresh for each experiment by adding 400 mg of root-bark powder to 10 mL deionized water. The solution was protected from light and allowed to nutate at room temperature for 1 hour, after which it was centrifuged at 4,200 rpm for 15 minutes to remove insoluble particles. The liquid was lyophilized to determine the concentration of dissolved analytes (7 mg/mL).

## Lipophilic Extraction of Bayberry and Isolation of (+)-aR, 11S-myricanol

50 g of raw bayberry root-bark powder was added to 150 mL of toluene (Fisher). The solution was protected from light and placed on a rotator (Thermo, ~100 rpm) for 6 hours at room temperature. The lipophilic solution was then centrifuged at 4,200 rpm for 15 min and the supernatant (toluene extract) was removed and set aside. The pellet was resuspended in 150 mL 95% ethanol and again protected from light and placed on the rotator for an additional 2 hours. The solution was again centrifuged at 4,200 rpm for 15 min and supernatant (the ethanol extract) was collected. The ethanol extract was dried in vacuo, yielding 5.64 g of crude extract.

Acid hydrolysis was performed on 2 g of crude extract to facilitate the conversion of myricitrin to myricetin (Hakkinen et al. *J. Agric. Food Chem.* 1999, 47, 2274-2279). Concentrated bayberry ethanol extract (2 g) was refluxed for 2 hrs in 16 mL 1:1 methanol:aqueous HCl (1.2M) with addition of t-butylhydroquinone (TBHQ) (32 mg) as an antioxidant. The mixture was then concentrated in vacuo and fractionated on a silica gel column using medium pressure liquid chromatography (MPLC, silica cartridge, Isco Combiflash Companion) with a linear gradient of 0-20% methanol:chloroform (0.1% TFA). UV-active bayberry fractions (F5-F13) were concentrated then resolubilized in dimethylsulfoxide (Fisher) at 40 mg/mL.

F8 was further purified using MPLC (silica, eluting with a linear gradient from 40-45% EtOAc hexane) to afford (+)-aR, 11S-myricanol 12 mg. The previously prepared toluene extract was concentrated and found to be composed of a mixture of compounds (~1.4 g) with H-aR, 11S-myricanol as a major constituent. A portion of the toluene extract was purified (430 mg) using silica gel, MPLC, eluting at 40% EtOAc: hexane to yield 180 mg (+)-S-myricanol: 86% ee; +48.0 (c 1.0, CHCl<sub>3</sub>); UV  $\lambda_{max}$  nm (MeOH) 215, 220, 260, 300; CD  $\lambda_{max}$  nm (MeOH) (As) 238 (+5.6), 258 (+6.1), 301 (+4.9). Identification of Myricanol Isolated from *M. cerifera*

Products were chromatographed on a Teledyne Isco Combiflash Companion MPLC instrument using an appropriately sized normal phase silica gel cartridge (4, 12, or 24 g) purchased from Teledyne Isco.

Specific rotations were measured on an Autopol IV automatic polarimeter using Na lamp corrected to 20° C. <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded on a Varian 400 MHz spectrometer using residual protonated solvent as <sup>1</sup>H internal standard or <sup>13</sup>C absorption lines of solvents for <sup>13</sup>C internal standard. NMR data were obtained in CDC<sub>13</sub> (purchased from Cambridge Isotope Labs). The specific rotation of myricanol was found to be  $[\alpha]_D^{20}=+48.0$  (c 1.0, CHCl<sub>3</sub>); <sup>1</sup>H NMR (400 MHz, CDC<sub>13</sub>)  $\delta$  (integration, multiplicity, J (Hz), position): 1.50-1.62 (3H, m, OH-11, H9a, H10a), 1.63-1.77 (2H, m, H9b, H12a), 1.89-1.99 (2H, m, H8, H10b), 2.35 (1H, m, 12b), 2.56 (1H, m, H-7a), 2.81 (1H, dt, 18.2, 3.0, H7b), 2.93 (2H, m, 13a, b), 3.89 (3H, s, H20), 4.01 (3H, s, H21), 4.10 (1H, t, 9.8, H11), 5.87 (1H, br s, OH5), 6.92 (1H, s, H19), 6.92 (1H, d, 8.1, H16), 7.10 (1H, dd, 8.1, 2.0, H15), 7.19 (1H, d, 2.0, H18), 7.68 (1H, s, OH7); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>)  $\delta$

(position): 22.9 (9C), 25.4 (C7), 25.8 (C8), 26.9 (C13), 34.7 (C12), 39.4 (C10), 61.4 (2C, C20 and C21), 68.6 (C11), 116.8 (C16), 122.6 (C6), 123.4 (C2), 124.7 (C1), 129.4 (C19), 129.9 (C15), 130.7 (C14), 133.1 (C18), 138.7 (C4), 145.9 (C3), 147.7 (C5), 151.4 (C17). Both <sup>1</sup>H and <sup>13</sup>C NMR data are in agreement with recent published data (Joshi et al. *J. Nat. Prod.* 1996, 59, 759-764; Kawai et al. *J. Wood Sci.* 2008, 54, 256-260), but differ from those NMR data published earlier (Sun et al. *J. Chem. Soc. C* 1971, 3634-3642).

Interestingly, it is observed that the specific rotation for myricanol isolated from *M. cerifera* (+48.0) was not in agreement with that of myricanol previously isolated from other *Myrica* species:  $[\alpha]_D^{275}=-65.6$  (c 3.0, CHCl<sub>3</sub>) from *M. nagi* (Begley et al. *J. Chem. Soc. C* 1971, 3634-3642),  $[\alpha]_D=-62.9$  from *M. rubra* (Inoue et al. *Chem. Pharm. Bull.* 1987, 35, 2569-2573),  $[\alpha]_D^{22}=-27.6$  (c 0.65, CHCl<sub>3</sub>) from *M. rubra* (Takeda et al. *Chem. Pharm. Bull.* 1987, 35, 2569-2573),  $[\alpha]_D=-64.0$  (c 0.05, CHCl<sub>3</sub>) from *M. esculenta* (Sun et al. *Phytochemistry* 1988, 27, 579-583) and  $[\alpha]_D=0.0$  (CHCl<sub>3</sub>) from *M. cerifera* (Joshi et al. *J. Nat. Prod.* 1996, 59, 759-764).

All previously isolated myricanol displayed negative rotations with the exception of one racemate. The negative rotation was correlated to an 11R configuration via X-ray crystallography of a brominated derivative of myricanol (Begley et al. *J. Chem. Soc. C* 1971, 3634-3642). (-)-R-Myricanol from *M. rubra*  $[\alpha]_D^{24}=-48.3$  along with (+)-S-myricanol 5-O- $\beta$ -D-glucopyranoside, which, upon hydrolysis yielded (+)-S-myricanol ( $[\alpha]_D^{22}=+37.3$ ), have been reported with the 11S configuration, confirmed by Mosher's analysis (Matsuda et al. *Chem. Pharm. Bull.* 2002, 50, 208-215).

## Degradation Pathway Experiments

To inhibit lysosomal-mediated degradation of protein tau, HeLa cells stably over-expressing tau were treated with ammonium chloride (20 mM) 48 hrs before myricanol treatment, as previously described (Mirnikjoo et al. *J. Biol. Chem.* 2001, 276, 10888-10896. NP100572Z). To inhibit proteasomal-mediated degradation of protein tau, another well of the same plate was treated with epoxomicin (0.3  $\mu$ M) 6 hrs before myricanol treatment.

## EXAMPLES

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

## Example 1

Preparation of *Myrica cerifera* Extract

This Example illustrates methods for preparation of *Myrica cerifera* extract using the toluene-ethanol sequential extraction procedure. Specifically, *Myrica cerifera* root barks were immersed in toluene for 8 hours with agitation at room temperature. This toluene fraction was then roto-vaporized and the resulting pellets were recovered. The pellets were re-suspended in 95% ethanol, and the ethanol fraction was then roto-vaporized and the resulting pellets were resuspended in CMT buffer comprising 30% chloroform, 10% methanol and 0.002% trifluoroacetic acid (TFA). The solvent fraction was then acid hydrolyzed with 4% sulfuric acid and TBHQ (tert-Butylhydroquinone) to remove carbohydrate moieties from chemical scaffolds. The CMT fraction was roto-vaporized and the resulting pellets were re-suspended in CMT buffer thereby forming a crude *Myrica cerifera* extract. The crude *Myrica cerifera* extract was fractionated using

## 19

medium pressure liquid chromatography (MPLC) with a linear gradient of 0-20% methanol:chloroform (0.1% TFA), yielding 8 UV-active *Myrica cerifera* fractions, including Fractions 5-9 and 11-13 that comprise flavonoids such as myricetin.

Surprisingly, Fractions 5, 7, 8, 9, 11, 12 and 13 effectively reduced levels of protein tau in vitro. Of these fractions, Fraction 8 most significantly reduced levels of protein tau. Liquid chromatography-mass spectrometry and NMR data (FIG. 6) revealed that the major components of Fraction 8 were myricanol and TBHQ.

## Example 2

#### Identification of Bayberry Extract as a Potent Tau-Reducing Agent Using Cell-Based Natural Product Screen

This Example demonstrates that the bayberry extract potentially reduces in vitro tau levels and has little toxicity. Briefly, a library of 190 natural product extracts was screened for efficacy in reducing levels of protein tau. M17 neuroblastoma cells were treated for 24 hrs and an In-Cell Western (ICW) screen was performed to assess tau and GAPDH levels. This M17 neuroblastoma cell line is derived from striatal neurons and has high levels of endogenous tau. Lack of signal in wells indicates toxicity. The entire assay was performed in a total of 10 plates. The results showed that eleven natural products reduced endogenous tau levels, but some of these extracts were also toxic to cells. The results from 10 random extracts, in triplicate, are shown in FIG. 1A. Wells lacking a GAPDH signal suggest toxicity. The entire assay was performed in a total of 10 plates. The extracts were categorized as having severe, mild or no toxicity based on their effects on GAPDH levels relative to vehicle (FIG. 1B). FIG. 1C shows toxicity levels of various extracts. Both trau oil and chapparal were extremely toxic, while trau oil still significantly reduced tau levels of the remaining viable cells. Analysis of this screen revealed that extract from *M. cerifera* (bayberry extract, Herb Pharm) was the most potent tau reducer lacking cellular toxicity (FIG. 1C). Of the extracts that reduced tau levels, the bayberry extract has little toxicity.

Anti-tau efficacy of the bayberry extract was confirmed using immunocytochemistry in a second neural cell line, H4 neuroglioma cells. H4 cells were chosen for secondary analysis not only because they have high levels of endogenous tau similar to M17 cells, but also because they are more adherent than M17 cells; as a result, H4 cells are very well-suited for fluorescent immunocytochemistry. H4 cells were treated with indicated amounts of bayberry extract. After 24 hours, immunofluorescent staining was performed. The results showed that treatment with bayberry extract resulted in a dose-dependent decrease in tau levels (FIG. 10). No toxicity was observed with LDH release analyses (data not shown).

## Example 3

#### Reduction of Tau Levels In Vitro and Ex-Vivo by Bayberry Aqueous Extract

This Example shows that the bayberry aqueous extract reduces tau levels. Briefly, raw bayberry root-bark powder was purchased and its solubility in water was tested. Raw bayberry root-bark powder was extracted in pure water, resulting in a solution that contained 7 mg extract/mL solution.

## 20

To assess the effects of the aqueous bayberry extract on overexpressed and endogenous tau levels, a HeLa cell line stably transfected with tau (HeLa—C3) and a human IMR32 cell line derived from hippocampal neurons were treated with the bayberry extract at indicated doses for four hours. The hippocampus is the region first affected by tau deposition in Alzheimer's disease.

Treatment with the aqueous bayberry preparation at 70 µg/mL for 4 hours reduced both overexpressed and endogenous tau levels by ~60% (FIGS. 2A & B). The results demonstrate that the bayberry extract can reduce tau levels, including in three brain-derived cell lines as well as a cell line overexpressing tau.

This Example also shows that bayberry aqueous extract significantly reduces tau levels in fresh forebrain tissue slices derived from mice. To confirm efficacy of the bayberry extract on reduction of neuronal tau, acute murine brain slices from wild type mice were treated with the aqueous preparation at six doses (9-140 µg/mL) for 4 hours (FIG. 2D). The results showed that total and phospho-tau levels were reduced by as much as 40%, reaching statistical significance at doses >70 µg/mL by volume (FIG. 2E).

## Example 4

#### Myricetin as a Major Component of Bayberry

The flavonoid myricetin was previously shown to be both a major constituent of bayberry and an inhibitor of Hsp70 ATPase activity that reduced tau levels. Thin layer chromatography (TLC) of crude bayberry extract was performed against a standard of myricetin. The results demonstrated that myricetin was present in high concentrations in bayberry (FIG. 3A). In addition, myricitrin, a glycoside of myricetin, is also present in bayberry.

A time course study was performed using commercially available myricitrin and myricetin. The anti-tau efficacy of each over a 24 h period in HeLa—C3 cells (FIGS. 6A and B), and the results showed that myricitrin and myricetin have similar anti-tau activity (FIG. 6C), indicating that the anti-tau efficacy of myricetin cannot be improved by rhamnosidation.

## Example 5

#### Highly Abundant Flavonoids are not the Primary Tau-Reducing Constituents of Bayberry

Ethanol extraction and fractionation of *M. cerifera* was performed to identify the primary tau-reducing constituent(s) of bayberry. The results show that the crude EtOH extraction did not reduce the potency of the bayberry extract. HeLa cells were treated with indicated concentrations of the EtOH extract of bayberry for four hours, and tau levels were assessed by Western blot. The aqueous EtOH bayberry extract reduced tau levels at all concentrations and in a dose-dependent manner (FIG. 5B).

This Example also reveals that flavonoids such as myricetin, which are abundantly present in bayberry, are not the primary chemical constituents contributing to reduction of tau levels. Briefly, the EtOH extract was subjected to acid hydrolysis to enrich the aglycone component of the extract, and was then fractionated via medium pressure liquid chromatography (MPLC). The bayberry crude extract was separated into 8 UV-active fractions by MPLC (FIG. 4A). The MPLC extraction method was designed to identify UV active flavonoids such as myricetin. Fractions 5-9 and 11-13 all showed UV activity. Treatment of HeLa—C3 cells with 400

## 21

μg/mL of each UVactive fraction from the hydrolyzed EtOH extract showed that Fraction 8 most effectively reduced tau levels (FIGS. 4C and 4D).

The results of liquid chromatography/mass spectrophotometry (LC/MS) showed that Fraction 8 contains little myricetin, most of which is present in Fraction 11 (FIG. 5).

## Example 6

## Identification of Myricanol as the Primary Anti-Tau Chemical Constituent of Bayberry

To identify the chemical constituent in F8 that most effectively reduces tau levels, <sup>13</sup>C NMR spectroscopy was performed. The diarylheptanoid myricanol was identified as the primary constituent of F8 (FIG. 6). This determination was in good agreement with previously reported assignments for myricanol (Lyketsos et al. *Neurology* 2007, 68, 1800-1808). Only trace amounts of myricanol were found in the other fractions (data not shown). The results also showed that myricanol isolated from *M. cerifera* is (+)-αR,11S-myricanol.

The results of chiral HPLC, as shown in FIGS. 8 and 9, revealed that myricanol isolated from *Myrica cerifera* (BB8-10) is predominantly present in “+” forms (93.4% in “+” forms v. 6.6% in “-” forms). The specific rotation of myricanol isolated from *M. cerifera* is  $[\alpha]_D^{20}=+48.0$ .

Specifically, the enantiomeric mixture was separated on a Whelk column with a 5% isopropanol:hexane solvent system. The “+” forms of BB8-10 are in 86% enantiomeric excess, as 93.4% of enantiomer in “+” forms eluted at ~34 min, while 6.6% of enantiomer in “-” forms eluted at ~45 min. In comparison, synthetic myricanol (Cas No. 33606-81-4) is also not a racemic mixture, but contains myricanol in 9.6% enantiomeric excess.

The CD spectrum of our (+)-S-myricanol displayed positive Cotton effects that mirror the negative Cotton effects previously reported for (-)-R-myricanol, confirming that myricanol isolated from bayberry not only has an 11S configuration, but also has equal and opposite biphenyl twisting. Joshi et al. reported an X-ray structure of the enantiomeric pair of (+)-αR,11S- and (-)-αS,11R-myricanol and also noted that the crystal structure of (-)-αS,11R myricanol closely resembled that of the previously reported brominated derivative of 11R-myricanol isolated by Begley et al. (+)-11S-Myricanol has been identified previously as a glycoside 41 and part of a racemic mixture. The (+)-11S-enantiomer of myricanol had not previously been found to occur naturally.

## Example 7

## Identification of Myricanol as a Potent Tau Modulator

This Example also shows that myricanol is a potent tau modulator in several models, including a brain tissue model. (+)-αR,11S-Myricanol was used to treat HeLa—C3 cells (FIG. 7A), IMR32 hippocampus-derived cells (FIG. 7B), and murine brain slices (FIG. 7C). The results showed that (+)-αR,11S-Myricanol potently reduced tau levels in all models with comparable potency (FIG. 7D).

Pre-treatment of HeLa—C3 cells with either an autophagy inhibitor ammonium chloride (NH<sub>4</sub>Cl; 20 mM) (19) or a proteasomal inhibitor epoxomicin (epox; 0.3 μM) abrogated tau reductions mediated by myricanol, suggesting that myricanol facilitates clearance of tau proteins via proteasomal- and lysosomal-mediated tau degradation pathways (FIG. 7E). In addition, a cleavage product of approximately 38 kDa (\*)

## 22

was not observed in the NH<sub>4</sub>Cl-treated cells, confirming NH<sub>4</sub>Cl effectively inhibits lysosomal-mediated tau degradation. Using molar concentrations of commercially available myricanol (FIG. 7E), The lactate dehydrogenase release assay also indicates that myricanol has little toxicity.

## Example 8

## Chirality Significantly Contributes to the Anti-Tau Efficacy of Myricanol

Chiral HPLC analysis revealed that myricanol isolated from *M. cerifera* is not enantiopure but, in fact, a scalemic mixture (86% ee, FIG. 11A; racemic myricanol is shown for comparison in 11B). To investigate the effect of chirality on anti-tau activity, HaLa-C3 cells were treated with the isolated (+)-αR,11S-myricanol (86% ee) and commercially available “racemic” myricanol (actually 9% ee) at the indicated concentrations. The results showed that only the isolated myricanol reduced tau levels (FIG. 11C). The EC<sub>50</sub> of (+)-αR,11S-myricanol (86% ee) is 35 μM (FIG. 11D).

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application. In addition, any elements or limitations of any invention or embodiment thereof disclosed herein can be combined with any and/or all other elements or limitations (individually or in any combination) or any other invention or embodiment thereof disclosed herein, and all such combinations are contemplated with the scope of the invention without limitation thereto.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables, to the extent they are not inconsistent with the explicit teachings of this specification.

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Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly
1          5          10          15

Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr Met His
          20          25          30

Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Ala Glu Glu Ala
          35          40          45

Gly Ile Gly Asp Thr Pro Ser Leu Glu Asp Glu Ala Ala Gly His Val
          50          55          60

Thr Gln Ala Arg Met Val Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp
65          70          75          80

Asp Lys Lys Ala Lys Gly Ala Asp Gly Lys Thr Lys Ile Ala Thr Pro
          85          90          95

Arg Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg
          100          105          110

Ile Pro Ala Lys Thr Pro Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly
          115          120          125

Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser
          130          135          140

Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro
145          150          155          160

Pro Thr Arg Glu Pro Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys
          165          170          175

Ser Pro Ser Ser Ala Lys Ser Arg Leu Gln Thr Ala Pro Val Pro Met
          180          185          190

Pro Asp Leu Lys Asn Val Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu
          195          200          205

Lys His Gln Pro Gly Gly Gly Lys Val Gln Ile Val Tyr Lys Pro Val
          210          215          220

Asp Leu Ser Lys Val Thr Ser Lys Cys Gly Ser Leu Gly Asn Ile His
225          230          235          240

His Lys Pro Gly Gly Gly Gln Val Glu Val Lys Ser Glu Lys Leu Asp
          245          250          255

Phe Lys Asp Arg Val Gln Ser Lys Ile Gly Ser Leu Asp Asn Ile Thr
          260          265          270

His Val Pro Gly Gly Gly Asn Lys Lys Ile Glu Thr His Lys Leu Thr

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275					280					285					
Phe	Arg	Glu	Asn	Ala	Lys	Ala	Lys	Thr	Asp	His	Gly	Ala	Glu	Ile	Val
290					295					300					
Tyr	Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu	Ser
305					310					315					320
Asn	Val	Ser	Ser	Thr	Gly	Ser	Ile	Asp	Met	Val	Asp	Ser	Pro	Gln	Leu
					325					330					335
Ala	Thr	Leu	Ala	Asp	Glu	Val	Ser	Ala	Ser	Leu	Ala	Lys	Gln	Gly	Leu
					340					345					350
<210> SEQ ID NO 2															
<211> LENGTH: 441															
<212> TYPE: PRT															
<213> ORGANISM: Homo sapiens															
<400> SEQUENCE: 2															
Met	Ala	Glu	Pro	Arg	Gln	Glu	Phe	Glu	Val	Met	Glu	Asp	His	Ala	Gly
1					5					10					15
Thr	Tyr	Gly	Leu	Gly	Asp	Arg	Lys	Asp	Gln	Gly	Gly	Tyr	Thr	Met	His
					20					25					30
Gln	Asp	Gln	Glu	Gly	Asp	Thr	Asp	Ala	Gly	Leu	Lys	Glu	Ser	Pro	Leu
					35					40					45
Gln	Thr	Pro	Thr	Glu	Asp	Gly	Ser	Glu	Glu	Pro	Gly	Ser	Glu	Thr	Ser
					50					55					60
Asp	Ala	Lys	Ser	Thr	Pro	Thr	Ala	Glu	Asp	Val	Thr	Ala	Pro	Leu	Val
					65					70					75
Asp	Glu	Gly	Ala	Pro	Gly	Lys	Gln	Ala	Ala	Ala	Gln	Pro	His	Thr	Glu
					85					90					95
Ile	Pro	Glu	Gly	Thr	Thr	Ala	Glu	Glu	Ala	Gly	Ile	Gly	Asp	Thr	Pro
					100					105					110
Ser	Leu	Glu	Asp	Glu	Ala	Ala	Gly	His	Val	Thr	Gln	Ala	Arg	Met	Val
					115					120					125
Ser	Lys	Ser	Lys	Asp	Gly	Thr	Gly	Ser	Asp	Asp	Lys	Lys	Ala	Lys	Gly
					130					135					140
Ala	Asp	Gly	Lys	Thr	Lys	Ile	Ala	Thr	Pro	Arg	Gly	Ala	Ala	Pro	Pro
					145					150					155
Gly	Gln	Lys	Gly	Gln	Ala	Asn	Ala	Thr	Arg	Ile	Pro	Ala	Lys	Thr	Pro
					165					170					175
Pro	Ala	Pro	Lys	Thr	Pro	Pro	Ser	Ser	Gly	Glu	Pro	Pro	Lys	Ser	Gly
					180					185					190
Asp	Arg	Ser	Gly	Tyr	Ser	Ser	Pro	Gly	Ser	Pro	Gly	Thr	Pro	Gly	Ser
					195					200					205
Arg	Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu	Pro	Lys
					210					215					220
Lys	Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys
					225					230					235
Ser	Arg	Leu	Gln	Thr	Ala	Pro	Val	Pro	Met	Pro	Asp	Leu	Lys	Asn	Val
					245					250					255
Lys	Ser	Lys	Ile	Gly	Ser	Thr	Glu	Asn	Leu	Lys	His	Gln	Pro	Gly	Gly
					260					265					270
Gly	Lys	Val	Gln	Ile	Ile	Asn	Lys	Lys	Leu	Asp	Leu	Ser	Asn	Val	Gln
					275					280					285
Ser	Lys	Cys	Gly	Ser	Lys	Asp	Asn	Ile	Lys	His	Val	Pro	Gly	Gly	Gly
					290					295					300

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Ser Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys Val Thr Ser  
 305 310 315 320  
 Lys Cys Gly Ser Leu Gly Asn Ile His His Lys Pro Gly Gly Gly Gln  
 325 330 335  
 Val Glu Val Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser  
 340 345 350  
 Lys Ile Gly Ser Leu Asp Asn Ile Thr His Val Pro Gly Gly Gly Asn  
 355 360 365  
 Lys Lys Ile Glu Thr His Lys Leu Thr Phe Arg Glu Asn Ala Lys Ala  
 370 375 380  
 Lys Thr Asp His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser  
 385 390 395 400  
 Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser  
 405 410 415  
 Ile Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val  
 420 425 430  
 Ser Ala Ser Leu Ala Lys Gln Gly Leu  
 435 440

<210> SEQ ID NO 3  
 <211> LENGTH: 383  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly  
 1 5 10 15  
 Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr Met His  
 20 25 30  
 Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Ala Glu Glu Ala  
 35 40 45  
 Gly Ile Gly Asp Thr Pro Ser Leu Glu Asp Glu Ala Ala Gly His Val  
 50 55 60  
 Thr Gln Ala Arg Met Val Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp  
 65 70 75 80  
 Asp Lys Lys Ala Lys Gly Ala Asp Gly Lys Thr Lys Ile Ala Thr Pro  
 85 90 95  
 Arg Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg  
 100 105 110  
 Ile Pro Ala Lys Thr Pro Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly  
 115 120 125  
 Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser  
 130 135 140  
 Pro Gly Thr Pro Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro  
 145 150 155 160  
 Pro Thr Arg Glu Pro Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys  
 165 170 175  
 Ser Pro Ser Ser Ala Lys Ser Arg Leu Gln Thr Ala Pro Val Pro Met  
 180 185 190  
 Pro Asp Leu Lys Asn Val Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu  
 195 200 205  
 Lys His Gln Pro Gly Gly Gly Lys Val Gln Ile Ile Asn Lys Lys Leu  
 210 215 220  
 Asp Leu Ser Asn Val Gln Ser Lys Cys Gly Ser Lys Asp Asn Ile Lys  
 225 230 235 240



His	Val	Pro	Gly	Gly	Gly	Ser	Val	Gln	Ile	Val	Tyr	Lys	Pro	Val	Asp
			245						250					255	
Leu	Ser	Lys	Val	Thr	Ser	Lys	Cys	Gly	Ser	Leu	Gly	Asn	Ile	His	His
			260					265					270		
Lys	Pro	Gly	Gly	Gly	Gln	Val	Glu	Val	Lys	Ser	Glu	Lys	Leu	Asp	Phe
			275				280					285			
Lys	Asp	Arg	Val	Gln	Ser	Lys	Ile	Gly	Ser	Leu	Asp	Asn	Ile	Thr	His
	290					295					300				
Val	Pro	Gly	Gly	Gly	Asn	Lys	Lys	Ile	Glu	Thr	His	Lys	Leu	Thr	Phe
305					310					315					320
Arg	Glu	Asn	Ala	Lys	Ala	Lys	Thr	Asp	His	Gly	Ala	Glu	Ile	Val	Tyr
			325						330					335	
Lys	Ser	Pro	Val	Val	Ser	Gly	Asp	Thr	Ser	Pro	Arg	His	Leu	Ser	Asn
			340					345					350		
Val	Ser	Ser	Thr	Gly	Ser	Ile	Asp	Met	Val	Asp	Ser	Pro	Gln	Leu	Ala
			355				360					365			
Thr	Leu	Ala	Asp	Glu	Val	Ser	Ala	Ser	Leu	Ala	Lys	Gln	Gly	Leu	
	370					375					380				

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<210> SEQ ID NO 4
<211> LENGTH: 758
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens
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<400> SEQUENCE: 4

Met 1	Ala	Glu	Pro	Arg 5	Gln	Glu	Phe	Glu	Val 10	Met	Glu	Asp	His	Ala 15	Gly
Thr	Tyr	Gly	Leu 20	Gly	Asp	Arg	Lys	Asp 25	Gln	Gly	Gly	Tyr	Thr 30	Met	His
Gln	Asp	Gln	Glu	Gly	Asp	Thr	Asp 40	Ala	Gly	Leu	Lys	Glu 45	Ser	Pro	Leu
Gln	Thr 50	Pro	Thr	Glu	Asp	Gly 55	Ser	Glu	Glu	Pro	Gly 60	Ser	Glu	Thr	Ser
Asp 65	Ala	Lys	Ser	Thr	Pro 70	Thr	Ala	Glu	Asp	Val 75	Thr	Ala	Pro	Leu	Val 80
Asp	Glu	Gly	Ala	Pro 85	Gly	Lys	Gln	Ala	Ala 90	Ala	Gln	Pro	His	Thr 95	Glu
Ile	Pro	Glu	Gly 100	Thr	Thr	Ala	Glu	Glu 105	Ala	Gly	Ile	Gly	Asp 110	Thr	Pro
Ser	Leu	Glu	Asp	Glu	Ala	Ala	Gly 120	His	Val	Thr	Gln	Glu 125	Pro	Glu	Ser
Gly	Lys 130	Val	Val	Gln	Glu	Gly 135	Phe	Leu	Arg	Glu	Pro 140	Gly	Pro	Pro	Gly
Leu 145	Ser	His	Gln	Leu	Met 150	Ser	Gly	Met	Pro	Gly 155	Ala	Pro	Leu	Leu	Pro 160
Glu	Gly	Pro	Arg	Glu 165	Ala	Thr	Arg	Gln	Pro 170	Ser	Gly	Thr	Gly	Pro 175	Glu
Asp	Thr	Glu	Gly 180	Gly	Arg	His	Ala	Pro 185	Glu	Leu	Leu	Lys	His 190	Gln	Leu
Leu	Gly	Asp	Leu	His	Gln	Glu	Gly 200	Pro	Pro	Leu	Lys	Gly 205	Ala	Gly	Gly
Lys 210	Glu	Arg	Pro	Gly	Ser	Lys 215	Glu	Glu	Val	Asp	Glu	Asp 220	Arg	Asp	Val
Asp	Glu	Ser	Ser	Pro	Gln	Asp	Ser	Pro	Pro	Ser	Lys	Ala	Ser	Pro	Ala

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225					230					235					240
Gln	Asp	Gly	Arg	Pro	Pro	Gln	Thr	Ala	Ala	Arg	Glu	Ala	Thr	Ser	Ile
				245					250					255	
Pro	Gly	Phe	Pro	Ala	Glu	Gly	Ala	Ile	Pro	Leu	Pro	Val	Asp	Phe	Leu
		260						265					270		
Ser	Lys	Val	Ser	Thr	Glu	Ile	Pro	Ala	Ser	Glu	Pro	Asp	Gly	Pro	Ser
	275						280					285			
Val	Gly	Arg	Ala	Lys	Gly	Gln	Asp	Ala	Pro	Leu	Glu	Phe	Thr	Phe	His
290						295					300				
Val	Glu	Ile	Thr	Pro	Asn	Val	Gln	Lys	Glu	Gln	Ala	His	Ser	Glu	Glu
305					310					315					320
His	Leu	Gly	Arg	Ala	Ala	Phe	Pro	Gly	Ala	Pro	Gly	Glu	Gly	Pro	Glu
				325					330					335	
Ala	Arg	Gly	Pro	Ser	Leu	Gly	Glu	Asp	Thr	Lys	Glu	Ala	Asp	Leu	Pro
			340					345					350		
Glu	Pro	Ser	Glu	Lys	Gln	Pro	Ala	Ala	Ala	Pro	Arg	Gly	Lys	Pro	Val
	355						360					365			
Ser	Arg	Val	Pro	Gln	Leu	Lys	Ala	Arg	Met	Val	Ser	Lys	Ser	Lys	Asp
370						375					380				
Gly	Thr	Gly	Ser	Asp	Asp	Lys	Lys	Ala	Lys	Thr	Ser	Thr	Arg	Ser	Ser
385					390					395					400
Ala	Lys	Thr	Leu	Lys	Asn	Arg	Pro	Cys	Leu	Ser	Pro	Lys	His	Pro	Thr
				405					410					415	
Pro	Gly	Ser	Ser	Asp	Pro	Leu	Ile	Gln	Pro	Ser	Ser	Pro	Ala	Val	Cys
		420						425					430		
Pro	Glu	Pro	Pro	Ser	Ser	Pro	Lys	Tyr	Val	Ser	Ser	Val	Thr	Ser	Arg
	435						440					445			
Thr	Gly	Ser	Ser	Gly	Ala	Lys	Glu	Met	Lys	Leu	Lys	Gly	Ala	Asp	Gly
450						455					460				
Lys	Thr	Lys	Ile	Ala	Thr	Pro	Arg	Gly	Ala	Ala	Pro	Pro	Gly	Gln	Lys
465					470					475					480
Gly	Gln	Ala	Asn	Ala	Thr	Arg	Ile	Pro	Ala	Lys	Thr	Pro	Pro	Ala	Pro
				485					490					495	
Lys	Thr	Pro	Pro	Ser	Ser	Gly	Glu	Pro	Pro	Lys	Ser	Gly	Asp	Arg	Ser
		500						505					510		
Gly	Tyr	Ser	Ser	Pro	Gly	Ser	Pro	Gly	Thr	Pro	Gly	Ser	Arg	Ser	Arg
	515					520						525			
Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu	Pro	Lys	Lys	Val	Ala
530						535						540			
Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys	Ser	Arg	Leu
545					550					555					560
Gln	Thr	Ala	Pro	Val	Pro	Met	Pro	Asp	Leu	Lys	Asn	Val	Lys	Ser	Lys
				565					570					575	
Ile	Gly	Ser	Thr	Glu	Asn	Leu	Lys	His	Gln	Pro	Gly	Gly	Gly	Lys	Val
			580					585					590		
Gln	Ile	Ile	Asn	Lys	Lys	Leu	Asp	Leu	Ser	Asn	Val	Gln	Ser	Lys	Cys
	595						600					605			
Gly	Ser	Lys	Asp	Asn	Ile	Lys	His	Val	Pro	Gly	Gly	Gly	Ser	Val	Gln
610						615					620				
Ile	Val	Tyr	Lys	Pro	Val	Asp	Leu	Ser	Lys	Val	Thr	Ser	Lys	Cys	Gly
625					630					635					640
Ser	Leu	Gly	Asn	Ile	His	His	Lys	Pro	Gly	Gly	Gly	Gln	Val	Glu	Val
				645					650					655	

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Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser Lys Ile Gly  
                   660                  665                  670  
 Ser Leu Asp Asn Ile Thr His Val Pro Gly Gly Gly Asn Lys Lys Ile  
                   675                  680                  685  
 Glu Thr His Lys Leu Thr Phe Arg Glu Asn Ala Lys Ala Lys Thr Asp  
                   690                  695                  700  
 His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr  
 705                  710                  715                  720  
 Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser Ile Asp Met  
                   725                  730                  735  
 Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val Ser Ala Ser  
                   740                  745                  750  
 Leu Ala Lys Gln Gly Leu  
                   755

<210> SEQ ID NO 5  
 <211> LENGTH: 776  
 <212> TYPE: PRT  
 <213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly  
 1                  5                  10                  15  
 Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr Met His  
                   20                  25                  30  
 Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Glu Ser Pro Leu  
                   35                  40                  45  
 Gln Thr Pro Thr Glu Asp Gly Ser Glu Glu Pro Gly Ser Glu Thr Ser  
                   50                  55                  60  
 Asp Ala Lys Ser Thr Pro Thr Ala Glu Asp Val Thr Ala Pro Leu Val  
 65                  70                  75                  80  
 Asp Glu Gly Ala Pro Gly Lys Gln Ala Ala Ala Gln Pro His Thr Glu  
                   85                  90                  95  
 Ile Pro Glu Gly Thr Thr Ala Glu Glu Ala Gly Ile Gly Asp Thr Pro  
                   100                  105                  110  
 Ser Leu Glu Asp Glu Ala Ala Gly His Val Thr Gln Glu Pro Glu Ser  
                   115                  120                  125  
 Gly Lys Val Val Gln Glu Gly Phe Leu Arg Glu Pro Gly Pro Pro Gly  
                   130                  135                  140  
 Leu Ser His Gln Leu Met Ser Gly Met Pro Gly Ala Pro Leu Leu Pro  
 145                  150                  155                  160  
 Glu Gly Pro Arg Glu Ala Thr Arg Gln Pro Ser Gly Thr Gly Pro Glu  
                   165                  170                  175  
 Asp Thr Glu Gly Gly Arg His Ala Pro Glu Leu Leu Lys His Gln Leu  
                   180                  185                  190  
 Leu Gly Asp Leu His Gln Glu Gly Pro Pro Leu Lys Gly Ala Gly Gly  
                   195                  200                  205  
 Lys Glu Arg Pro Gly Ser Lys Glu Glu Val Asp Glu Asp Arg Asp Val  
                   210                  215                  220  
 Asp Glu Ser Ser Pro Gln Asp Ser Pro Pro Ser Lys Ala Ser Pro Ala  
 225                  230                  235                  240  
 Gln Asp Gly Arg Pro Pro Gln Thr Ala Ala Arg Glu Ala Thr Ser Ile  
                   245                  250                  255  
 Pro Gly Phe Pro Ala Glu Gly Ala Ile Pro Leu Pro Val Asp Phe Leu

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260							265					270				
Ser	Lys	Val	Ser	Thr	Glu	Ile	Pro	Ala	Ser	Glu	Pro	Asp	Gly	Pro	Ser	
		275					280					285				
Val	Gly	Arg	Ala	Lys	Gly	Gln	Asp	Ala	Pro	Leu	Glu	Phe	Thr	Phe	His	
		290				295					300					
Val	Glu	Ile	Thr	Pro	Asn	Val	Gln	Lys	Glu	Gln	Ala	His	Ser	Glu	Glu	
		305			310					315				320		
His	Leu	Gly	Arg	Ala	Ala	Phe	Pro	Gly	Ala	Pro	Gly	Glu	Gly	Pro	Glu	
				325					330					335		
Ala	Arg	Gly	Pro	Ser	Leu	Gly	Glu	Asp	Thr	Lys	Glu	Ala	Asp	Leu	Pro	
			340					345					350			
Glu	Pro	Ser	Glu	Lys	Gln	Pro	Ala	Ala	Ala	Pro	Arg	Gly	Lys	Pro	Val	
		355					360					365				
Ser	Arg	Val	Pro	Gln	Leu	Lys	Ala	Arg	Met	Val	Ser	Lys	Ser	Lys	Asp	
		370				375					380					
Gly	Thr	Gly	Ser	Asp	Asp	Lys	Lys	Ala	Lys	Thr	Ser	Thr	Arg	Ser	Ser	
		385			390					395					400	
Ala	Lys	Thr	Leu	Lys	Asn	Arg	Pro	Cys	Leu	Ser	Pro	Lys	His	Pro	Thr	
				405				410						415		
Pro	Gly	Ser	Ser	Asp	Pro	Leu	Ile	Gln	Pro	Ser	Ser	Pro	Ala	Val	Cys	
			420					425					430			
Pro	Glu	Pro	Pro	Ser	Ser	Pro	Lys	Tyr	Val	Ser	Ser	Val	Thr	Ser	Arg	
		435					440					445				
Thr	Gly	Ser	Ser	Gly	Ala	Lys	Glu	Met	Lys	Leu	Lys	Gly	Ala	Asp	Gly	
		450				455					460					
Lys	Thr	Lys	Ile	Ala	Thr	Pro	Arg	Gly	Ala	Ala	Pro	Pro	Gly	Gln	Lys	
		465			470					475					480	
Gly	Gln	Ala	Asn	Ala	Thr	Arg	Ile	Pro	Ala	Lys	Thr	Pro	Pro	Ala	Pro	
				485					490					495		
Lys	Thr	Pro	Pro	Ser	Ser	Ala	Thr	Lys	Gln	Val	Gln	Arg	Arg	Pro	Pro	
		500						505					510			
Pro	Ala	Gly	Pro	Arg	Ser	Glu	Arg	Gly	Glu	Pro	Pro	Lys	Ser	Gly	Asp	
		515					520					525				
Arg	Ser	Gly	Tyr	Ser	Ser	Pro	Gly	Ser	Pro	Gly	Thr	Pro	Gly	Ser	Arg	
		530				535					540					
Ser	Arg	Thr	Pro	Ser	Leu	Pro	Thr	Pro	Pro	Thr	Arg	Glu	Pro	Lys	Lys	
		545			550					555					560	
Val	Ala	Val	Val	Arg	Thr	Pro	Pro	Lys	Ser	Pro	Ser	Ser	Ala	Lys	Ser	
				565				570						575		
Arg	Leu	Gln	Thr	Ala	Pro	Val	Pro	Met	Pro	Asp	Leu	Lys	Asn	Val	Lys	
			580					585					590			
Ser	Lys	Ile	Gly	Ser	Thr	Glu	Asn	Leu	Lys	His	Gln	Pro	Gly	Gly	Gly	
		595					600					605				
Lys	Val	Gln	Ile	Ile	Asn	Lys	Lys	Leu	Asp	Leu	Ser	Asn	Val	Gln	Ser	
		610				615						620				
Lys	Cys	Gly	Ser	Lys	Asp	Asn	Ile	Lys	His	Val	Pro	Gly	Gly	Gly	Ser	
		625			630					635					640	
Val	Gln	Ile	Val	Tyr	Lys	Pro	Val	Asp	Leu	Ser	Lys	Val	Thr	Ser	Lys	
				645				650						655		
Cys	Gly	Ser	Leu	Gly	Asn	Ile	His	His	Lys	Pro	Gly	Gly	Gly	Gln	Val	
			660					665					670			
Glu	Val	Lys	Ser	Glu	Lys	Leu	Asp	Phe	Lys	Asp	Arg	Val	Gln	Ser	Lys	
		675					680						685			

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Ile Gly Ser Leu Asp Asn Ile Thr His Val Pro Gly Gly Gly Asn Lys
 690                               695                               700

Lys Ile Glu Thr His Lys Leu Thr Phe Arg Glu Asn Ala Lys Ala Lys
 705                               710                               715                               720

Thr Asp His Gly Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly
                               725                               730                               735

Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser Ile
                               740                               745                               750

Asp Met Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val Ser
                               755                               760                               765

Ala Ser Leu Ala Lys Gln Gly Leu
 770                               775

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<210> SEQ ID NO 6
<211> LENGTH: 412
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

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<400> SEQUENCE: 6

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Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly
 1                               5                               10                               15

Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr Met His
 20                               25                               30

Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Glu Ser Pro Leu
 35                               40                               45

Gln Thr Pro Thr Glu Asp Gly Ser Glu Glu Pro Gly Ser Glu Thr Ser
 50                               55                               60

Asp Ala Lys Ser Thr Pro Thr Ala Glu Ala Glu Glu Ala Gly Ile Gly
 65                               70                               75                               80

Asp Thr Pro Ser Leu Glu Asp Glu Ala Ala Gly His Val Thr Gln Ala
 85                               90                               95

Arg Met Val Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp Asp Lys Lys
 100                              105                              110

Ala Lys Gly Ala Asp Gly Lys Thr Lys Ile Ala Thr Pro Arg Gly Ala
 115                              120                              125

Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg Ile Pro Ala
 130                              135                              140

Lys Thr Pro Pro Ala Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro
 145                              150                              155                              160

Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser Pro Gly Thr
 165                              170                              175

Pro Gly Ser Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg
 180                              185                              190

Glu Pro Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser
 195                              200                              205

Ser Ala Lys Ser Arg Leu Gln Thr Ala Pro Val Pro Met Pro Asp Leu
 210                              215                              220

Lys Asn Val Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys His Gln
 225                              230                              235                              240

Pro Gly Gly Gly Lys Val Gln Ile Ile Asn Lys Lys Leu Asp Leu Ser
 245                              250                              255

Asn Val Gln Ser Lys Cys Gly Ser Lys Asp Asn Ile Lys His Val Pro
 260                              265                              270

Gly Gly Gly Ser Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys

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275	280	285
Val Thr Ser Lys Cys Gly	Ser Leu Gly Asn Ile	His His Lys Pro Gly
290	295	300
Gly Gly Gln Val Glu Val	Lys Ser Glu Lys Leu Asp	Phe Lys Asp Arg
305	310	315 320
Val Gln Ser Lys Ile Gly	Ser Leu Asp Asn Ile Thr	His Val Pro Gly
	325	330 335
Gly Gly Asn Lys Lys Ile	Glu Thr His Lys Leu Thr	Phe Arg Glu Asn
	340	345 350
Ala Lys Ala Lys Thr Asp	His Gly Ala Glu Ile Val	Tyr Lys Ser Pro
	355	360 365
Val Val Ser Gly Asp Thr	Ser Pro Arg His Leu Ser	Asn Val Ser Ser
	370	375 380
Thr Gly Ser Ile Asp Met	Val Asp Ser Pro Gln Leu	Ala Thr Leu Ala
385	390	395 400
Asp Glu Val Ser Ala Ser	Leu Ala Lys Gln Gly Leu	
	405	410

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What is claimed is:

1. A method of preparing a *Myrica* extract comprising myricanol, comprising:

- a) providing a sufficient quantity of raw material of a *Myrica* species;
- b) extracting the raw material with toluene to yield a toluene fraction and a first residue;
- c) mixing the first residue with ethanol to yield an ethanol fraction and a second residue;
- d) mixing the second residue with CMT buffer comprising about 30% chloroform, 10% methanol and

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0.002% trifluoroacetic acid (TFA) to obtained a CMT extract; and

- e) fractionating the CMT extract using MPLC and recovering the fraction that comprises myricanol.

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2. The method of claim 1, wherein the myricanol is in predominant form of (+)- $\alpha$ R,11S-myricanol as compared to (-)- $\alpha$ S,11R-myricanol, wherein the ( $\pm$ )- $\alpha$ R,11S form is in at least 10% enantiomeric excess of the (-)- $\alpha$ S,11R foam.

3. The method of claim 2, wherein the (+)- $\alpha$ R,11S form is in about 86% enantiomeric excess of the (-)- $\alpha$ S,11R form.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 8,940,945 B2  
APPLICATION NO. : 13/810362  
DATED : January 27, 2015  
INVENTOR(S) : Chad Dickey et al.

Page 1 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the specification

Column 3,

Line 67, “(+)-aR” should read -- (+)- $\alpha$ R --

Column 4,

Line 4, “(+)-aR,” should read -- (+)- $\alpha$ R, --

Column 12,

Line 47, “pal lidopontonigral” should read -- pallidopontonigral --

Column 16,

Line 10, “VS-tagged” should read -- V5-tagged --

Column 17,

Line 11, “(+)-aR,” should read -- (+)- $\alpha$ R, --

Line 38, “(+)-aR,” should read -- (+)- $\alpha$ R, --

Line 41, “H-aR,” should read -- (+)- $\alpha$ R, --

Lines 41-42, “myricanolas a major” should read -- myicanol as a major --

Line 46, “(As)” should read -- ( $\Delta\epsilon$ ) --

Column 18,

Line 13, “[ $\alpha$ ]<sub>D</sub><sup>275</sup> =” should read -- [ $\alpha$ ]<sub>D</sub><sup>27.5</sup> = --

Column 19,

Line 37, “Both trau” should read -- Both tau --

Line 38, “trau oil” should read -- tau oil --

Signed and Sealed this  
Eighth Day of December, 2015



Michelle K. Lee  
Director of the United States Patent and Trademark Office

**CERTIFICATE OF CORRECTION (continued)**

Page 2 of 2

**U.S. Pat. No. 8,940,945 B2**

Column 21,

Line 21, “is (+)- $\alpha$ R” should read -- is (+)- $\alpha$ R --

Line 41, “(+)-aR, ... (-)-aS,” should read -- (+)- $\alpha$ R, ... (-)- $\alpha$ S --

Line 42, “of (-)-aS,” should read -- of (-)- $\alpha$ S, --

Line 56, “(+)-aR,” should read -- (+)- $\alpha$ R, --

Lines 58-59, “(+)-aR, 11SMyricanol” should read -- (+)- $\alpha$ R, 11S - Myricanol --

Column 22,

Line 18, “(+)-aR,” should read -- (+)- $\alpha$ R, --

Line 21, “(+)-aR,” should read -- (+)- $\alpha$ R, --

In the claims

Column 40,

Line 31, Claim 2, “-( $\pm$ )- $\alpha$ R” should read -- (+)- $\alpha$ R --

Line 32, Claim 2, “foam.” should read -- form. --