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(54) Title: NEEM COMPOSITIONS USED FOR THE TREATMENT OF CANCER

(57) Abstract: The present invention includes compositions and methods of treating one or more symptom of cancer by administering a therapeutically effective dose of a pharmaceutical formulation, selected from Nimbolide, Nimbandiol, 2', 3 ' dihydro Nimbolide, 28 dihydro Nimbolide, or a combination thereof to the patient to ameliorate one or more symptoms of the cancer; and monitoring the one or more symptom of the cancer.



NEEM COMPOSITIONS USED FOR THE TREATMENT OF CANCER

TECHNICAL FIELD OF THE INVENTION

The present invention relates generally to methods and compositions derived from neem extracts used in the treatment of cancer and specifically used in the treatment of prostate cancer.

5 BACKGROUND OF THE INVENTION

Without limiting the scope of the invention, its background is described in connection with neem extract compositions used in the treatment of cancer and specifically used in the treatment of prostate cancer.

Products made from Neem trees have been used in India and throughout the world as
10 anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive and sedative agents as well as being used for healthy hair, to improve liver function, detoxify the blood, balance blood sugar levels and to treat skin diseases. However, there are numerous compounds in Neem trees which includes limonoids (i.e., 4 six-membered rings and a furan ring) also classified as tetranortriterpenes; and terpenes or triterpenoid
15 (i.e., azadirachtin A, azadirachtin B, nimbin, salannin, nimbidin, meliantriol, glycyrrhizin, citral, taxol, α -pinene, l-menthol, camphor, and ginkgolide). For example, nimbin is a chemical compound classified as a triterpenoid isolated from *Azadirachta indica* (Neem tree) and is reported to have anti-inflammatory, antipyretic, antifungal, antihistamine and antiseptic properties. Although, neem extracts and terpenes in general may be known in
20 the treatment of cancer due to their toxic effects on cancer cells, these substances are often toxic to noncancerous cells as well.

For example, U.S. Patent No. 5,602,184, entitled, "Monoterpenes, Sesquiterpenes and Diterpenes as Cancer Therapy," discloses methods of treating cancer including administering an effective amount of selected terpenes to a mammal having cancer when
25 the cancer is prostate cancer, colon cancer, astrocytoma, or sarcoma. The terpene is selected from the group consisting of a cyclic monoterpene, a noncyclic monoterpene, a noncyclic sesquiterpene and a noncyclic diterpene. The invention also provides a method of sensitizing a cancer to radiation including administering an effective amount of a terpene to a mammal having the cancer. Additionally, the invention provides methods of
30 inhibiting the growth of cancer cells.

U.S. Patent No. 5,370,873, entitled, "Therapeutic Compounds Derived from the Neem Tree" discloses purified extracts from neem leaves. The extracts inhibit the adhesion of infectious cells and cancer cells to endothelial cells. The extracts also inhibit viruses, and malaria parasites in both the asexual and sexual forms.

- 5 U.S. Patent Application Publication No. 2012/0058113, entitled, "Cancer Treatment Method," discloses a method of treating cancer by administration of a 4-quinazolineamine and at least one other anti-neoplastic agent as well as a pharmaceutical combination including the 4-quinazolineamines.

- U.S. Patent Application Publication No. 2010/0216801, entitled, "Thiazolones for use as
10 PI3 Kinase Inhibitors," discloses a method of inhibiting the activity/function of PI3 kinases using substituted thiazolones and a method of treating one or more disease states selected from: autoimmune disorders, inflammatory diseases, cardiovascular diseases, neurodegenerative diseases, allergy, asthma, pancreatitis, multiorgan failure, kidney diseases, platelet aggregation, cancer, sperm motility, transplantation rejection, graft
15 rejection and lung injuries by the administration of substituted thiazolones.

- Similarly, U.S. Patent Application Publication No. 2010/0179143, entitled, "Naphthyridine, Derivatives as PI3 Kinase Inhibitors," discloses a method of inhibiting the activity/function of PI3 kinases using naphthyridine derivatives and a method of treating one or more disease states selected from: autoimmune disorders, inflammatory
20 diseases, cardiovascular diseases, neurodegenerative diseases, allergy, asthma, pancreatitis, multiorgan failure, kidney diseases, platelet aggregation, cancer, sperm motility, transplantation rejection, graft rejection and lung injuries by the administration of naphthyridine derivatives.

SUMMARY OF THE INVENTION

- 25 Although terpenes (e.g., naphthyridines, quinazolines, quinazolineamines, thiazolones pyrrolopyrimidines and derivatives) have been shown to be useful in treating cancer, inhibiting kinases and as treatments of colorectal cancer, specific pharmaceutical compositions including the compounds (i.e., Nimbin, salannin, Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, and 28 dihydro Nimbolide and analogs thereof) have not been
30 shown for cancer treatments. Similarly, specific pharmaceutical compositions including the compounds that inhibit integrin $\beta 1$, calreticulin (CRT) and/or focal adhesion kinase (FAK) activation in LNCaP-luc2 and PC3 prostate cancer cells have not been shown.

The present invention provides bioactive compounds from supercritical Neem leaf extract (SENL) that exerts antitumor activity. For example, the present invention provides a method of treating one or more symptom of cancer by identifying a patient suffering from cancer; administering a therapeutically effective dose of a pharmaceutical formulation to the patient to ameliorate one or more symptoms of the cancer, wherein the pharmaceutical formulation comprises Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, 28 dihydro Nimbolide, or a combination thereof; and monitoring the one or more symptoms of the cancer. The cancer may be prostate cancer, colon cancer, astrocytoma, and sarcoma and the pharmaceutical formulation may modulate at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activity.

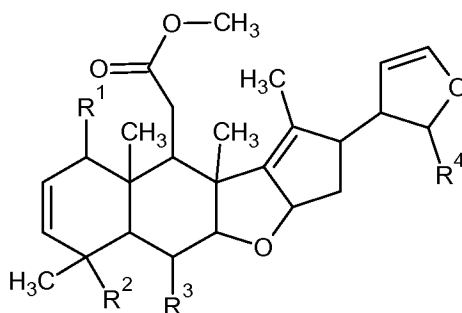
The present invention provides a method for reducing the number of cancer cells in a mammal by identifying a mammal having one or more cancer cells; administering a therapeutically effective dose of a pharmaceutical formulation to the mammal to reduce the number of cancer cells, wherein the pharmaceutical formulation comprises Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof; and monitoring the number of cancer cells in the mammal.

The present invention also provides a method for inhibiting at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activation in one or more cells by administering a therapeutically effective dose of a pharmaceutical formulation to one or more cells to inhibit at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activity, wherein the pharmaceutical formulation comprises Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof.

The present invention provides a pharmaceutical formulation for inhibiting at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activity in cancer cells wherein the pharmaceutical formulation comprises: a physiologically effective amount of Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof, in a pharmaceutical carrier to inhibit at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activity. The Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination may be isolated and purified by supercritical extraction.

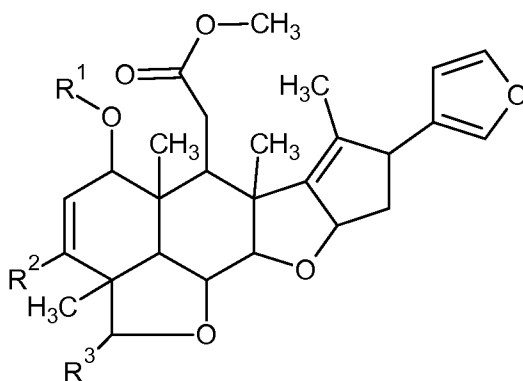
The present invention provides a pharmaceutical formulation for inhibiting at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activity, in prostate

cancer cells wherein the pharmaceutical formulation comprises: a physiologically effective amount of a composition having the formula:



in a pharmaceutical carrier sufficient to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein R^1 , R^2 , R^3 , and R^4 are independently may be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group. For example, the R^1 may be a O, R^2 may be a $-OH$, R^3 may be a $-OH$, R^4 may be a H; or R^1 may be a O, R^2 may be a $-OH$, R^3 may be a $-OCOCH_3$, R^4 may be a H; or R^1 may be a O, R^2 may be a $-COOCH_3$, R^3 may be a $-OH$; R^4 may be a H.

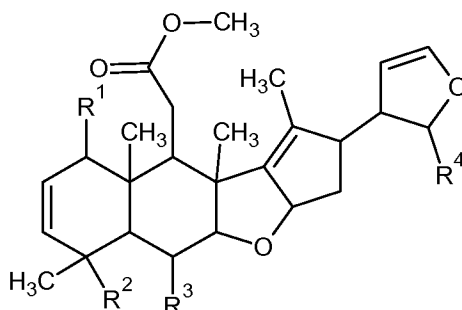
In addition, the present invention provides a pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in prostate cancer cells wherein the pharmaceutical formulation comprises: a physiologically effective amount of a composition having the formula:



in a pharmaceutical carrier sufficient to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein R^1 , R^2 , R^3 , and R^4 are independently may be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group. For example R^1 is not present, R^2 may be a $-H$, and R^3 may be a $-O$; R^1 may be a $-COCCH_3CHCH_3$, R^2 may be a $-$

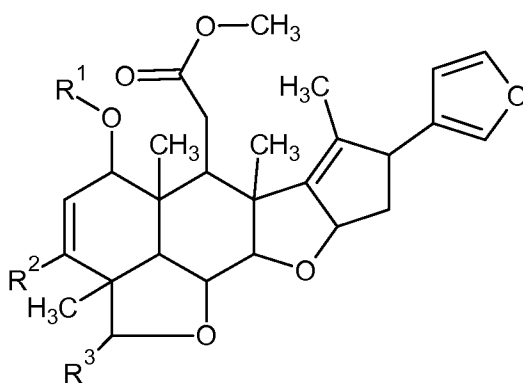
OCOCH₃, and R³ may be a -H; R¹ may be not present, R² may be a -H, and R³ may be a -H; R¹ may be a -COCCH₃CHCH₃, R² may be a -H, and R³ may be a -H; and R¹ may be a -COCCH₃CHCH₃, R² may be a -OCOCH₃, and R³ may be a -H.

The present invention provides a method of treating prostate cancer comprising: administering to a patient a therapeutically effective dose of a composition having the formula:



to reduce the number of prostate cancer cells in the patient, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group, e.g., R¹ is a O, R² is a -OH, R³ is a -OH, R⁴ is a H; or R¹ is a O, R² is a -OH, R³ is a -OCOCH₃, R⁴ is a H; or R¹ is a O, R² is a -COOCH₃, R³ is a -OH; R⁴ is a H.

The present invention also provides a method of treating prostate cancer comprising: administering to a patient a therapeutically effective dose of a composition having the formula:



to reduce the number of prostate cancer cells in the patient, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group, e.g., R¹ is not present, R² is a -H, and R³ is a -O: R¹ is a -COCCH₃CHCH₃, R² is a -OCOCH₃, and R³ is a -H; R¹ is not

present, R^2 is a $-H$, and R^3 is a $-H$; R^1 is a $-COCCH_3CHCH_3$, R^2 is a $-H$, and R^3 is a $-H$; and R^1 is a $-COCCH_3CHCH_3$, R^2 is a $-OCOCH_3$, and R^3 is a $-H$.

The present invention provides a method of performing a clinical trial to evaluate a candidate drug believed to be useful in treating prostate or colon cancer. The method includes measuring an enzyme activity from tissue suspected of having prostate or colon cancer from a set of patients, wherein the enzyme activity is at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity; administering a candidate drug to a first subset of the patients, and a placebo to a second subset of the patients; repeating after the administration of the candidate drug or the placebo; and determining if the candidate drug statistically significantly modulates the enzyme activity as compared to any reduction occurring in the second subset of patients, wherein a statistically significant reduction indicates that the candidate drug is useful in treating said disease state, for example, the candidate drug is selected from Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, 28 dihydro Nimbolide, or a combination thereof.

The present invention provides a pharmaceutical formulation for treating one or more symptom of cancer, wherein the pharmaceutical formulation comprises a therapeutically effective amount of Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, 28 dihydro Nimbolide, or a combination thereof to ameliorate one or more symptoms of the cancer.

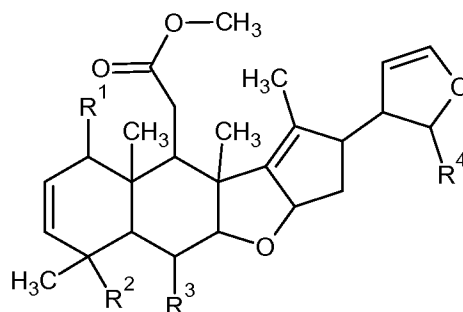
The present invention also provides a Nimbolide composition for reducing the number of cancer cells, wherein the Nimbolide composition comprises a therapeutically effective amount of Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof to reduce the number of the one or more cancer cells.

The present invention provides a method for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activation in one or more cells comprising the steps of: administering a therapeutically effective dose of a pharmaceutical formulation to one or more cells to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein the pharmaceutical formulation comprises Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof.

The present invention provides a pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in cancer cells wherein the pharmaceutical formulation comprises: a physiologically effective amount of

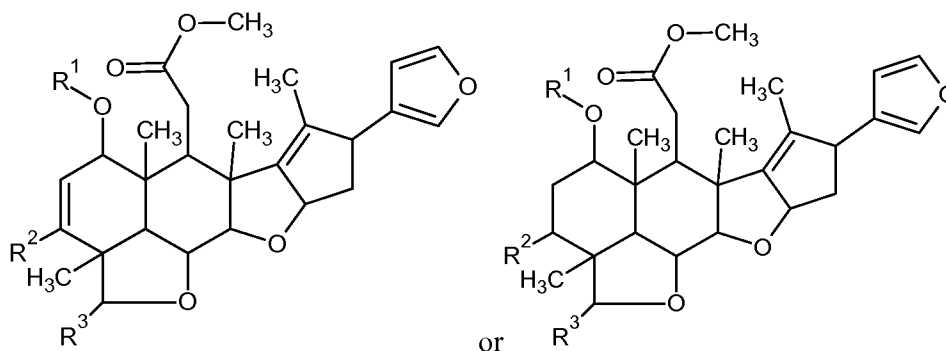
Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof, in a pharmaceutical carrier to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity. The pharmaceutical formulation comprises of Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro
 5 Nimbolide; or a combination thereof is isolated and purified by supercritical extraction.

The present invention provides a pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in prostate cancer cells wherein the pharmaceutical formulation comprises: a physiologically effective amount of a composition having the formula:



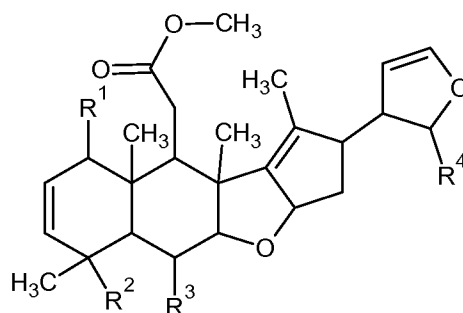
in a pharmaceutical carrier sufficient to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein R^1 , R^2 , R^3 , and R^4 are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group. For example, R^1 is a O, R^2 is a –
 15 OH, R^3 is a –OH, R^4 is a H; or R^1 is a O, R^2 is a –OH, R^3 is a –OCOCH₃, R^4 is a H; or R^1 is a O, R^2 is a –COOCH₃, R^3 is a –OH; R^4 is a H.

The present invention provides a pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in prostate cancer cells wherein the pharmaceutical formulation comprises: a physiologically
 20 effective amount of a composition having the formula:



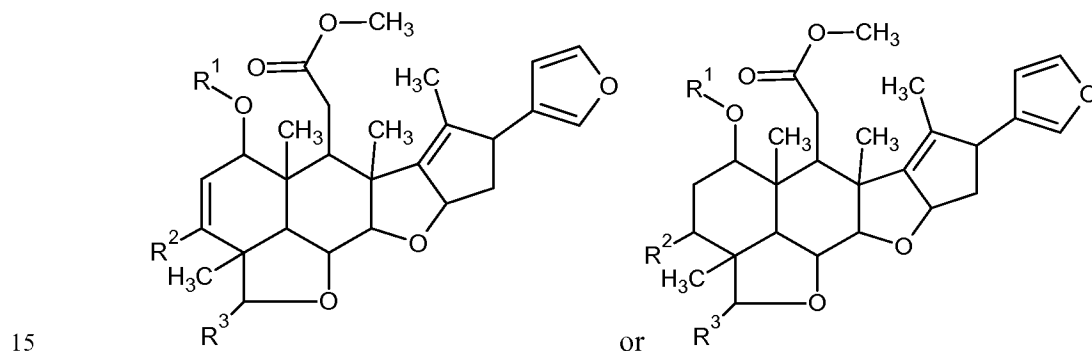
in a pharmaceutical carrier sufficient to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein R^1 , R^2 , R^3 , and R^4 are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

- 5 The present invention provides pharmaceutical formulation for treating prostate cancer wherein the pharmaceutical formulation comprises a therapeutically effective dose of a composition having the formula:



- 10 to reduce the number of prostate cancer cells in the patient, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

The present invention provides a pharmaceutical formulation for treating prostate cancer wherein the pharmaceutical formulation comprises a therapeutically effective dose of a composition having the formula:



to reduce the number of prostate cancer cells in the patient, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

BRIEF DESCRIPTION OF THE DRAWINGS

For a more complete understanding of the features and advantages of the present invention, reference is now made to the detailed description of the invention along with the accompanying figures and in which:

FIGURE 1A is a graph of the relative cell proliferation as a function of SENL.

5 FIGURE 1B is an image of a western blot analysis of AR protein levels.

FIGURE 1C is a graph of LNCaP-luc2 cells supernatants analyzed for PSA levels by ELISA.

FIGURE 1D is an image of a western blot analysis of LNCaP-luc2 and PC3 prostate cancer cells treated with SENL.

10 FIGURES 2A-2D are immunofluorescence images of the expression of FAK and integrin β 1 in prostate cancer cells.

FIGURE 3A shows bioluminescence imaging of mice implanted with LNCaP-luc2 tumors.

15 FIGURE 3B is a graph of the tumor volume changes of mice treated by oral gavage with vehicle or SENL. FIGURE 3C is a graph of the tumor weight of the mice after 9 weeks of treatment in different groups. FIGURE 3D is a plot of the body weight of mice in different groups over the treatment period.

FIGURES 4A-4D are images of the histological changes of LNCaP-luc2 tumor tissues of mice treated with SENL.

20 FIGURE 5A is an image of the chromatogram of the fractionation of SENL. FIGURE 5B is an image of the activity of the peaks labeled in the HPLC chromatogram evaluated for cytotoxic activity against LNCaP-luc2 cells. FIGURES 5C-5M are images of the mass spectrometric analysis of SENL fractions.

25 FIGURES 6A-6I are images of the structures of the neem compounds identified in the mass spectra.

FIGURES 7A-7B are images of HPLC chromatograms of mice plasma and tumor tissues for the bioavailability of SENL compounds after oral administration of SENL. FIGURES 7C-7D are images of mass spectrometric analysis of mice plasma and tumor tissues for the bioavailability of SENL compounds after oral administration of SENL.

30 DETAILED DESCRIPTION OF THE INVENTION

While the making and using of various embodiments of the present invention are discussed in detail below, it should be appreciated that the present invention provides many applicable inventive concepts that can be embodied in a wide variety of specific contexts. The specific embodiments discussed herein are merely illustrative of specific
5 ways to make and use the invention and do not delimit the scope of the invention.

As used herein, the term “alkyl” denotes branched or unbranched hydrocarbon chains, preferably having about 1 to about 8 carbons, such as, methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, iso-butyl, tert-butyl, octa-decyl and 2-methylpentyl. These groups can be optionally substituted with one or more functional groups which are attached
10 commonly to such chains, such as, hydroxyl, bromo, fluoro, chloro, iodo, mercapto or thio, cyano, alkylthio, heterocyclyl, aryl, heteroaryl, carboxyl, carbalkoyl, alkyl, alkenyl, nitro, amino, alkoxy, amido, and the like to form alkyl groups such as trifluoro methyl, 3-hydroxyhexyl, 2-carboxypropyl, 2-fluoroethyl, carboxymethyl, cyanobutyl and the like.

As used herein, the term “aryl” denotes a chain of carbon atoms which form at least one
15 aromatic ring having between about 4-14 carbon atoms, such as phenyl, naphthyl, and the like, and which may be substituted with one or more functional groups which are attached commonly to such chains, such as hydroxyl, bromo, fluoro, chloro, iodo, mercapto or thio, cyano, cyanoamido, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carbalkoyl, alkyl, alkenyl, nitro, amino, alkoxy, amido, and the like to form aryl groups such as
20 biphenyl, iodobiphenyl, methoxybiphenyl, anthryl, bromophenyl, iodophenyl, chlorophenyl, hydroxyphenyl, methoxyphenyl, formylphenyl, acetylphenyl, trifluoromethylthiophenyl, trifluoromethoxyphenyl, alkylthiophenyl, trialkylammoniumphenyl, amidophenyl, thiazolylphenyl, oxazolylphenyl, imidazolylphenyl, imidazolylmethylphenyl, and the like.

25 As used herein, the term “carboxyl” denotes --C(O)O-- , and the term “carbonyl” denotes --C(O)-- . The term “cycloalkyl” signifies a saturated, cyclic hydrocarbon group with 3-8, preferably 3-6 carbon atoms, i.e. cyclopropyl, cyclobutyl, cyclopentyl, and cyclohexyl and the like.

As used herein, the term “alkoxy” denotes --OR-- , wherein R is alkyl. As used herein, the
30 term “hydroxy” denotes --OH . As used herein, the term “carboxylic acid” denotes C(O)OH .

As used herein, the term “pharmaceutically acceptable” or “pharmacologically acceptable” is meant a material which is not biologically or otherwise undesirable, i.e., the material may be administered to an individual in a formulation or composition without causing any undesirable biological effects or interacting in a deleterious manner with any of the components of the composition in which it is contained. It is one that is suitable for use with humans and/or animals without undue adverse side effects (e.g., toxicity, irritation, and allergic response) commensurate with a reasonable benefit/risk ratio.

As used herein, the term “therapeutically effective amount” denotes an amount of a compound of the present invention effective to yield a desired therapeutic response. For example to prevent cancer or treat the symptoms of cancer in a host or an amount effective to treat cancer. The specific “therapeutically effective amount” will, obviously, vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal being treated, the duration of the treatment, the nature of concurrent therapy (if any), and the specific formulations employed and the structure of the compounds or its derivatives.

As used herein, a “pharmaceutical carrier” is a pharmaceutically acceptable solvent, suspending agent or vehicle for delivering the anti-cancer agent to the animal or human. The carrier may be liquid or solid and is selected with the planned manner of administration in mind.

As used herein, “a subject in need thereof” is a patient, animal, mammal or human, who will benefit from the method of this invention. This patient may be a person genetically disposed to cancer or a patient who is believed to be at risk for developing cancer.

As used herein, the term “treatment,” “treating,” “palliating,” and “ameliorating” are used interchangeably. These terms refer to an approach for obtaining beneficial or desired results including but not limited to therapeutic benefit and/or a prophylactic benefit. By therapeutic benefit is meant eradication or amelioration of the underlying disorder being treated. Also, a therapeutic benefit is achieved with the eradication or amelioration of one or more of the physiological symptoms associated with the underlying disorder such that an improvement is observed in the patient, notwithstanding that the patient may still be afflicted with the underlying disorder. For prophylactic benefit, the compositions may be administered to a patient at risk of developing a particular disease, or to a patient

reporting one or more of the physiological symptoms of a disease, even though a diagnosis of this disease may not have been made.

The present invention provides bioactive compounds from supercritical Neem leaf extract (SENL) that exert antitumor activity. The present invention examines the molecular targets of supercritical Neem leaf extract (SENL) *in vitro*, and evaluates the *in vivo* efficacy to inhibit tumor growth. The instant invention shows that treatment of LNCaP-luc2 prostate cancer cells with SENL suppresses dihydrotestosterone (DHT)-induced androgen receptor (AR) and prostate specific antigen (PSA) levels. SENL inhibits integrin $\beta 1$, calreticulin (CRT) and focal adhesion kinase (FAK) activation in LNCaP-luc2 and PC3 prostate cancer cells. Oral administration of SENL significantly reduced the LNCaP-luc2 xenograft tumor growth in mice with the formation of hyalinized fibrous tumor tissue. To identify the active anticancer compounds, SENL was fractionated by high pressure liquid chromatography (HPLC) and the resulting 16 peaks evaluated for cytotoxic activity. Four of the sixteen peaks exhibited significant cytotoxic activity against prostate cancer cells. Mass spectrometry of the isolated peaks suggests the compounds with cytotoxic activity as nimbandiol, nimbolide, 2', 3'- dihydronimbolide and 28-deoxonimbolide. Analysis of tumor tissues and plasma indicates 28-deoxonimbolide and nimbolide as the major compounds in mice treated with SENL. The bioactive SENL compounds of the present invention provide anticancer activity mediated through alteration in AR and CRT levels in prostate cancer.

Prostate cancer is the most frequently diagnosed malignancy among men in the Western society. Tumor development and progression involves multiple cellular processes which include cell transformation, deregulation of programmatic cell death, proliferation, invasion, angiogenesis and metastasis. Targeting a single molecule in the treatment of cancer has shown limited promise due to the diversity of deregulated pathways in cancer. The initial effect of drugs currently approved by the FDA (e.g., docetaxel and abiraterone) are often amazing to see tumor decline in advanced cancers, but many of these therapies are short-lived because the cancer cells develop resistance and most of them exhibit chemotoxicities to patients. However, despite the incorporation of new chemotherapies and regimens into prostate cancer clinical trials, the response rate and median overall survival for treated patients has not significantly improved; therefore, newer therapeutic approaches to improve upon the medication selection process are warranted. An approach in overcoming such a problem is the development of new agents

that can be used in combination with chemotherapeutic agents, encompassing better results compared to chemotherapeutic agents alone. Accumulating evidence suggests that many natural products may have the potential to interact with multiple targets in the network of pathways which support numerous molecular cascades involved in controlling the progression of cancer. Therefore, the present invention provides natural compounds that possess antitumor effects and may be used as a treatment for cancer to overcome the above-mentioned problems.

Neem leaves have been used widely in Southeast Asia for treatment of many diseases including cancer. Neem leaf glycoprotein exhibits antitumor activity by activation of cytotoxic T lymphocytes and natural killer cells in patients with head and neck squamous cell carcinoma. Although Neem leaves have been used for treatment of many diseases including cancer, bioactive neem compounds and their molecular basis of actions are not well studied. The present invention provides compositions extracted (i.e., ethanol) from Neem leaves that show antitumor activity *in vitro* and *in vivo* against prostate cancer models. The present invention demonstrates that ethanol extract of Neem leaves can regulate cell proliferation and migration, attenuate the stimulatory effects of VEGF and exert antiangiogenic effects in human umbilical vein endothelial cells (18). To obtain bioactive compounds with higher extraction efficiencies, supercritical fluid extraction method was used and the anticancer activity of supercritical Neem leaf extract (SENL) in prostate cancer was examined. Supercritical fluid extraction with carbon dioxide allows the isolation of compounds minimizing thermal and chemical degradation. The relatively low temperature of the process and the stability of CO₂ allow most compounds to be extracted with little damage or denaturing. The extraction process facilitates collection of volatile oils from the processing material. Volatile oils reduce acute and chronic inflammation and more importantly help in absorption of other compounds in humans. Extracts obtained with application of supercritical fluid extraction are very stable and can be used directly in diet supplementation.

The present invention provides SENL compositions with potential antitumor activity and methods of treating prostate cancer using these SENL compositions. DHT induced androgen receptor and PSA levels were suppressed in LNCaP-luc2 cells with SENL treatment. Oral administration of SENL dramatically reduces LNCaP-luc2 prostate cancer xenograft tumor growth in nude mice. SENL down-regulates CRT levels and inhibits FAK activation in LNCaP-luc2 and PC3 prostate cancer cells. We fractionated

SENL by high pressure liquid chromatography (HPLC) and tested the individual fraction for cytotoxic activity. HPLC fractions were analyzed on a mass selective detector time-of-flight (MSD-TOF) system to identify the compounds that exhibit anticancer activity. In the present study, we have shown for the first time molecular targets of SENL,
5 evaluated the efficacy to inhibit tumor growth and identified bioactive compounds of anticancer activity.

Supercritical extract of fresh Neem leaf was prepared in our laboratory using the Spe-ed SFE-2 system (Applied Separations, Allentown, PA). Fresh Neem leaves were purchased from Neem Tree Farms, a U.S. Department of Agriculture (USDA)-certified organic
10 Neem Farm (Brandon, FL). Supercritical fluid grade carbon dioxide was obtained from Praxair, Inc., (Rochester, MN). Supercritical CO₂ is a fluid state of CO₂ that is held at or above its critical temperature (31.1 C) and critical pressure (72.9 atm/7.39 MPa). Neem leaves of the same age were washed with distilled water, air-dried, and 100 grams of the pulverized leaves were used for extraction using 300 mL vessel. The extraction
15 parameters used were: 9000 psi, 50°C for 1 hour static and 2 hour dynamic flow of liquid CO₂ at 3 liters per minute. The collection glass vial was cooled to -49°C with dry ice/acetone bath to collect volatile oils. The process yielded approximately 5.0g of the extract. An aliquot of the extract was dissolved in dimethyl sulfoxide (DMSO) plus ethanol to make a stock solution of 200 µg/µL as described. The final concentration of
20 DMSO in the culture medium never exceeded 0.01%. The effect of the extract on cell viability and HPLC profiles described below were assessed to standardize the method of extraction. We obtained consistent results with different lots of the extract.

LNCaP-luc2, originated from lymph node derived LNCaP, is a luciferase expressing androgen dependent prostate cancer cell line stably transfected with firefly luciferase
25 gene (luc2), was purchased from Caliper LifeScience (Hopkinton, MA). PC3, a bone-derived prostate cancer cell line was purchased from the American Type Tissue Collection (Manassas, VA). LNCaP-luc2 cells were cultured in RPMI 1640 medium and PC3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) media with 10% fetal bovine serum, 25 unit/ml penicillin, and 25 µg/ml streptomycin as described
30 previously.

To determine cell growth, LNCaP-luc2 and PC3 cells were seeded at a density 3×10^3 and 1.5×10^3 per well as described. Cells were treated with 5.0 to 25.0 µg/mL of the SENL, or serial dilutions of the HPLC fractions 0.468 µg/ml to 15.0 µg/ml or with the vehicle

(ethanol + DMSO) control for 24 hours. Cell medium was replenished and cell growth was determined by MTS-formazan reduction using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA) as described.

LNCaP-luc2 cells were treated with SENL (12.0 $\mu\text{g/ml}$) in the presence or absence of 10
5 nM DHT (Sigma-Aldrich, St. Louis, MO) for 24 hrs. PSA assay was performed using the supernatants collected from LNCaP-luc2 cells. PSA secretion (ng/mL) was determined using a Human PSA ELISA Kit purchased from Abnova (Walnut, CA). For western blotting, LNCaP-luc2 and PC3 prostate cancer cells were treated with SENL (12.0 $\mu\text{g/mL}$ and 15 $\mu\text{g/mL}$) for 24 hours. Total proteins were extracted using RIPA buffer and
10 immunoblotting was performed as described previously (30). The primary antibodies for phospho-FAK (Y397) (Abcam #ab4803); FAK (Abcam #ab131435); Integrin β 1 (Cell Signaling #4706); CRT (Cell Signaling #2891); Rab5 (Cell Signaling #2143); AR (Cell Signaling #3202); and GAPDH (Santa Cruz #sc-137179) were purchased from companies. For immunofluorescence, LNCaP-luc2 and PC3 cells were grown on
15 coverslips at a density of 4×10^4 and 3×10^4 cell per well respectively. Coverslips were precoated with fibronectin for LNCaP-luc2 cell adhesion. After treatment of cells with SENL for 24 hours fixation and permeabilization of the cells were performed as described. Primary antibodies FAK rabbit polyclonal antibody (Abcam #ab131435) and Integrin β 1 mouse monoclonal antibody (BD Biosciences #610467) and secondary
20 antibodies Alexa Fluor® 488(green, Cy5 labeled) goat anti-mouse IgG and Alexa FLUOR® 594 (red, FITC labeled) goat anti-rabbit IgG were used for immunostaining. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and confocal images were taken on LSM 780 at 100 x magnification.

All of the procedures involving animals were reviewed and approved by Mayo Clinic
25 Institutional Animal Care and Use Committee. Male athymic *nu/nu* mice, 5-week-old (Charles River Laboratories, Wilmington, MA) were implanted subcutaneously in the flank with LNCaP-luc2 cells (2×10^6 cells/100 μL /mouse) suspended in 50% matrigel in RPMI medium. Tumor dimensions were measured 2 to 3 times per week, and volume was calculated as length $\times$ width $\times$ height $\times$ 0.52. Once tumors reached 30 mm³, the mice
30 were randomly assigned to 3 groups of 8 each. Group 1 received vehicle (olive oil, Sigma-O1514); Group 2 received 100 mg/kg body weight of SENL; Group 3 received 200 mg/kg body weight of SENL. Animals in each groups received the same volume of vehicle (olive oil) or SENL in 100 μL of olive oil orally by gavage, 6 days per week for 9

weeks. Tumor growth, tumor imaging and body weights were determined as described. At the end of the study, two hours before the mice were euthanized, they were given SENL, and blood was collected from each mouse *via* retro-orbital plexus into heparinized tubes; plasma was separated and stored at -80°C until used for analysis. The mice were sacrificed by CO₂ inhalation, xenograft tumor tissues and the organs including heart, lungs, liver, kidneys, and spleen were excised for fixation and hematoxylin and eosin (H&E) staining or stored frozen at -80°C for analysis of SENL compounds.

For analysis of SENL compounds in plasma and tissues, we initially performed spike and recovery studies using human plasma samples to obtain maximum recovery rates for selection of the suitable method for extraction. Extraction of spiked SENL from human plasma treated with methanol and chloroform in 1:3:1 ratio yielded maximum recovery. Mice plasma and tumor tissues collected from SENL treated groups were analyzed for bioavailability of SENL compounds and compared the profiles with the vehicle treated group as control. Mice plasma or tumor tissues were treated with methanol and chloroform in 1:3:1 ratio for deprotonation, after incubation at room temperature for 5 minutes the mixture was centrifuged for 3,500 rpm for 15 minutes. The extraction procedure was performed in glass tubes. The supernatant was evaporated using a speed vac at 4°C, reconstituted in methanol, and analyzed by HPLC and ESI-MS.

Since SENL contains a mixture of compounds, active anticancer compounds present in the SENL were separated by HPLC to evaluate the effect on growth inhibitory activity. A Shimadzu LC-10ATvP system (Columbia, MD) was used. Separation was performed on a Kinetex core-shell silica column (C18, 250 mm × 4.6 mm, particle size 5 µm, pore size 100 Å, Phenomenex, USA). Column temperature was maintained at 40°C. The autosampler injected 20 µL of sample. Mobile phase A was 100% water and B was 100% methanol. A flow rate of 1.0 ml/min starting with a 50 minute linear gradient from 50% to 100% B, 50 to 56 minutes with 100% B and 56 to 58 minutes to 10% B, the total run time of 60 min was used with UV detection at 254 nm. Methanol and water used for analysis were HPLC grade obtained from Sigma-Aldrich (St. Louis, MO).

The fractions collected from HPLC were separated on a reversed-phase C18 analytical column (Zorbax Eclipse 300SB-C18, 2.1 x 30 mm; 3.5 micron) on a mass selective detector time-of-flight (MSD-TOF) instrument (Agilent Technologies) in a positive mode ESI as described. The instrument was operated with mobile phase A as water and B as methanol with a gradient 50% B to 100% B over 10 minutes; 100% B for 5 minutes and 5

minutes to equilibrate and a flow rate of 300 μ L/minute. The scan range for acquisition was 300-1000 m/z range; scan rate: 1 spectra/sec; gas temperature 325°C; gas flow 7L/min; neb: 40 psi; Vacuum pressure: 3800V; Fragmentor: 140; Skimmer: 65; Oct: 750. Nimbolide from BioVision (Mountain View, CA) and 2',3'-dehydrosalannol from Sigma-Aldrich were used as standards.

Data are presented as the means $\pm$ SDs for the indicated number of separate studies. Statistical significance was tested by using Student's *t*-test, one-way ANOVA, Fisher's exact test and Kruskal–Wallis non-parametric ANOVA based on ranks with a Dunn's multiple comparison tests were used to compare the different experimental groups. P value < 0.05 was considered significant. Fifty percent inhibition concentration (IC₅₀) values were calculated by Probit regression.

SENL suppressed LNCaP-luc2 cell growth and inhibited DHT induced AR and PSA levels in prostate cancer cells. SENL suppresses growth, inhibits AR, PSA and CRT expression, and FAK activation in prostate cancer cells.

FIGURE 1A is a graph of the relative cell proliferation as a function of SENL. FIGURE 1A shows the antiproliferative effect of SENL on LNCaP-luc2 and PC3 prostate cancer cells was evaluated by using the MTS viability assay after 24 hours of treatment. FIGURE 1B is an image of a western blot analysis of AR protein levels. LNCaP-luc2 cells were treated with SENL (12.0 μ g/mL) in the presence and absence of 10 nM DHT for 24 hours. AR protein levels were measured with specific antibodies by Western blot analysis with GAPDH loaded as a control. FIGURE 1C is a graph of LNCaP-luc2 cells supernatants analyzed for PSA levels by ELISA. FIGURE 1D is an image of a western blot analysis of LNCaP-luc2 and PC3 prostate cancer cells treated with SENL (12.0 μ g/mL and 15 μ g/mL) for 24 hours. Protein levels were measured with specific antibodies by Western blot analysis. All experiments were performed in triplicate; data are expressed as the mean $\pm$ SD of the triplicate determinations of a representative experiment. *p < 0.05. Vehicle treated cells were used as control. GAPDH was the loading control for western blot analysis and the representative blot was shown. To assess anticancer activity, we investigated the cell death effects induced by SENL in LNCaP-luc2 and PC3 prostate cancer cell lines. The antiproliferative activity of SENL was measured by MTS assay. Exposure of cells to SENL for 24 hours exhibited a dose-dependent inhibition of LNCaP-luc2 and PC3 cell growth. LNCaP-luc2 cells exhibited

IC₅₀ of 12.0 µg/mL, while PC3 had IC₅₀ of 15 µg/mL. Next the effects of SENL was investigated on the expression levels of PSA in LNCaP-luc2 cells. PSA in the cell supernatants were analyzed by ELISA. Treatment with DHT (10 nM) for 24 hours induced PSA expression, whereas SENL significantly reduced DHT induced PSA levels in LNCaP-luc2 cells. To ascertain the effect is mediated by AR regulation, western blotting was performed to monitor the expression levels of AR after SENL treatment. DHT induced AR expression was significantly inhibited in LNCaP-luc2 cells after SENL treatment. This data supports the possibility that SENL effects are mediated through AR regulated gene expression.

FIGURES 2A-2D are immunofluorescence images of expression of FAK and integrin β 1 expression in prostate cancer cells. LNCaP-luc2 and PC3 cells were treated with SENL for 24 hours and the immunofluorescence was performed for FAK and integrin β 1 localization. Cy5 labeled secondary antibody was used for FAK staining and FITC labeled secondary antibody was used for integrin β1 co-staining and the nuclei were stained with DAPI, shown in blue. Confocal images were taken on LSM 780 at 100 x magnification. LNCaP-luc2 and PC3 cells treated with the vehicle control shows the extensions of the focal adhesion in red and integrin β1 expression at the focal adhesions as green (as seen in FIGURES 2A and 2B). Treatment with SENL caused cell rounding, with significant reduction in the FAK and integrin β1 expression (as seen in FIGURES 2C and 2D).

SENL inhibits the formation of focal adhesions in LNCaP-luc2 and PC3 prostate cancer cells. The present invention provides the inhibition of migration, invasion and angiogenesis of human umbilical vein endothelial cells after treatment with ethanol extract of Neem leaf. FAK involved in endothelial cell proliferation, migration and survival is up-regulated in many cancers. FAK activation signaling in prostate cancer cells after SENL treatment was accessed to examine growth inhibition of prostate cancer cells mediated through FAK modulation. LNCaP-luc2 and PC3 cells were examined for integrin β1 and phosphorylation of FAK at Y-397. Western blot showed that the levels of integrin β1 and phosphorylation of FAK at Y397 are reduced with SENL treatment, suggesting that FAK signaling is inhibited. Immunofluorescence for integrin β1 and FAK further demonstrate the suppression of formation of the focal adhesions after SENL treatment in LNCaP-luc2 and PC3 cells. Since integrin/FAK changes may be affected by alterations in calcium signaling protein CRT and membrane traffic regulating protein

Rab5, we then examined the SENL effects on these two proteins. SENL down-regulated CRT levels in both LNCaP-luc2 and PC3 cells whereas modest inhibition of Rab5 was observed in LNCaP-luc2 compared to PC3 cells. The reduction in the CRT and Rab5 by SENL could probably destabilize integrins-extracellular matrix complexes and inhibit FAK activation.

FIGURES 3A-3D show SENL induces hyalinization of tumor tissue and inhibits the growth of human LNCaP-luc2 prostate cancer xenografts in nude mice. FIGURE 3A shows bioluminescence imaging of mice implanted with LNCaP-luc2 tumors. Group I animals were orally administered with vehicle control (olive oil) while Group II and III animals were administered with 100 or 200 mg/kg body weight of SENL 6 days a week. A representative image of the mice from each group at the end of 9 weeks of treatment is shown in FIGURE 3A. FIGURE 3B is a graph of the tumor volume changes of mice treated by oral gavage with vehicle, SENL 100 or SENL 200 mg/kg body weight for 9 weeks. FIGURE 3C is a graph of the tumor weight of the mice after 9 weeks of treatment in different groups. FIGURE 3D is a plot of the body weight of mice in different groups over the treatment period. Data represent mean $\pm$ SD of tumor volume, tumor weight and body weight changes of 8 mice per group. $*p < 0.05$.

We next evaluated whether administration of SENL *via* gavage exhibits anticancer activity *in vivo* in xenograft tumor model. Male nude mice bearing LNCaP-luc2 xenografts were treated with SENL or vehicle control. In the beginning of the treatments, mean LNCaP-luc2 tumor volumes were similar in three groups. SENL treatments significantly suppressed the tumor growth compared to control (as seen in FIGURES 3A and B), mean tumor volume in SENL treated (200 mg/kg) group 189 mm³ compared to control group was 712 mm³ by 9 weeks (* ; $p \leq 0.05$). Significant inhibition of tumor growth was observed in the 200 mg and 100 mg/kg body weight of the SENL treated groups from 6 and 7 weeks onwards, respectively. At the end of the study, the excised tumor tissues showed significant decrease in the tumor weight with SENL treatments (as seen in FIGURE 3C). There was no significant change in body weight in any of the groups after treatment which suggests that oral dose of SENL causes no major toxicity to mice (see FIGURE 3D).

FIGURES 4A-4D are images of the histological changes of LNCaP-luc2 tumor tissues of mice treated with SENL (200 mg/kg body weight). At the end of 9 weeks xenograft tumor tissues were collected and stained with hematoxylin and eosin. Two sections of

tumor tissue from each mouse and 6 mice in a group were examined for histological changes. FIGURE 4A is an image of the tumor tissue of vehicle treated mice shows dense tumor cells, and the arrow points to an area of tumor necrosis at 40x magnification. FIGURE 4B is an image of a tumor tissue of vehicle treated mice showing mitotic figures (thick arrow), apoptosis (thin row) and necrosis (double arrow) at 400x magnification. FIGURE 4C is an image of the tumor tissue of mice treated with SENL and shows tumor cells separated by hyalinized connective tissue indicated by an arrow at 20x magnification, indication of treatment effect. FIGURE 4D is an image of the tumor tissue of SENL treated mice and shows nests of tumor cells separated by hyalinization at 400x magnification. Histological examination revealed hyalinization and apoptosis of the tumor tissues in SENL treated mice. Hyalinization (>50%) of tumor tissues was observed in 7 out of 8 mice treated with SENL in the group (200 mg/kg body weight) (Fig. 4). These data demonstrate that oral administration of SENL causes regression of the tumor tissue and inhibits tumor growth by promoting apoptosis. There was no significant change in the histology of the heart, lungs, liver, kidneys, and spleen after 9 weeks of SENL treatments compared to the control group which indicates that SENL has no adverse effects on these vital organs.

FIGURE 5A is an image of the chromatogram of the fractionation of SENL performed using a Kinetex C18 column on a Shimadzu HPLC system. Although the studies were performed in triplicate; a representative HPLC chromatogram of SENL is depicted. SENL fractions showed 2 major groups of compounds, terpenoids eluted around 70 to 80% methanol gradient at retention time of 22 to 34 minutes and yellow colored eluted with 100% methanol at 52 to 57 minutes. FIGURE 5B is an image of the activity of the peaks labeled in the HPLC chromatogram evaluated for the cytotoxic activity against LNCaP-luc2 cells. Terpenoid fractions 2 to 5 exhibited significant cytotoxic activity in prostate cancer cells. To our knowledge, nimbolide is the only neem compound being reported for anticancer activity in the literature. In a recent study, nimbolide has shown to inhibit colorectal cancer cell growth by suppressing nuclear factor-kappa B activation. The fractions collected from HPLC were concentrated using speed vac at 4°C, equal weight of the fractions were reconstituted in ethanol. LNCaP-luc2 cells were treated with serial dilutions of the fractions for 24 hours and the antiproliferative activity was determined using the MTS viability assay. FIGURE 5C is an image of the mass spectrometric analysis of SENL fraction #2 obtained from HPLC. The total ion

chromatogram the retention time for fraction #2 shows four major peaks labeled as 2A to 2D. The mass spectrum of the peak 2B depict by mass and retention time as nimbandiol at 456.2148 m/z , sodium salt of nimbandiol 478.1968 m/z , and by mass alone as nimbolide at 466.1991 m/z , ammonium salt of nimbolide 483.2256 m/z at the retention
5 time of 4.5 minutes. The mass spectrum of the peak 2C depict by mass alone as photo-oxidized salannin 610.2779 m/z , and sodium salt of photo-oxidized salannin 632.2598 m/z . FIGURE 5D is an image of the mass spectrometric analysis of SENL fraction #5 obtained from HPLC. The total ion chromatogram the retention time for fraction #5 shows two peaks labeled as 5A and 5B. The mass spectrum of the peak 5A depict by
10 mass alone as 6-acetyl nimbandiol at 498.2253 m/z and sodium salt of 6-acetyl nimbandiol at 520.2073 m/z at retention time of 5.3 minutes. The mass spectrum of the peak 5B depict by mass alone as 28-deoxonimbolide 452.2199 m/z , and sodium salt of 28-deoxonimbolide at 474.2019 m/z .

The HPLC separated SENL compounds were evaluated for cytotoxic activity on prostate
15 cancer cells *in vitro*. Chromatographic separation of compounds in SENL was performed using HPLC column. Methanol gradient was used for fractionation, HPLC profile of the SENL is illustrated in FIGURE 5A. UV absorption of the compounds at 254 nm showed a total of 16 peaks. The fractions 1 to 11 eluted with methanol gradient were colorless and 12 to 16 fractions eluted with 100% methanol were yellow colored. The fractions
20 were concentrated at 4⁰C using a speed vac, and equal weight fractions were reconstituted in ethanol. Serial dilutions of the fractions 0.468 $\mu\text{g/ml}$ to 15.0 $\mu\text{g/ml}$ were used to determine the efficacy to inhibit cell growth. The cytotoxic activity was measured by MTS assay after treatment of PC3 prostate cancer cells for 24 hours with the isolated fractions. Vehicle treated cells were included as controls. Although fractions 2 to 11 may
25 all have certain growth inhibitory effects, fractions 2, 3, and 5 exhibited a strong dose-dependent cell growth inhibition. The IC₅₀ concentration to inhibit cell growth for fractions 2, 3 and 5 were < 1.85 $\mu\text{g/ml}$. Fractions 2, 3 and 5 are the most active fractions to inhibit prostate cancer cell growth (as seen in FIGURE 5B).

FIGURE 5C is an image of the mass spectrometric analysis of SENL fraction #2 obtained
30 from HPLC. FIGURE 5D is an image of the mass spectrometric analysis of SENL fraction #3 obtained from HPLC. FIGURE 5E is an image of the mass spectrometric analysis of SENL fraction #4 obtained from HPLC. FIGURE 5F is an image of the mass spectrometric analysis of SENL fraction #5 obtained from HPLC. FIGURE 5G is an

image of the mass spectrometric analysis of SENL fraction #6 obtained from HPLC. FIGURE 5H is an image of the mass spectrometric analysis of SENL fraction #7 obtained from HPLC. FIGURE 5I is an image of the mass spectrometric analysis of SENL fraction #9 obtained from HPLC. FIGURE 5J is an image of the mass spectrometric analysis of SENL fraction #11 obtained from HPLC. FIGURE 5K is an image of the mass spectrometric analysis of SENL fraction #12 obtained from HPLC. FIGURE 5L is an image of the mass spectrometric analysis of SENL fraction #14 obtained from HPLC. FIGURE 5M is an image of the mass spectrometric analysis of nimbolide and 2', 3' dehydro salannol standards.

The compounds in SENL were identified by ESI-MS analysis. ESI-MS analysis was performed on the above HPLC fractions to identify the potential active anticancer compounds in the SENL. Fraction # 2 contained major peaks corresponding to masses of nimbandiol and nimbolide and a minor peak corresponding to photo-oxidized salannin. Fraction # 3 had a major peak suggested by mass as 2, 3-dihydronimbolide. Fraction # 4 contained peaks corresponding to desacetyl nimbion, nimbolide and 2, 3-dihydronimbolide. Fraction # 5 showed a major peak which suggests as 28-deoxonimbolide and a minor peak as 6-acetyl nimbandiol. Fraction # 6 had a major peak suggested as 28-deoxonimbolide and a minor peak as desacetyl salannin. Fraction # 7 showed major peaks corresponds to masses of desacetyl salannin and salannin. Mass spectra of the fractions 2 and 5 are shown in the FIGURES 5C and 5F. Mass spectra of all other fractions and nimbolide and 2', 3'-dehydro salannol used as standards were obtained.

The structures of the neem compounds identified by mass spectra are presented in the FIGURE 6A-6I. Mass spectrometric analysis of standard nimbolide and mice plasma and tumor tissues for the bioavailability of SENL compounds after oral administration with 200 mg/kg bw of SENL was performed. FIGURE 6A illustrates nimbolide and the mass spectrum depicted (not shown) the M ion at 466.2003 m/z , $M+Na^+$ at 483.2268 m/z and $M+NH_3^+$ at 488.1822 m/z . The mass spectrum of mice plasma (not shown) depicted the M ion at 488.1825 m/z suggested by mass alone as sodium salt of nimbolide as seen in FIGURE 6B. The mass spectrum of mice tumor tissue (not shown) depicted the M ion at 488.1824 m/z suggested by mass alone as sodium salt of nimbolide as seen in FIGURE 6C. The mass spectrum of mice tumor tissue (not shown) depicted the M ion at 452.2195 m/z suggested by mass alone as 28-deoxonimbolide as seen in FIGURE 6D. The

difference between the measured value for sodium salt of nimbolide standard from the measured value in plasma and tumor tissue is < 2.8 ppm and for 28-deoxonimbolide in the tumor tissues from the calculated value < 1 ppm. FIGURE 6E illustrates desacetyl nimbin. FIGURE 6F illustrates 6-acetyl nimbolide. FIGURE 6G illustrates 28-deoxonimbolide. FIGURE 6H illustrates desacetyl salannin. FIGURE 6I illustrates salannin.

Bioavailability of SENL compounds in mice plasma and tumor tissues. We examined the availability of SENL compounds in the plasma and tumor tissues of the treated mice. The extraction recoveries for SENL with methanol and chloroform were 82-92%. Vehicle treated group and SENL treated group (200 mg/kg bw) samples were used to study the bioavailability of SENL compounds by ESI-MS analysis. The plasma and tumor tissues were collected from 4 mouse of each group and the samples were analyzed in duplicate. SENL compounds were extracted from plasma and tumor tissues with methanol and chloroform. Mass spectra of tumor tissues treated with SENL suggest the presence of both 28-deoxonimbolide and sodium salt of nimbolide, whereas the plasma shows only the sodium salt of nimbolide. Overall, these results indicate that 28-deoxonimbolide and nimbolide could be the most active compounds bioavailable for antitumor activity. Further studies are required to identify metabolites of these compounds *in vivo*.

Natural products are an important source of potential cancer chemotherapeutic and anti-metastasis agents. Ethanol extract of neem leaf inhibits tumor growth and angiogenesis. The present invention provides a supercritical extraction method which is free of any chemical solvent and preserves the natural state of compounds. We collected the volatile oil from the neem leaf during the supercritical extraction procedure. Natural essential oils from aromatic herbs and dietary plants play an important role in cancer prevention and treatment. The present invention provides a molecular mechanism for anticancer activity of the SENL. The present invention utilizes a well-established prostate cancer model for demonstration of the *in vivo* efficacy. The present invention also provides several important active anticancer compounds in the SENL, some of which can be detected in plasma and tissues after treatment.

FIGURES 7A-7B are images of HPLC chromatograms of mice plasma and tumor tissues for the bioavailability of SENL compounds after oral administration of SENL, 200 mg/kg body weight.. Specifically, FIGURE 7A is a HPLC chromatogram of mice plasma treated with SENL. FIGURE 7B is a HPLC chromatogram of LNCaP xenograft tumor tissue of

mice treated with SENL. FIGURES 7C-7D are images of mass spectrometric analysis of mice plasma and tumor tissues for the bioavailability of SENL compounds after oral administration of SENL, 200 mg/kg body weight. FIGURE 7C is the mass spectrometric analysis of mice tumor tissue fraction 1 obtained from HPLC depicts the M ion at 488.1824 m/z, suggestive by mass alone as the sodium salt of nimbolide. FIGURE 7D is the mass spectrometric analysis of mice tumor tissue fraction 2 obtained from HPLC depicts the M ion at 452.2195 m/z, suggestive by mass alone as the 28-deoxonimbolide. HPLC and mass spectrometric analysis was performed in duplicate, representative chromatograms were shown. The difference between the measured value for the sodium salt of nimbolide standard from the measured value in plasma and tumor tissue is less than 2.8 ppm and for 28-deoxonimbolide in the tumor tissues from the calculated value is less than 1 ppm.

The present invention demonstrates SENL inhibits LNCaP-luc2 and PC3 cell growth. Compared to androgen independent PC3 cells, androgen dependent LNCaP cells were slightly more sensitive to achieve lower IC₅₀ effect. We have also demonstrated that treatment of castration resistant prostate cancer cells with ethanol extract of Neem leaf increases the expression of AKR1C2 enzyme levels in C4-2B and PC3 cells and suppresses DHT levels in C4-2B xenograft tumor tissues of mice. AKR1C2 plays a prominent role in DTH catabolism. DHT is the most effective androgen to activate the AR functions. The AR plays a critical role in prostate cancer development and progression. To unravel the effect of SENL on DHT, we analyzed AR expression levels in the LNCaP cells stimulated with DHT and the expression of PSA, a downstream target of AR. There was a significant decrease in the expression of DHT induced AR and PSA levels after SENL treatment. SENL abrogates the DHT effects to induce AR expression by promoting DHT catabolism through activation of AKR1C2 enzyme levels. The effect of SENL on the inhibition of androgen dependent LNCaP-luc2 cells growth is at least partially mediated by down-regulation of the androgen regulated pathways; however, inhibition of AR-negative PC3 cells growth indicates that AR independent mechanisms may be affected by SENL treatment.

The present invention has shown inhibition of migration, invasion and angiogenesis of human umbilical vein endothelial cells after treatment with ethanol extract of Neem leaf. Many studies have reported FAK as a positive regulator of tumor invasion and progression and it is up-regulated in a wide variety of malignancies, such as prostate,

breast and pancreatic cancer. FAK is the primary enzyme involved in the engagement of integrins and assembly of focal adhesion through the activation of several downstream signals. Phosphorylation of FAK at Y-397 is prerequisite for its activated state, and phosphorylated FAK was shown to be elevated in highly invasive cancer cells and lymph
5 node metastasis. The present invention provides compositions and methods of making and using the compositions to effect FAK signaling.

The present invention shows that the activation of FAK at Y-397 and integrin $\beta 1$ levels was reduced in SENL treated cells (see FIGURE 1D). Further evidence of inactivation of FAK by SENL is demonstrated by the immunofluorescence staining, which reveals
10 inhibition in the formation of focal adhesions in LNCaP-luc2 and PC3 cells (FIGURE 2). This data indicates that SENL may contribute to both anti-migration/invasion and growth inhibitory effects by suppressing integrin/FAK signaling.

CRT is a unique endoplasmic reticulum luminal protein that participates in many cellular functions including calcium signaling and cell adhesion. CRT interacts with integrins and
15 plays an important role in attachment of cells to the extracellular matrix for focal adhesion assembly and disassembly process during invasion and migration of cells. Elevated CRT expression promotes the migration and invasion of cancer cells. SENL treatment decreased CRT expression in LNCaP-luc2 and PC3 cells (see FIGURE 1D). Suppression of CRT expression with SENL is at least partially contributed to the
20 inhibition of focal adhesions in prostate cancer cells. The formation of endoplasmic reticulum tubules *in vitro* requires a Rab family GTPase Rab-5. SENL did not substantially alter Rab-5 in LNCaP-luc2 cells, whereas it decreased significantly in PC3 cells. The inhibition of invasion/migration abilities of SENL in prostate cancer cells may be beneficial in preventing metastasis.

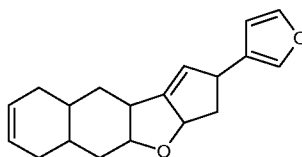
25 Oral administration of SENL compositions suppressed xenograft tumor growth. There were no significant changes in the body weight in the SENL treated groups compared to control group, which confirms that SENL at 100 and 200 mg/kg body weight has no adverse effects. The most significant histological change observed after SENL treatment in mice is the formation of hyalinized tumor tissue in more than 80% of mice (200 mg/kg
30 bw group). Similar change were seen in the C4-2B and PC3 xenograft tumor tissue of mice treated with ethanol extract of Neem leaf after i.p. administration. It is reported that fibrous tissue formation is an indicator of decreased tumor invasiveness and improved tumor regression. This histological feature of hyalinization confirms the tumor regression

with SENL treatment. Mass spectrometric analysis of tumor tissues and plasma suggests 28-deoxonimbolide and nimbolide as the bioavailable compound after SENL treatment. The present invention provides SENL that mediate antitumor activity *in vivo* by modulating multiple pathways in tumor development and progression. The present invention provides compositions (including nimbolide, nimbandiol, 2',3'-dihydronimbolide, and 28-deoxynimbolide) that display anticancer activity in prostate cancer cells.

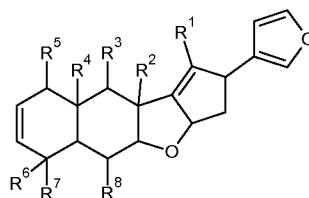
Although it may be known that flavonoids as secondary metabolites are present in several plants including Neem leaves and can serve as markers of the crude extract that possess anti-inflammatory, anti-lipidemic, anti-hyperglycemic and anti-cancer properties, examination of the fractions 12 to 16 have not shown significant cytotoxic activity, it is suggested that the interaction between the active compounds and its coexisting constituents may have an impact on the ultimate pharmacokinetics and pharmacological effects. In support a recent study has shown that the oral absorption and bioavailability of paclitaxel in *Taxus yunnanensis* extract were remarkably higher when compared with the pure paclitaxel.

The present invention provides terpenoids from SENL that are active compounds for inhibition of cancer growth and the anticancer activities of SENL are mediated through regulation of CRT, AR and FAK levels. The present invention has demonstrated the *in vivo* therapeutic potential of SENL in preclinical prostate cancer models.

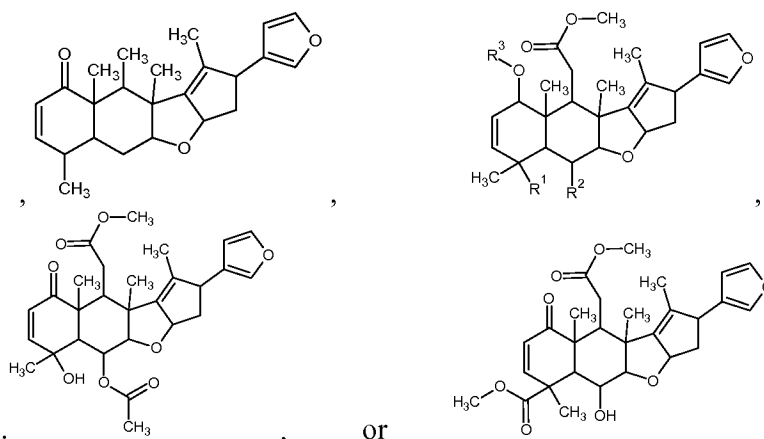
Although the composition may have a variety of structures one composition has the general structure including 4 fused rings with a pendent ring structure. More specifically, the composition includes a 2 fused 5 member rings and 2 fused 6 membered rings wherein one of the fused 5 member rings is an oxolane and the pendent ring is a furan. For example:



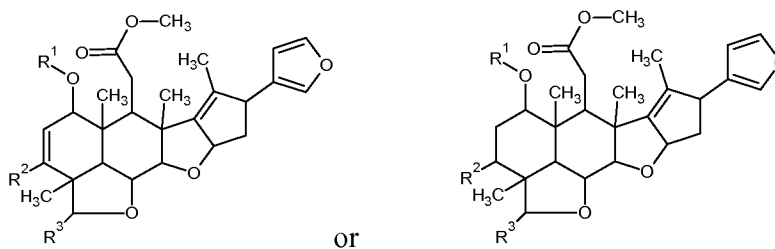
In addition, the core structure described herein may have dependent groups (R groups) distributed from the core structure. This provides a vast number of compositions and derivative composition that may be included in the present invention.



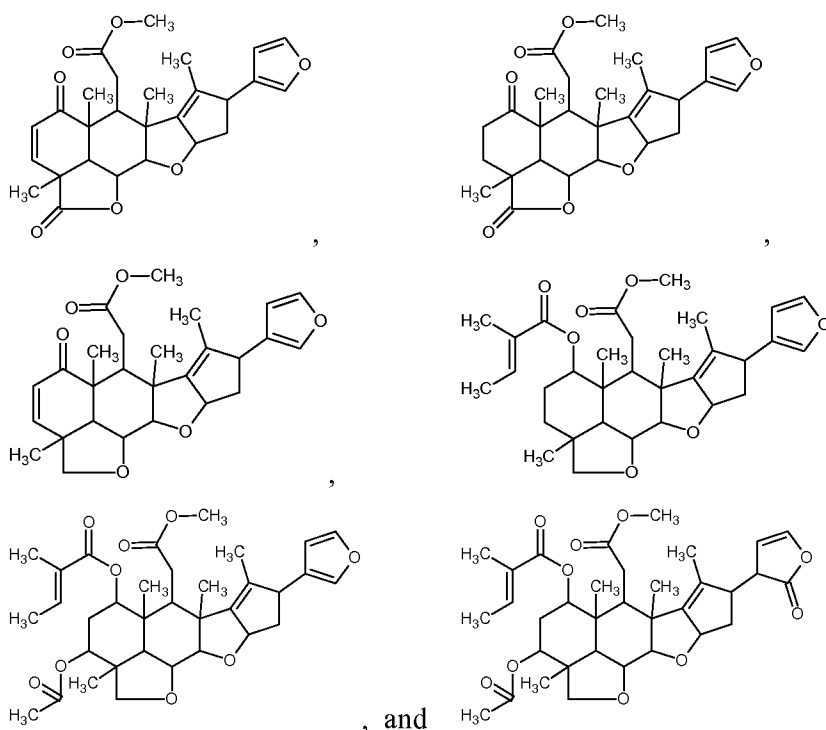
In these examples, the R groups may be a variety of independent groups. In many cases the R groups may independent be hydrogens or methyl groups; however, the R groups may independently be an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, or an alkoxy group. In the most basic terms the compounds share a core structure



and include various R groups substitutions to form the represented compositions above. In addition, it can be seen that the core structure can be modified through the addition of multiple connected R groups to form additional ring structures fused to the core composition as seen below:



Specific examples of the compositions are seen below:



The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, solution, suspension, pellet gel capsules, liquid syrups, soft gels and other formulations known to the skilled artisan. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Other additives conventionally used in pharmaceutical compositions may be included, which are well known in the art. Such additives include, e.g.,: anti-adherents (anti-sticking agents, glidants, flow promoters, lubricants) such as talc, magnesium stearate, fumed silica), micronized silica, polyethylene glycols, surfactants, waxes, stearic acid, stearic acid salts, stearic acid derivatives, starch, hydrogenated vegetable oils, sodium benzoate, sodium acetate, leucine, PEG-4000 and magnesium lauryl sulfate. Other additives include, binders (adhesives), i.e., agents that impart cohesive properties to powdered materials through particle-particle bonding, such as matrix binders (dry starch, dry sugars), film binders (PVP, starch paste, celluloses, bentonite and sucrose), and chemical binders (polymeric cellulose derivatives, such as carboxy methyl cellulose, HPC and HPMC; sugar syrups; corn syrup; water soluble polysaccharides such as acacia,

tragacanth, guar and alginates; gelatin; gelatin hydrolysate; agar; sucrose; dextrose; and non-cellulosic binders, such as PVP, PEG, vinyl pyrrolidone copolymers, pregelatinized starch, sorbitol, and glucose).

For certain compositions it may be useful to provide buffering agents (or bufferants),
5 where the acid is a pharmaceutically acceptable acid, such as hydrochloric acid, hydrobromic acid, hydriodic acid, sulfuric acid, nitric acid, boric acid, phosphoric acid, acetic acid, acrylic acid, adipic acid, alginic acid, alkanesulfonic acid, amino acids, ascorbic acid, benzoic acid, boric acid, butyric acid, carbonic acid, citric acid, fatty acids, formic acid, fumaric acid, gluconic acid, hydroquinosulfonic acid, isoascorbic acid, lactic
10 acid, maleic acid, methanesulfonic acid, oxalic acid, para-bromophenylsulfonic acid, propionic acid, p-toluenesulfonic acid, salicylic acid, stearic acid, succinic acid, tannic acid, tartaric acid, thioglycolic acid, toluenesulfonic acid and uric acid, and where the base is a pharmaceutically acceptable base, such as an amino acid, an amino acid ester, ammonium hydroxide, potassium hydroxide, sodium hydroxide, sodium hydrogen
15 carbonate, aluminum hydroxide, calcium carbonate, magnesium hydroxide, magnesium aluminum silicate, synthetic aluminum silicate, synthetic hydrotalcite, magnesium aluminum hydroxide, diisopropylethylamine, ethanolamine, ethylenediamine, triethanolamine, triethylamine, triisopropanolamine, or a salt of a pharmaceutically acceptable cation and acetic acid, acrylic acid, adipic acid, alginic acid, alkanesulfonic
20 acid, an amino acid, ascorbic acid, benzoic acid, boric acid, butyric acid, carbonic acid, citric acid, a fatty acid, formic acid, fumaric acid, gluconic acid, hydroquinosulfonic acid, isoascorbic acid, lactic acid, maleic acid, methanesulfonic acid, oxalic acid, para-bromophenylsulfonic acid, propionic acid, p-toluenesulfonic acid, salicylic acid, stearic acid, succinic acid, tannic acid, tartaric acid, thioglycolic acid, toluenesulfonic acid, and
25 uric acid.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitables. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. Preferred oral
30 formulations are those in which oligonucleotides of the invention are administered in conjunction with one or more penetration enhancers, surfactants and chelators. Preferred surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Preferred bile acids/salts include chenodeoxycholic acid (CDCA) and

ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucolic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate, sodium glycodihydrofusidate. Preferred fatty acids include arachidonic acid, undecanoic acid, 5 oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprinate, tricaprinate, monoolein, dilaurin, glyceryl 1-monocaprinate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g. sodium). Also preferred are combinations of penetration enhancers, for example, fatty acids/salts in combination with bile acids/salts. A particularly preferred combination is the sodium salt 10 of lauric acid, capric acid, and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. Oligonucleotides of the invention may be delivered orally in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. Oligonucleotide complexing agents include 15 poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pullulans, celluloses and starches. Particularly preferred complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, 20 polyspermines, protamine, polyvinylpyridine, polythiodiethylamino-methylethylene P(TDAE), polyaminostyrene (e.g. p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcyanoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, 25 poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG).

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, 30 carrier compounds and other pharmaceutically acceptable carriers or excipients.

It will be understood that particular embodiments described herein are shown by way of illustration and not as limitations of the invention. The principal features of this invention can be employed in various embodiments without departing from the scope of the

invention. Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures described herein. Such equivalents are considered to be within the scope of this invention and are covered by the claims.

- 5 The use of the word “a” or “an” when used in conjunction with the term “comprising” in the claims and/or the specification may mean “one,” but it is also consistent with the meaning of “one or more,” “at least one,” and “one or more than one.” The use of the term “or” in the claims is used to mean “and/or” unless explicitly indicated to refer to alternatives only or the alternatives are mutually exclusive, although the disclosure
10 supports a definition that refers to only alternatives and “and/or.” Throughout this application, the term “about” is used to indicate that a value includes the inherent variation of error for the device, the method being employed to determine the value, or the variation that exists among the study subjects.

- As used in this specification and claim(s), the words “comprising” (and any form of comprising, such as “comprise” and “comprises”), “having” (and any form of having, such as “have” and “has”), “including” (and any form of including, such as “includes” and “include”) or “containing” (and any form of containing, such as “contains” and “contain”) are inclusive or open-ended and do not exclude additional, unrecited elements or method steps.

- 20 The term “or combinations thereof” as used herein refers to all permutations and combinations of the listed items preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, CBA, BCA, ACB, BAC, or CAB. Continuing with this example, expressly included are combinations
25 that contain repeats of one or more item or term, such as BB, AAA, AB, BBC, AAABCCCC, CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

- All of the compositions and/or methods disclosed and claimed herein can be made and
30 executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied

to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by
5 the appended claims.

What is claimed is:

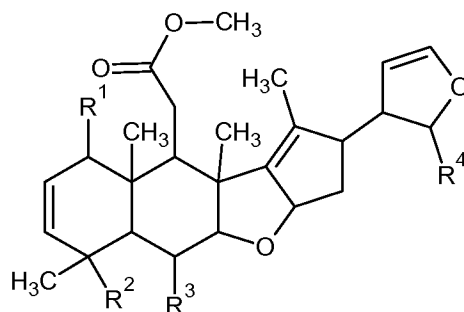
1. A pharmaceutical formulation for treating one or more symptom of cancer, wherein the pharmaceutical formulation comprises a therapeutically effective amount of Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, 28 dihydro Nimbolide, or a
5 combination thereof to ameliorate one or more symptoms of the cancer.
2. A Nimbolide composition for reducing the number of cancer cells, wherein the Nimbolide composition comprises a therapeutically effective amount of Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof to reduce the number of the one or more cancer cells.
- 10 3. The pharmaceutical formulation of claims 1 or 2, wherein the cancer is prostate cancer, colon cancer, astrocytoma, and sarcoma.
4. The pharmaceutical formulation of claims 1 or 2, wherein the pharmaceutical formulation modulates at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity.
- 15 5. The pharmaceutical formulation of claims 1 or 2, wherein the Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, 28 dihydro Nimbolide, or a combination thereof is purifying by supercritical extraction.
6. A method for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activation in one or more cells comprising the steps of:
20 administering a therapeutically effective dose of a pharmaceutical formulation to one or more cells to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein the pharmaceutical formulation comprises Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof.
- 25 7. A pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in cancer cells wherein the pharmaceutical formulation comprises:
a physiologically effective amount of Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof, in a pharmaceutical carrier

to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity.

8. The composition of claim 7, wherein the physiologically effective amount of Nimbolide; Nimbandiol; 2', 3' dihydro Nimbolide; 28 dihydro Nimbolide; or a combination thereof is isolated and purified by supercritical extraction.

9. A pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in prostate cancer cells wherein the pharmaceutical formulation comprises:

a physiologically effective amount of a composition having the formula:

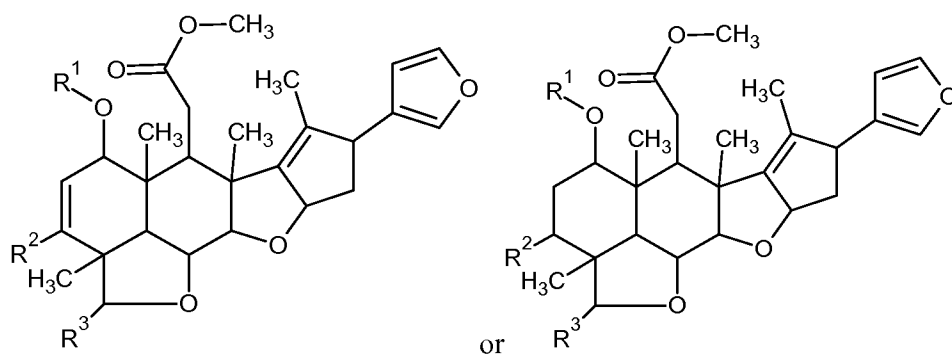


in a pharmaceutical carrier sufficient to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein R^1 , R^2 , R^3 , and R^4 are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

10. The composition of claim 9, wherein R¹ is a O, R² is a -OH, R³ is a -OH, R⁴ is a H; or R¹ is a O, R² is a -OH, R³ is a -OCOCH₃, R⁴ is a H; or R¹ is a O, R² is a -COOCH₃, R³ is a -OH; R⁴ is a H.

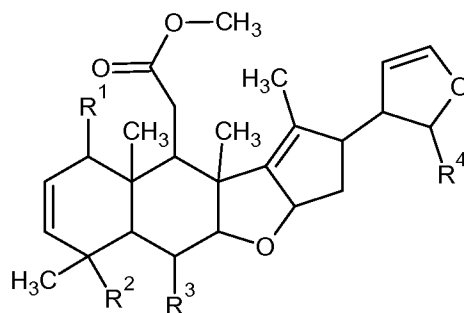
11. A pharmaceutical formulation for inhibiting at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity in prostate cancer cells wherein the pharmaceutical formulation comprises:

a physiologically effective amount of a composition having the formula:



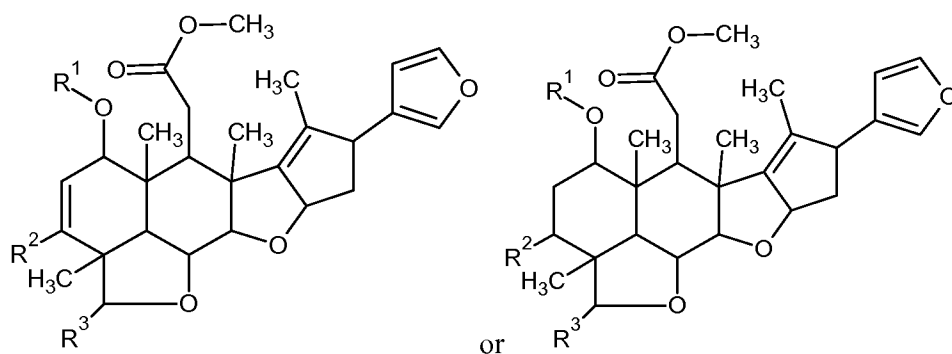
in a pharmaceutical carrier sufficient to inhibit at least one of integrin $\beta 1$ activity, calreticulin activity, and focal adhesion kinase activity, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

12. A pharmaceutical formulation for treating prostate cancer wherein the pharmaceutical formulation comprises a therapeutically effective dose of a composition having the formula:



to reduce the number of prostate cancer cells in the patient, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

13. A pharmaceutical formulation for treating prostate cancer wherein the pharmaceutical formulation comprises a therapeutically effective dose of a composition having the formula:



to reduce the number of prostate cancer cells in the patient, wherein R¹, R², R³, and R⁴ are independently be a hydrogen, an alkyl group, a carbonyl group, a hydroxyl, a ester, a ether, a methoxy, an acetoxy group or an alkoxy group.

14. The composition of claims 11, 12 or 13, wherein R¹ is not present, R² is a -H, and R³ is a -O; R¹ is a -COCCH₃CHCH₃, R² is a -OCOCH₃, and R³ is a -H; R¹ is not present, R² is a -H, and R³ is a -H; R¹ is a -COCCH₃CHCH₃, R² is a -H, and R³ is a -H; and R¹ is a -COCCH₃CHCH₃, R² is a -OCOCH₃, and R³ is a -H.

15. A method of performing a clinical trial to evaluate a candidate drug believed to be useful in treating prostate or colon cancer, the method comprising the steps of:

- a) measuring an enzyme activity from tissue suspected of having prostate or colon cancer from a set of patients, wherein the enzyme activity is at least one of integrin β 1 activity, calreticulin activity, and focal adhesion kinase activity;
- b) administering a candidate drug to a first subset of the patients, and a placebo to a second subset of the patients;
- c) repeating step a) after the administration of the candidate drug or the placebo; and
- d) determining if the candidate drug statistically significantly modulates the enzyme activity as compared to any reduction occurring in the second subset of patients, wherein a statistically significant reduction indicates that the candidate drug is useful in treating said disease state.

16. The composition of claim 15, wherein the candidate drug is selected from Nimbolide, Nimbandiol, 2', 3' dihydro Nimbolide, 28 dihydro Nimbolide, or a combination thereof.

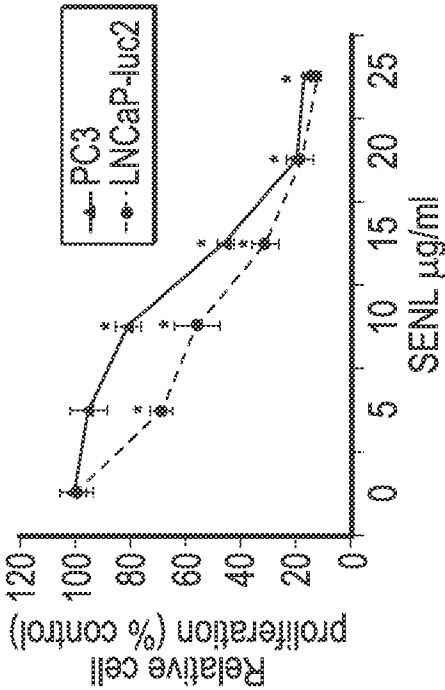


FIG. 1A

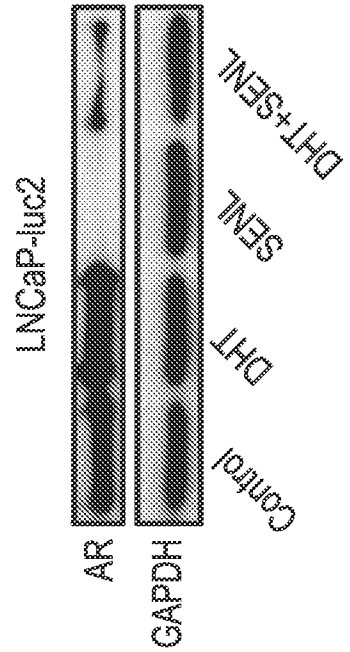


FIG. 1B

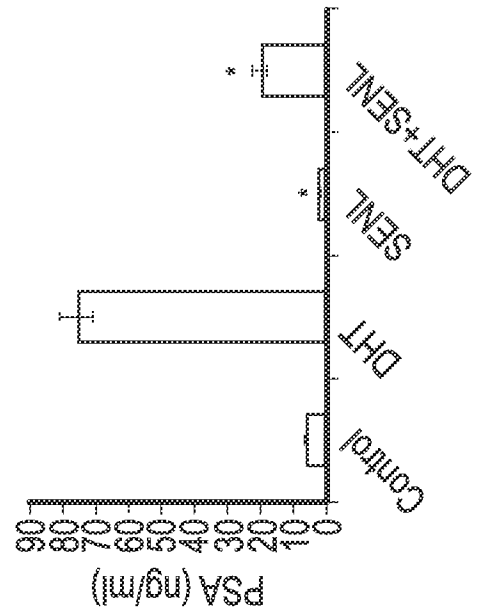


FIG. 1C

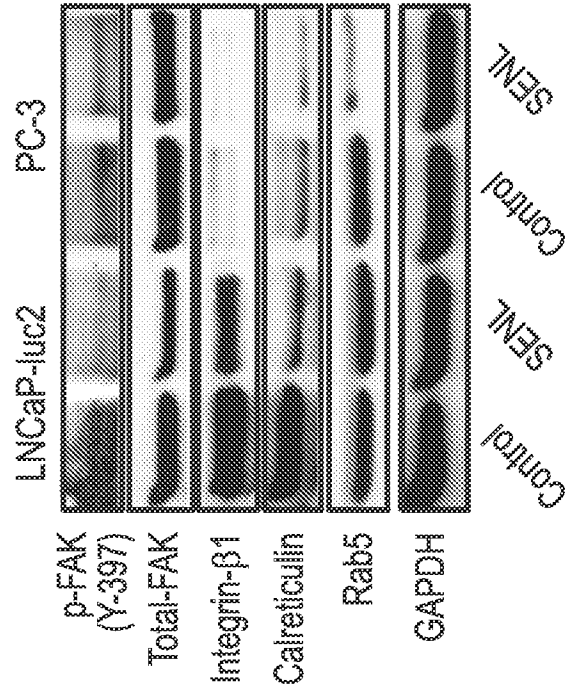


FIG. 1D

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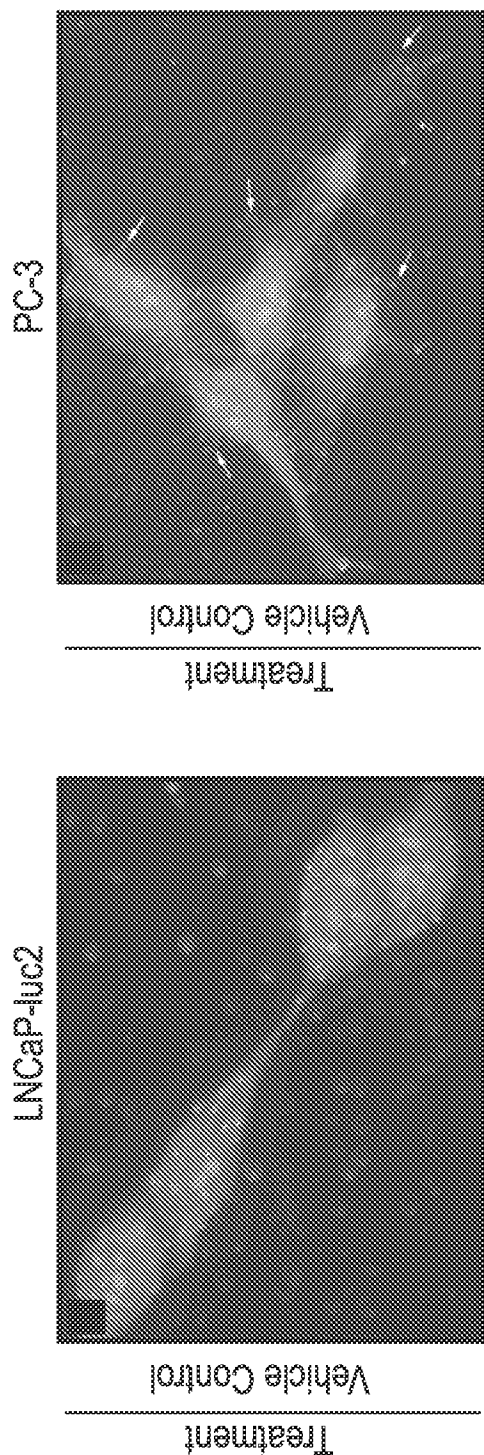


FIG. 2A

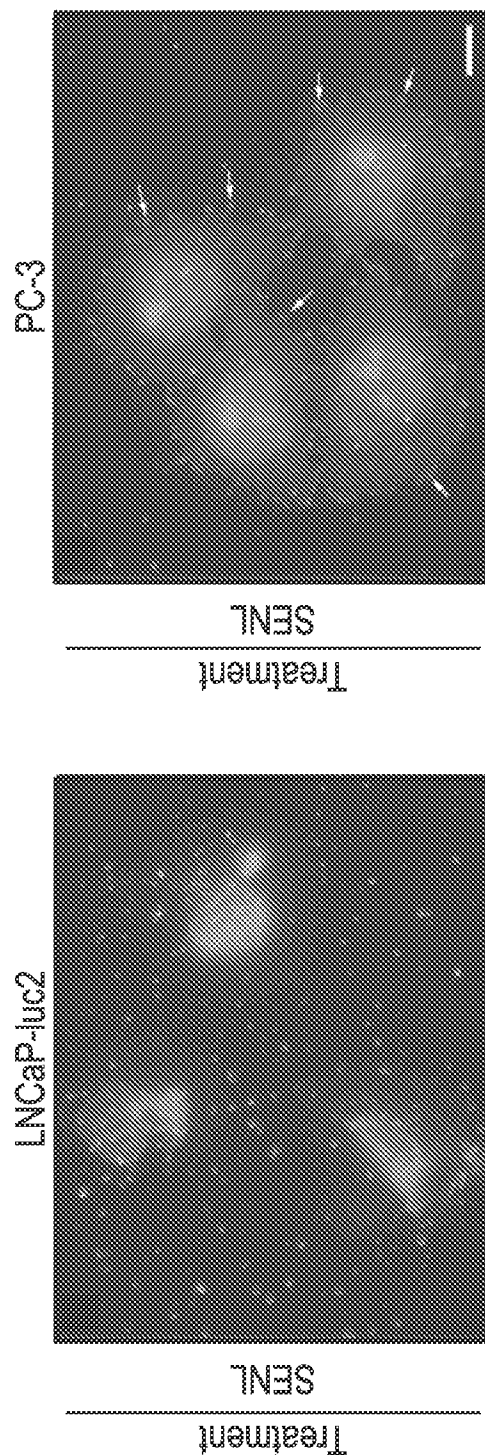


FIG. 2B

FIG. 2C

Red=FAK, GREEN=Integrin b1, Blue=DAPI

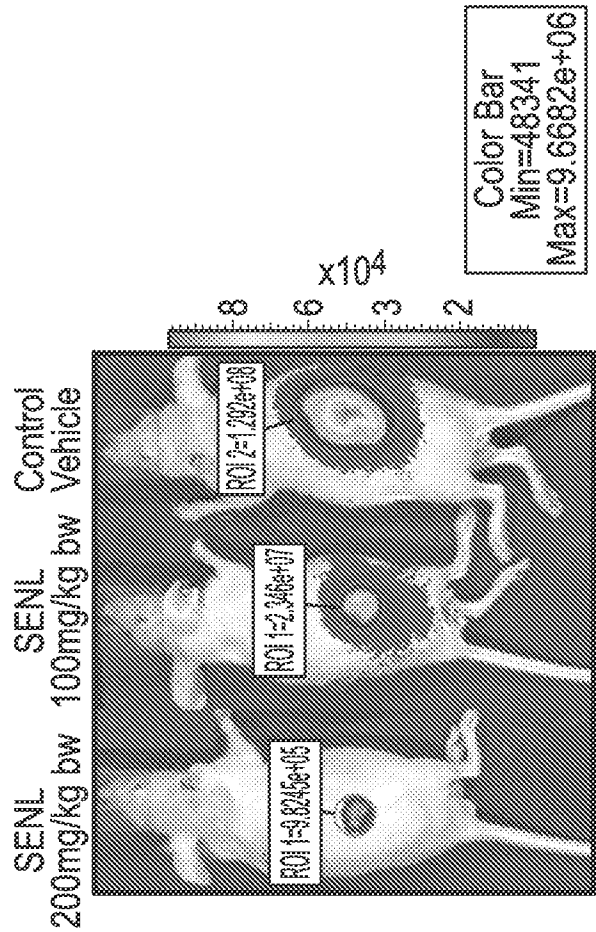


FIG. 3A

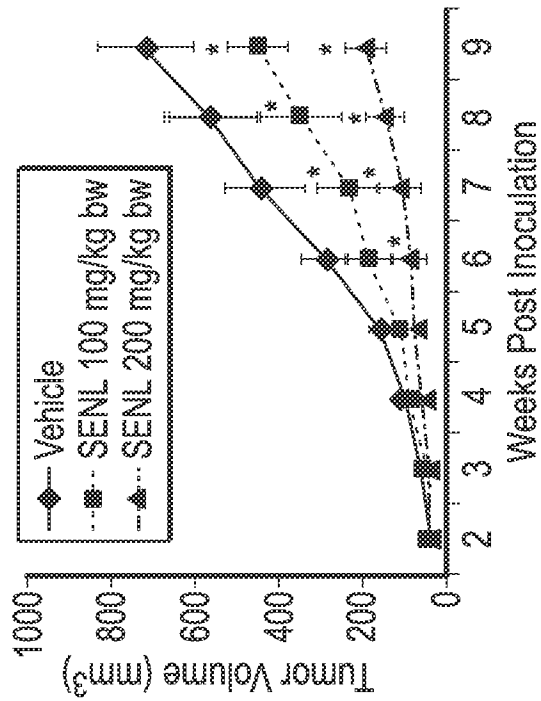


FIG. 3B

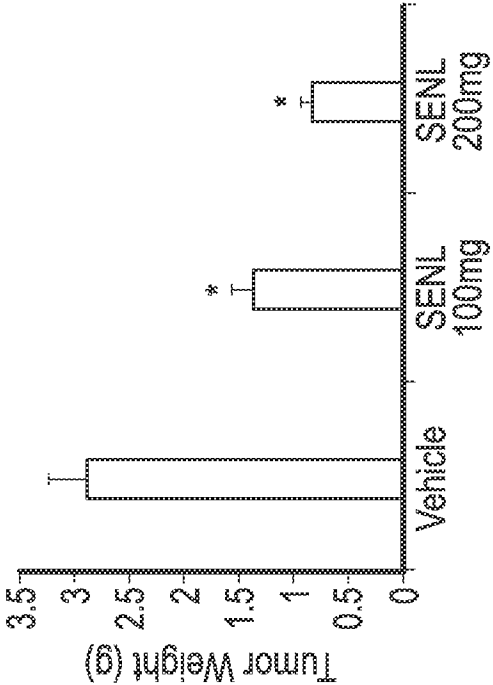


FIG. 3C

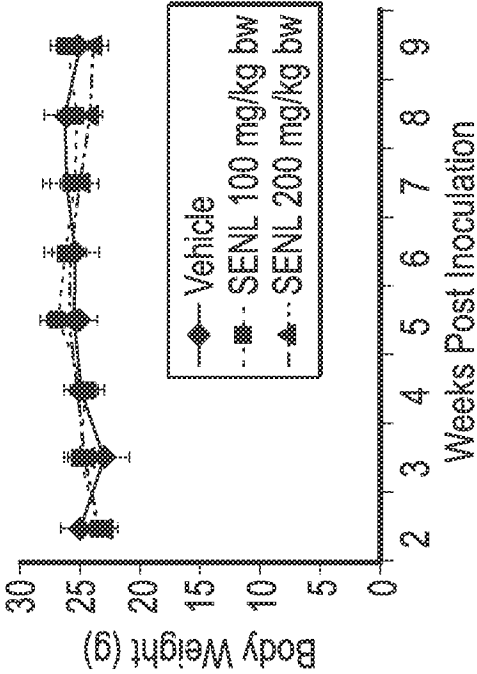


FIG. 3D

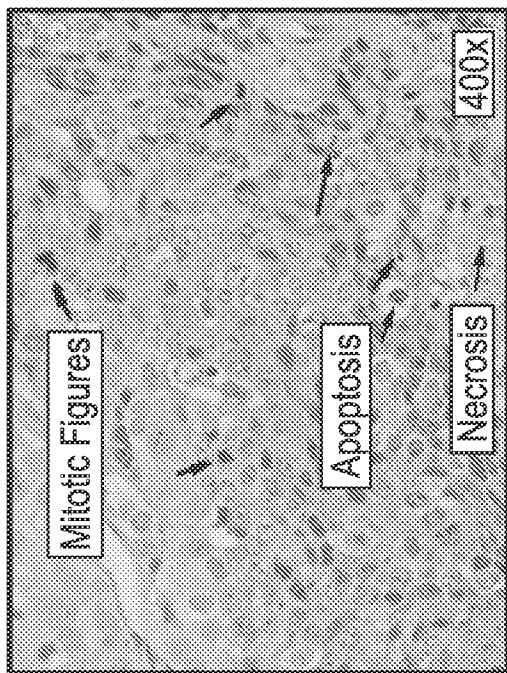


FIG. 4B

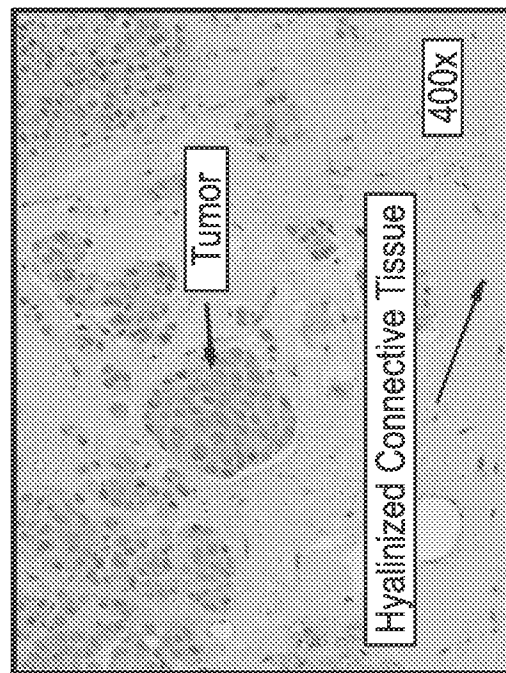


FIG. 4D

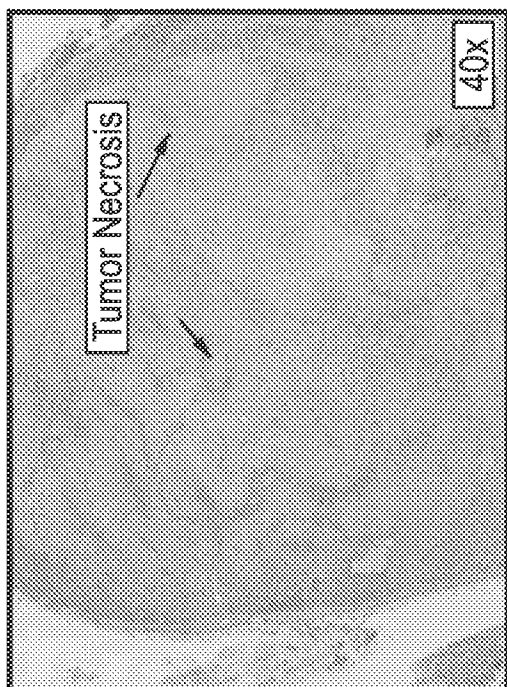


FIG. 4A

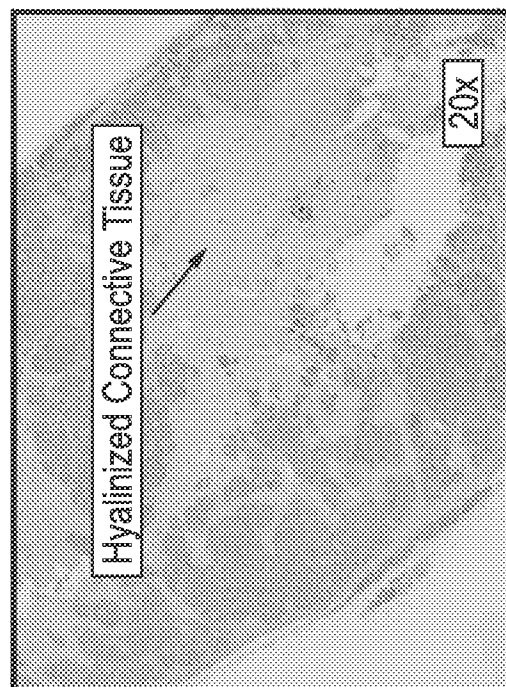


FIG. 4C

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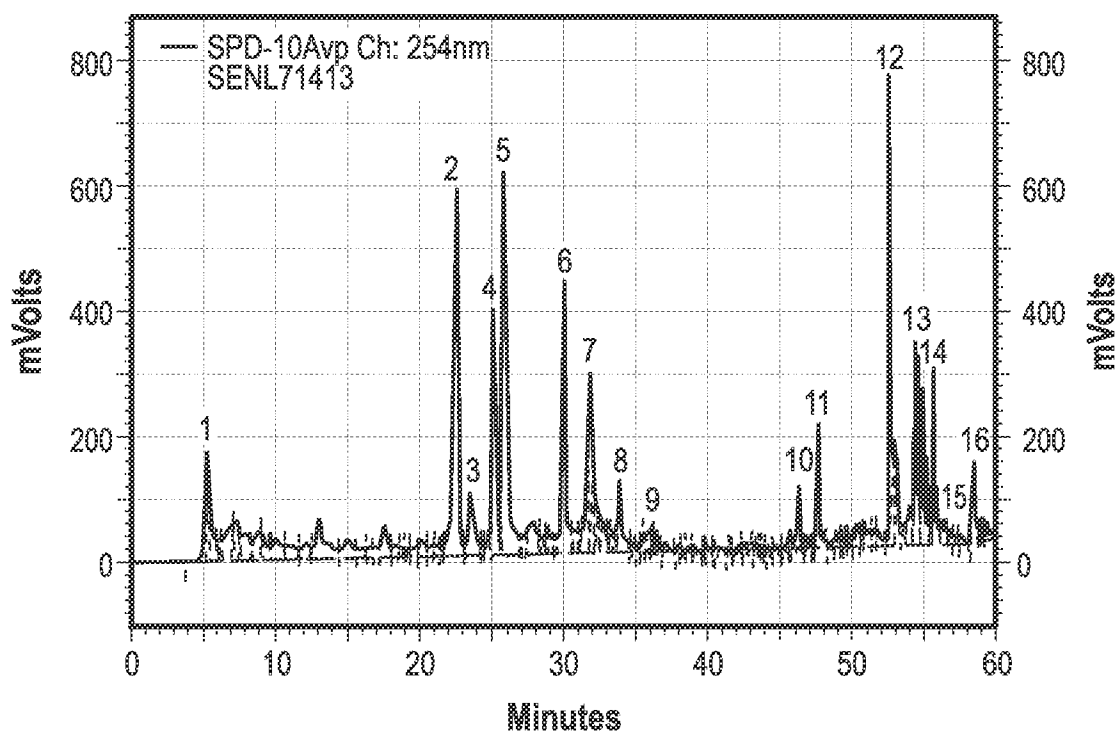


FIG. 5A

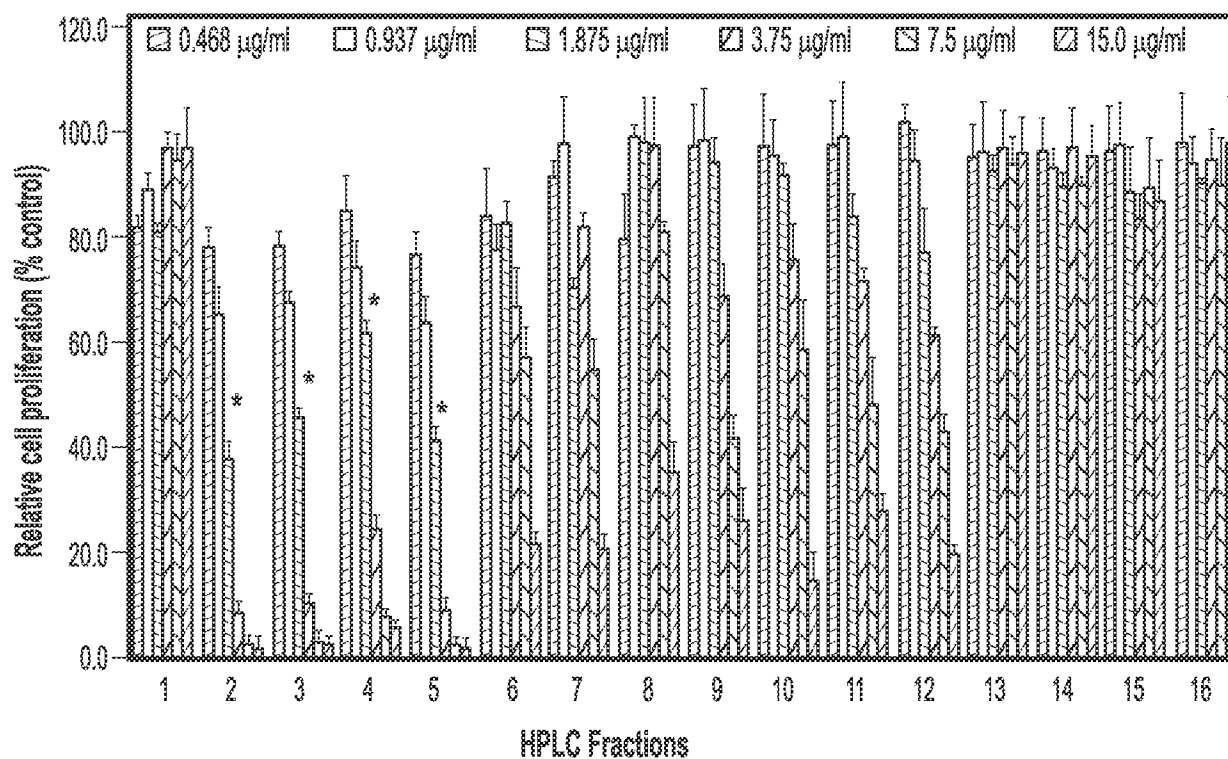


FIG. 5B

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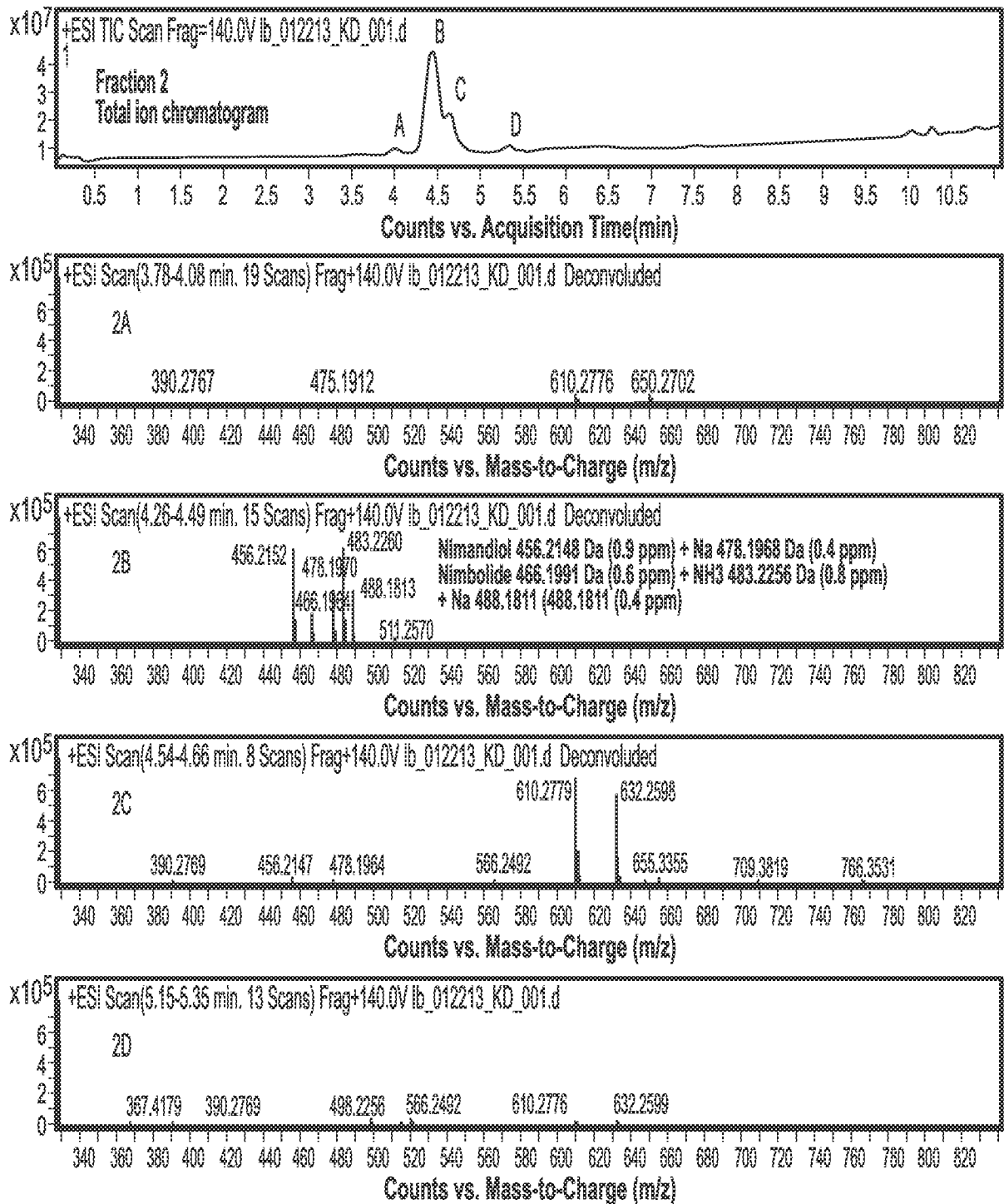


FIG. 5C

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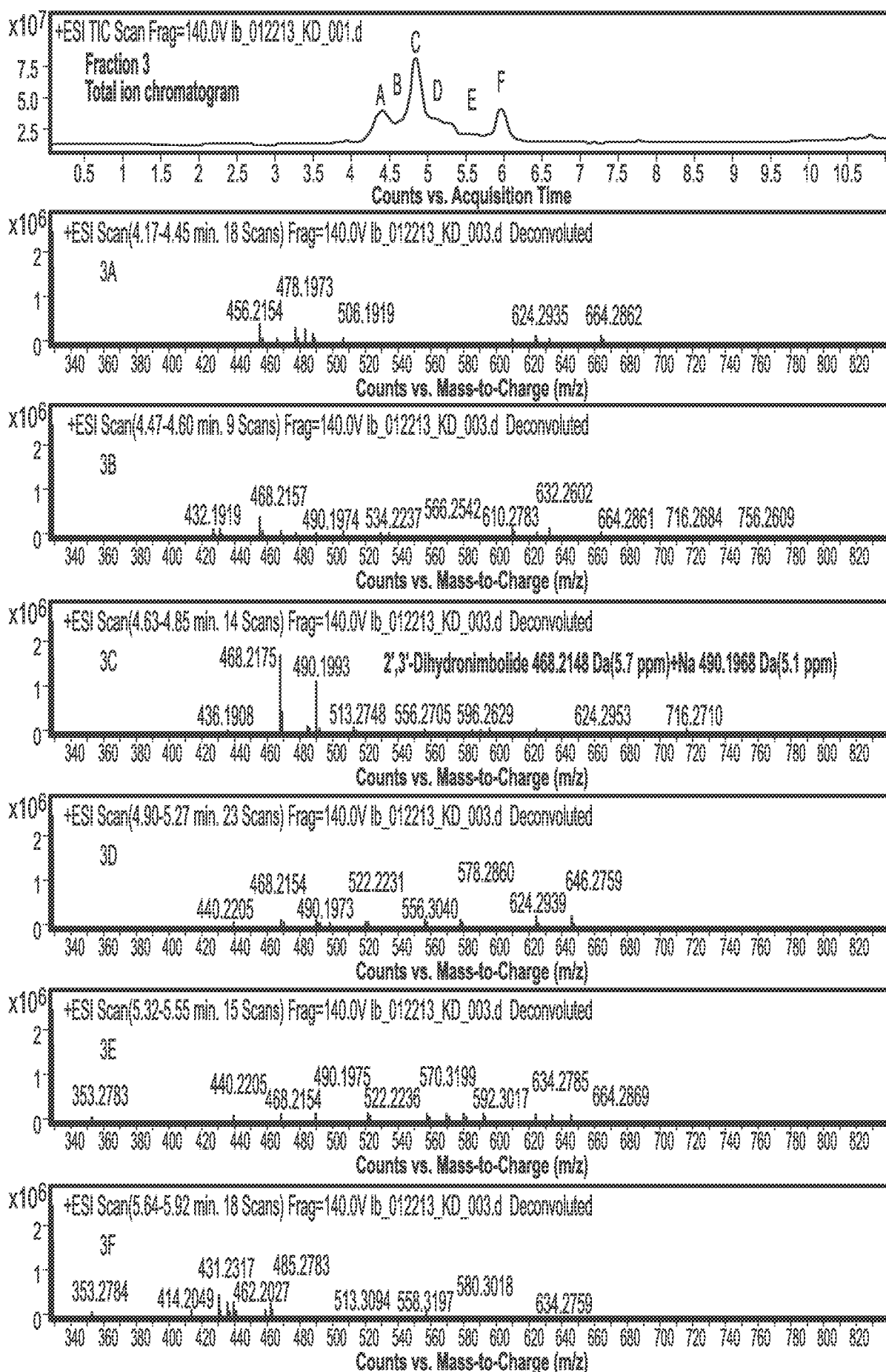


FIG. 5D

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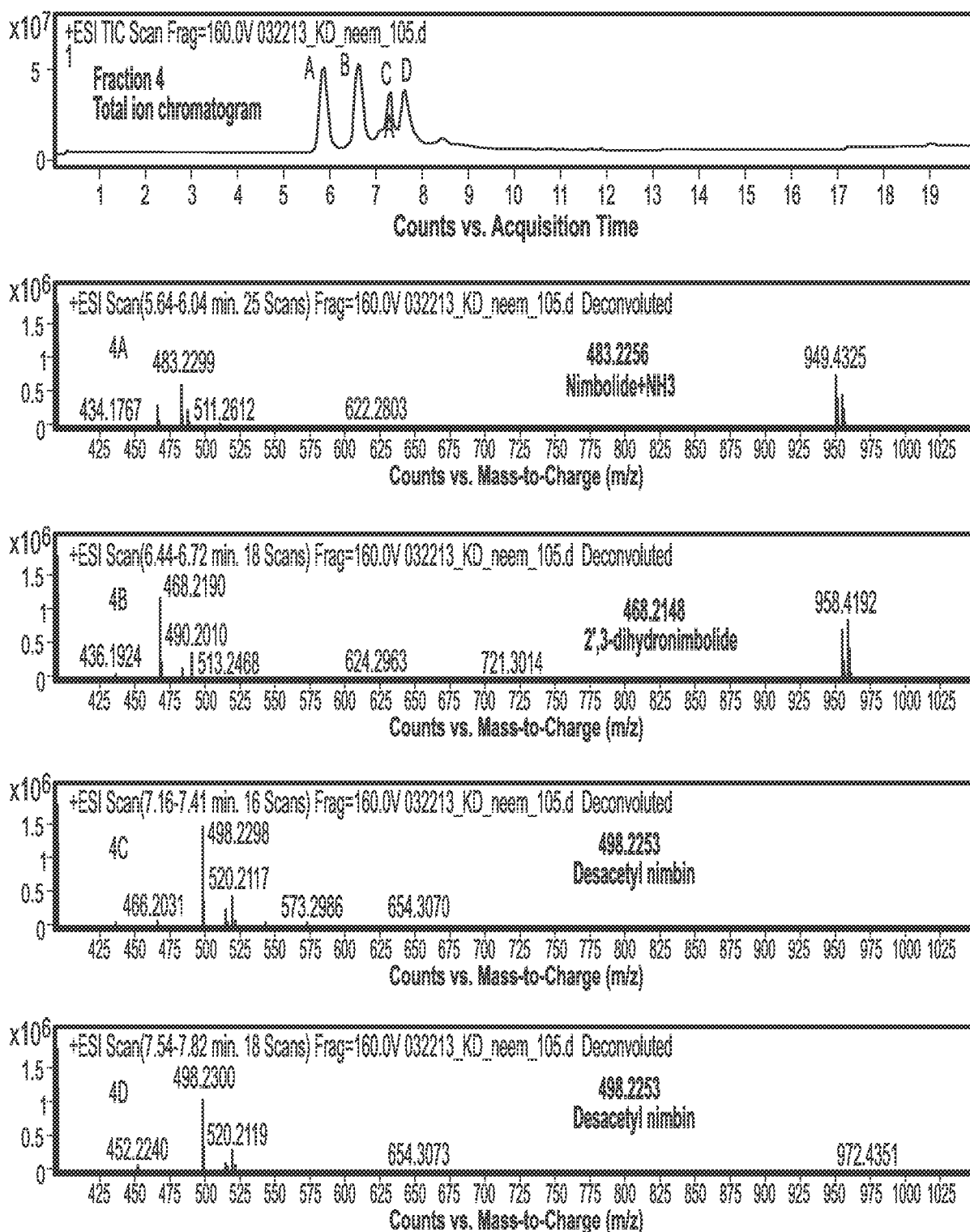


FIG. 5E

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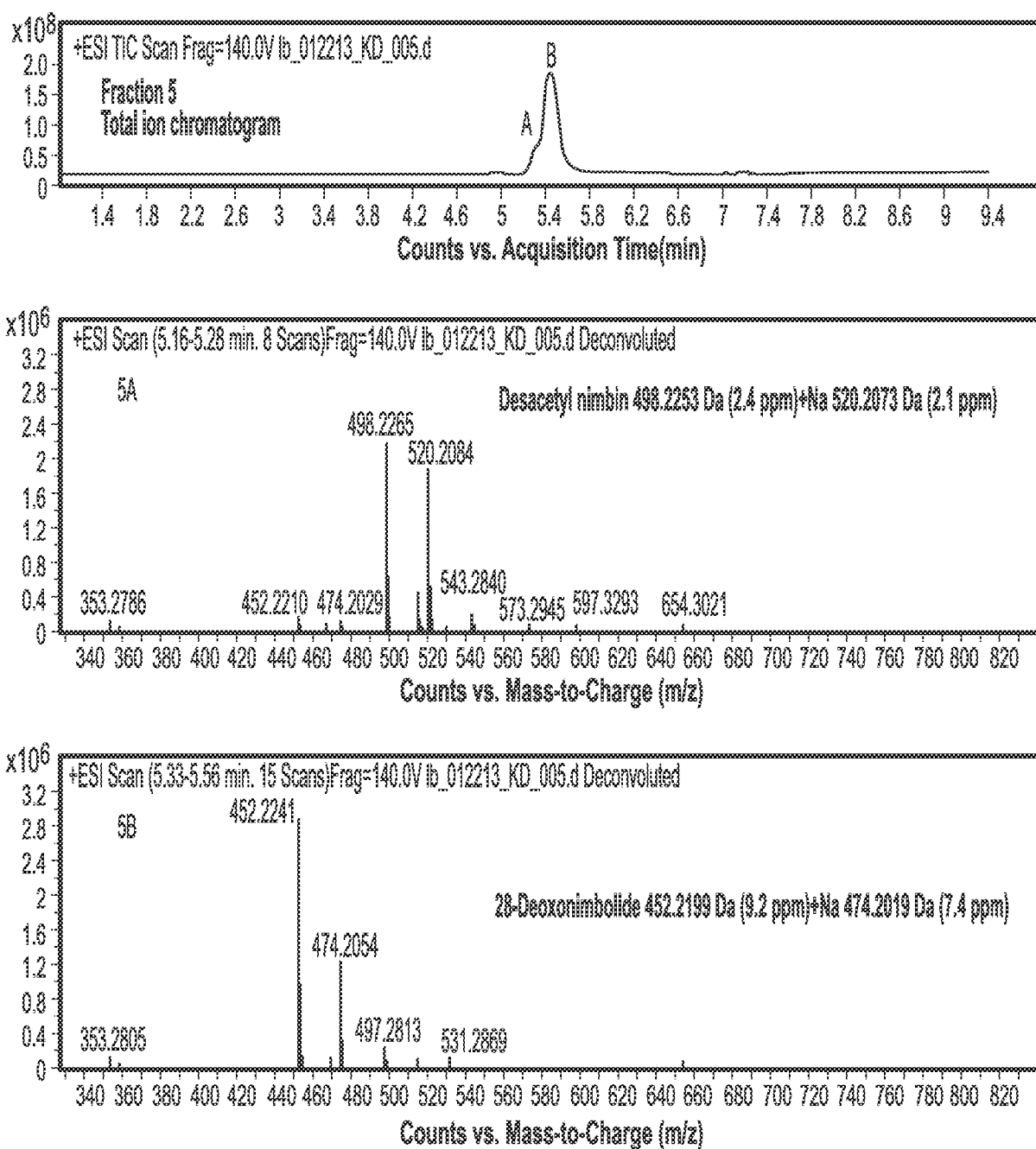


FIG. 5F

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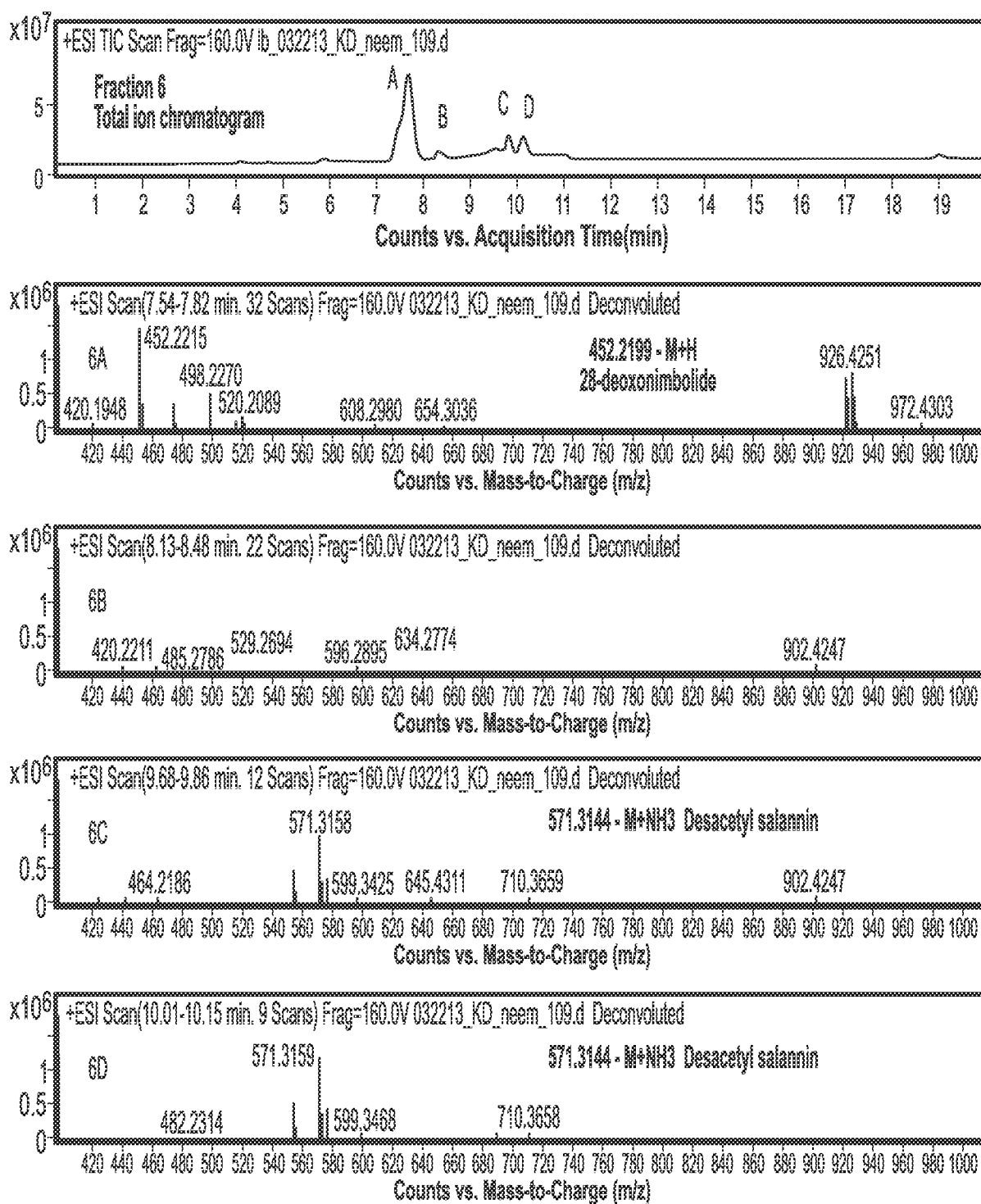


FIG. 5G

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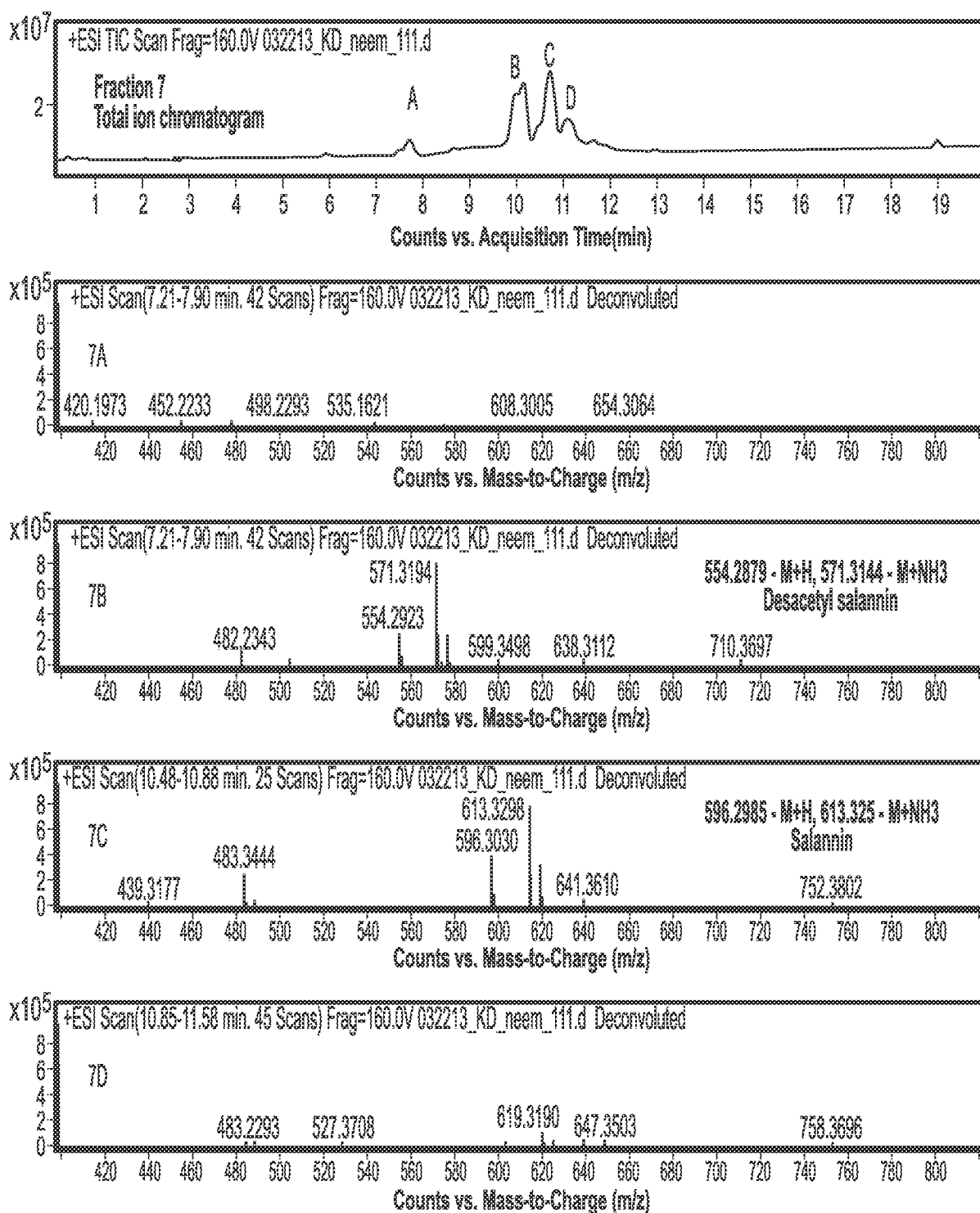


FIG. 5H

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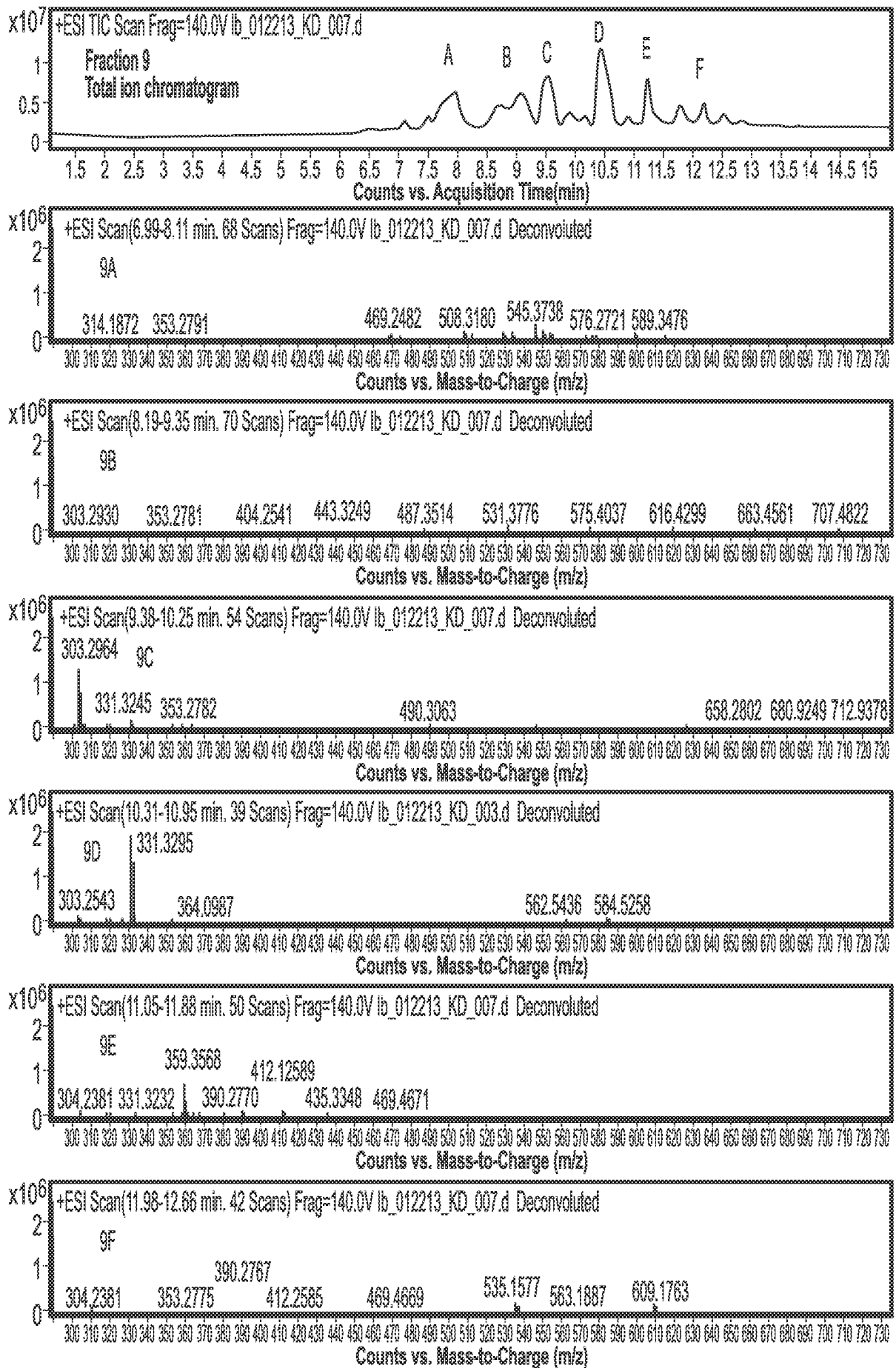


FIG. 5I

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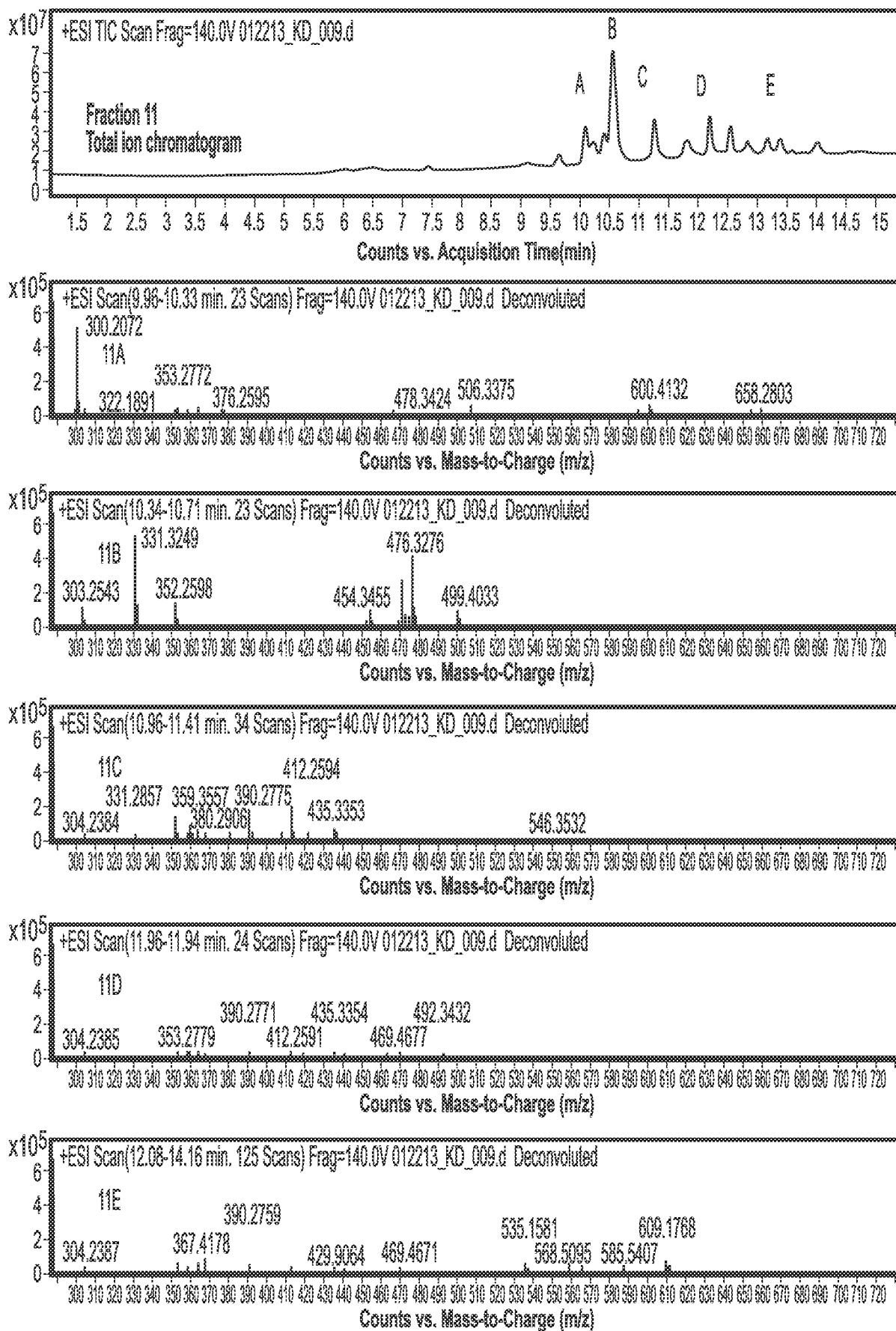


FIG. 5J

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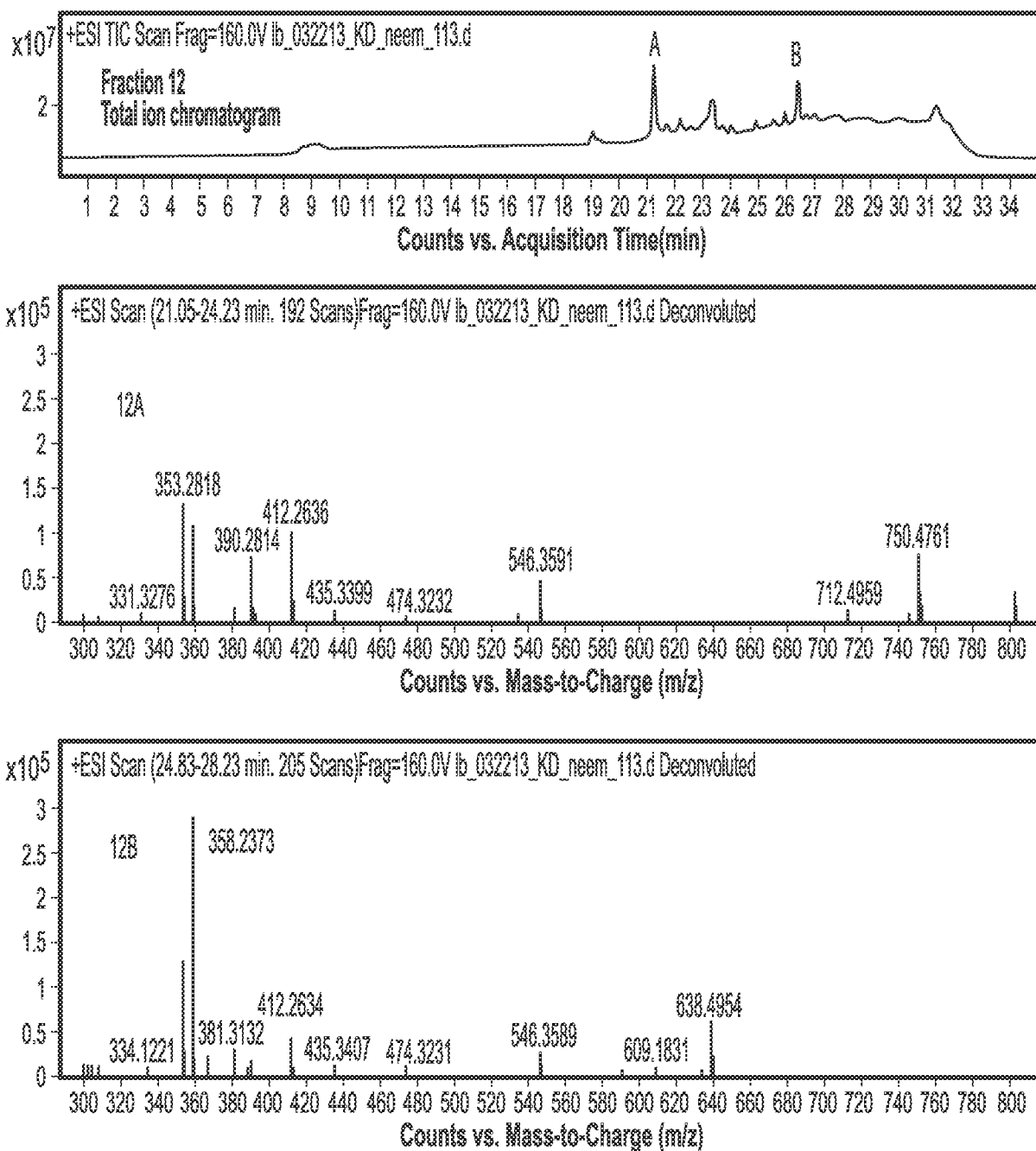


FIG. 5K

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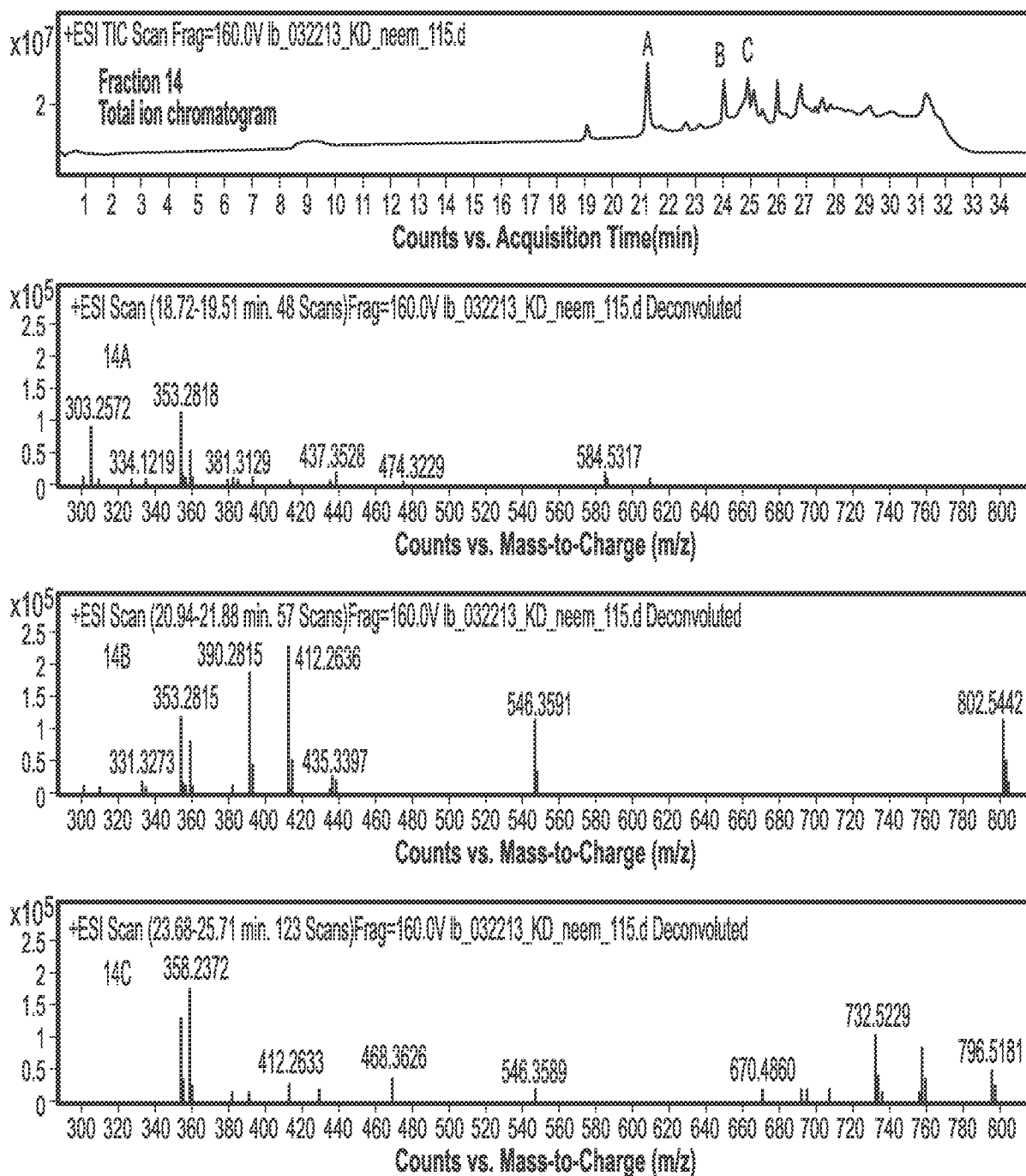


FIG. 5L

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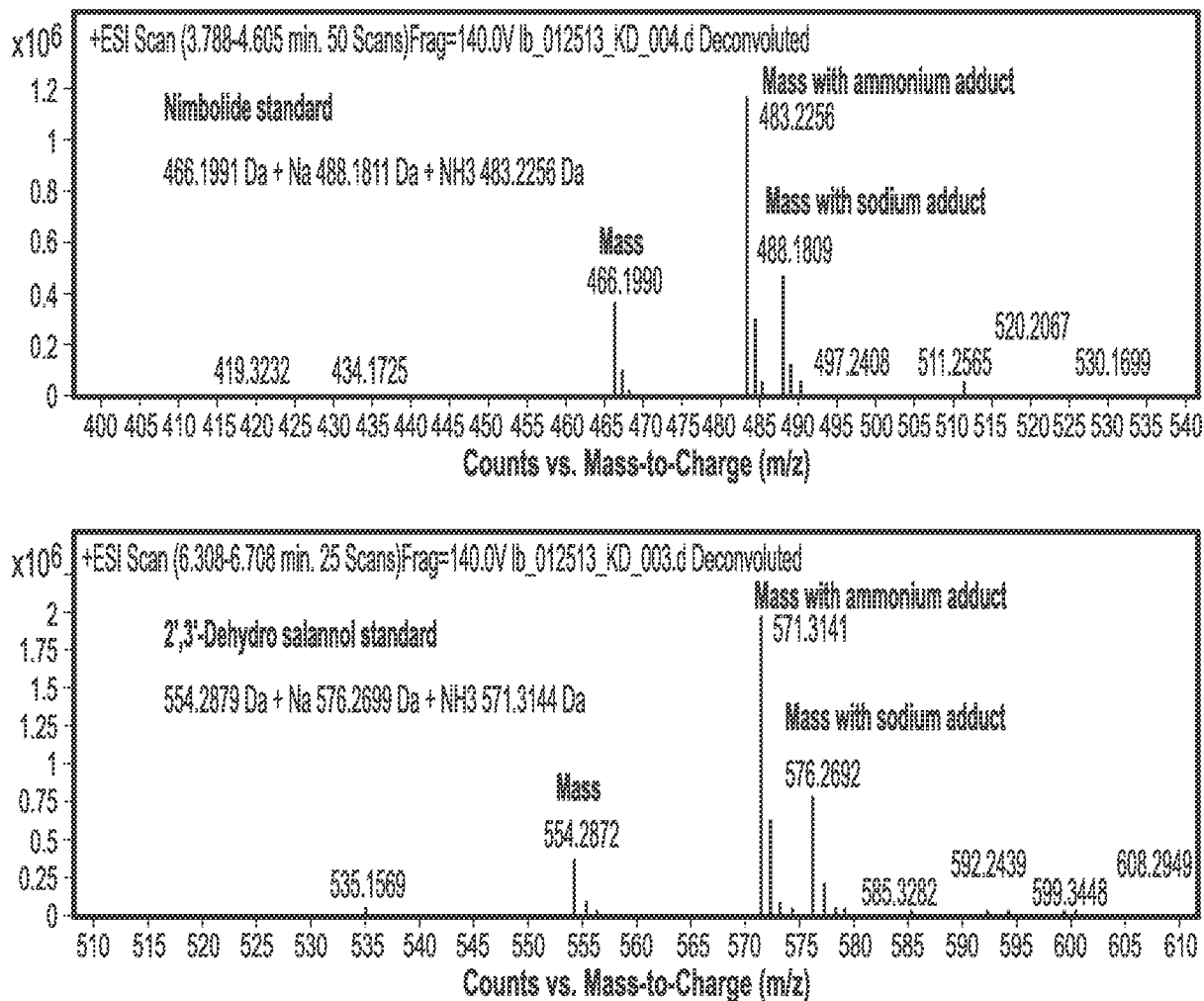
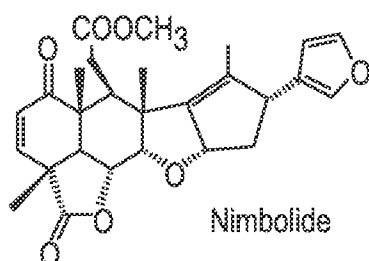
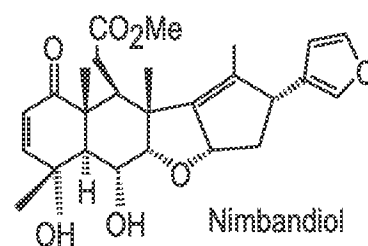
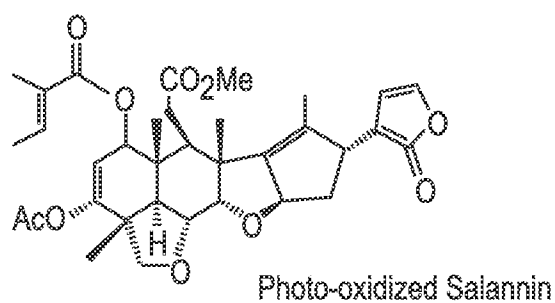
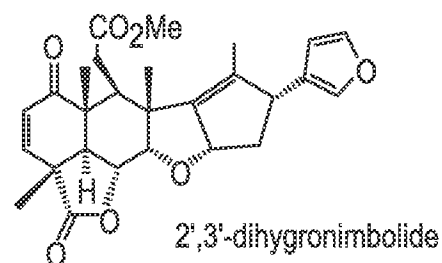
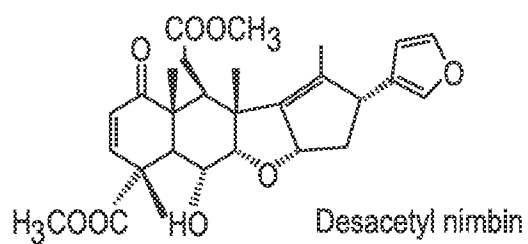
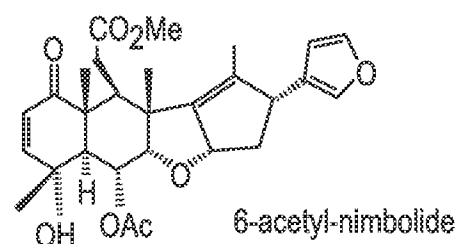
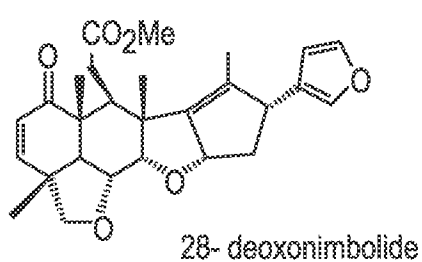
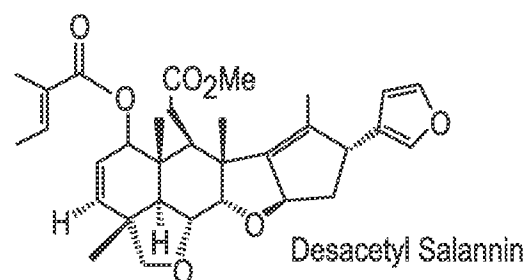
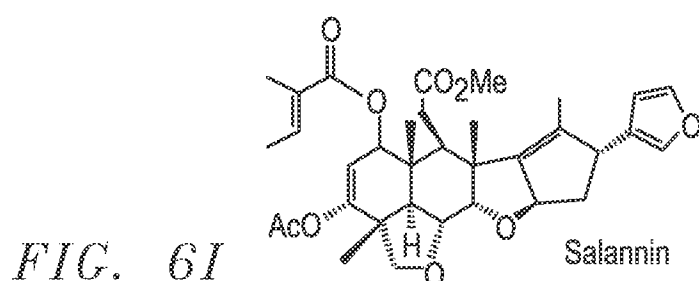


FIG. 5M

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*FIG. 6A**FIG. 6B**FIG. 6C**FIG. 6D**FIG. 6E**FIG. 6F**FIG. 6G**FIG. 6H**FIG. 6I*

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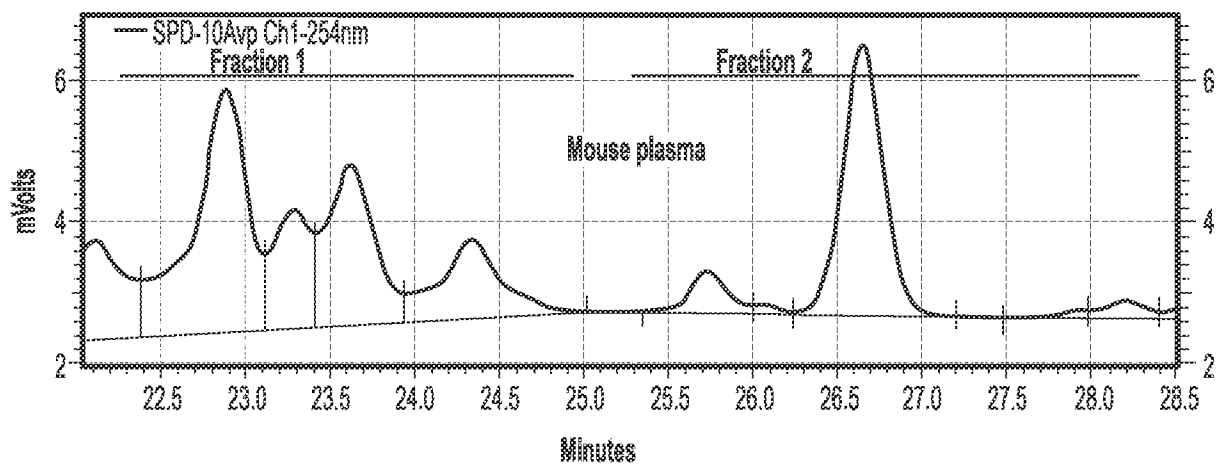


FIG. 7A

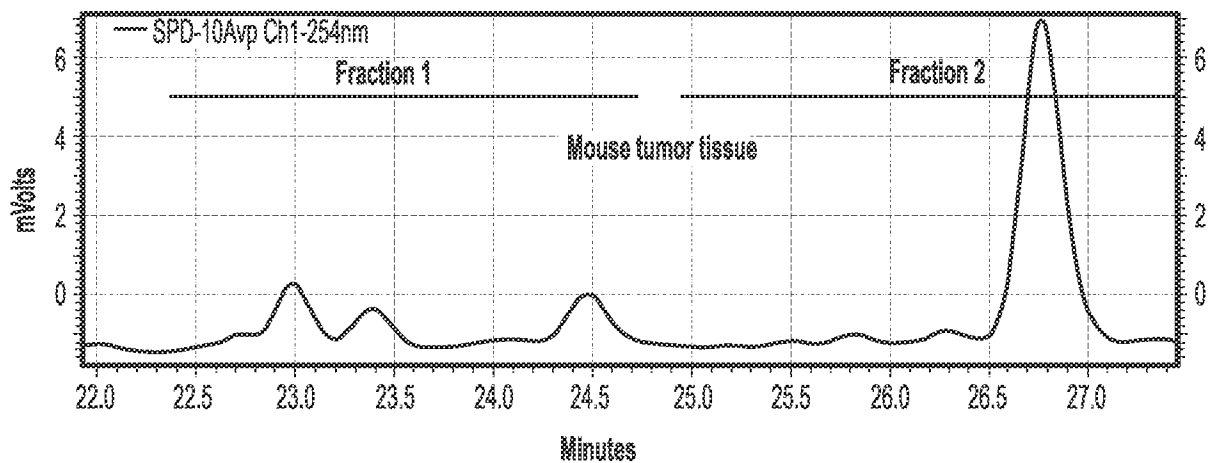


FIG. 7B

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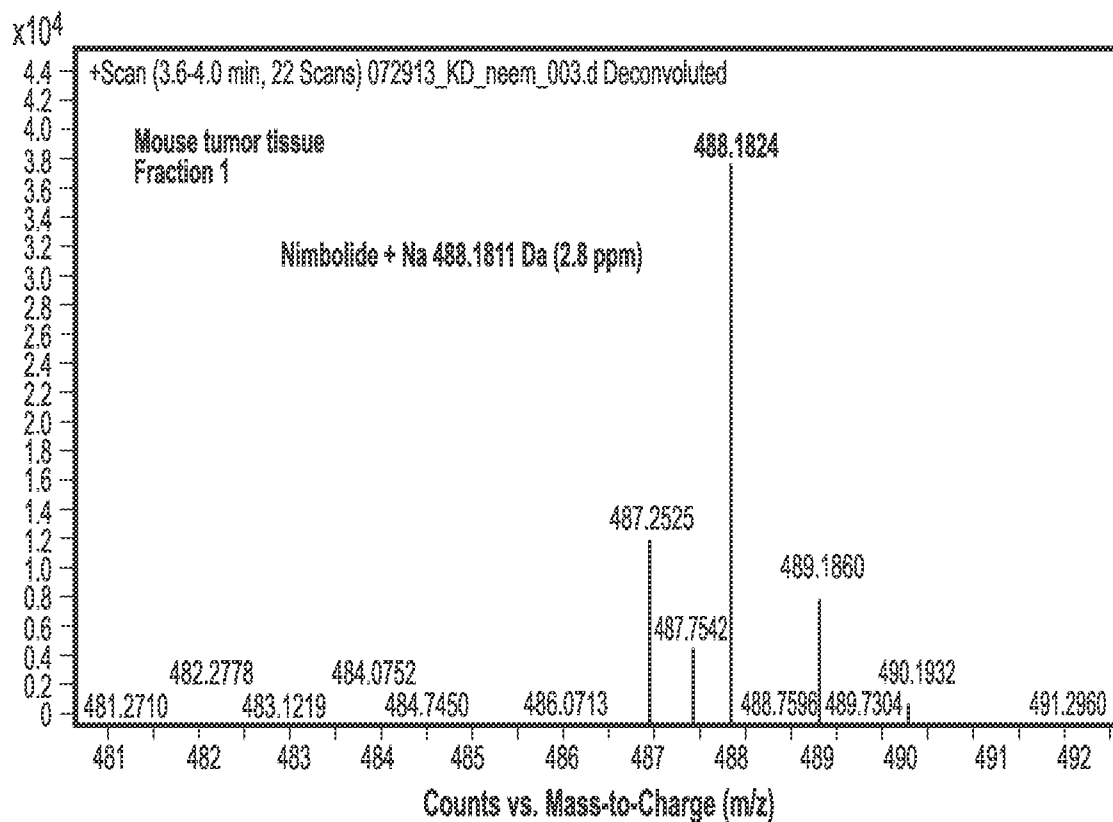


FIG. 7C

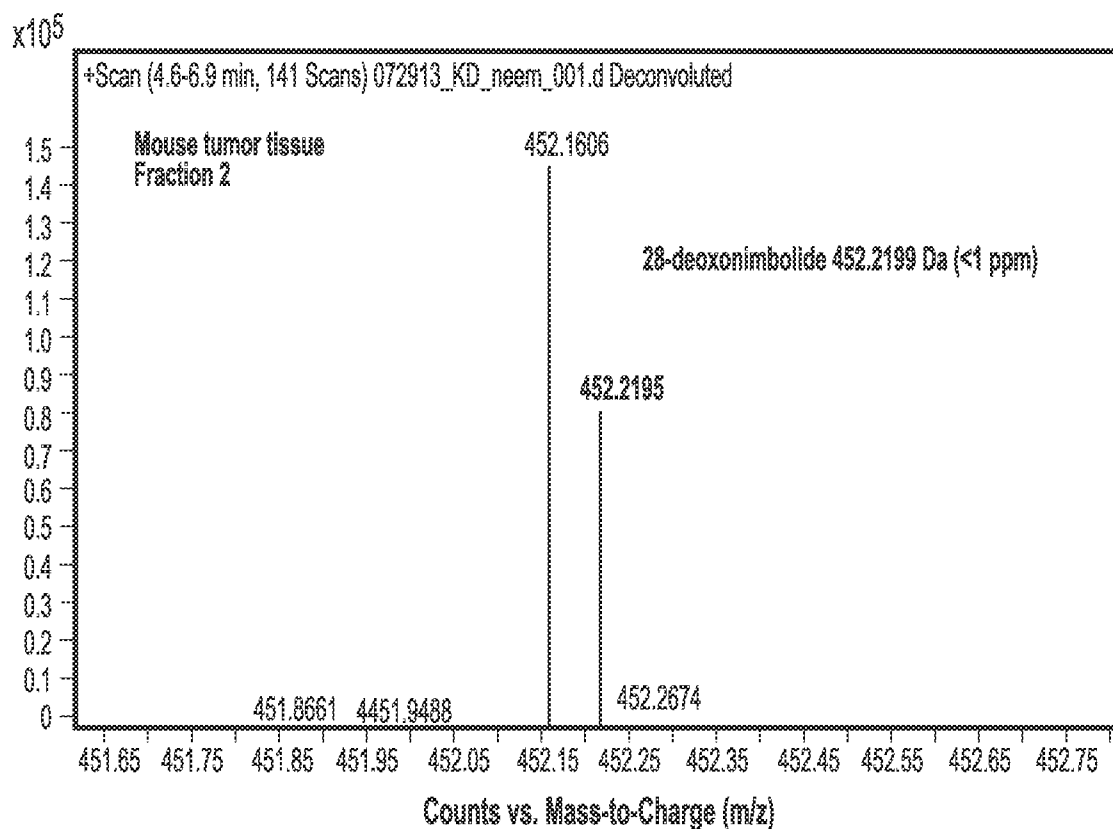


FIG. 7D

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 14/54338

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/343, 36/58; A61P 35/00; C07D 493/04 (2014.01)

CPC - A61K 36/58; A61K 31/343; C07D 493/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61K 31/343, 36/58; A61P 35/00; C07D 493/04 (2014.01)

CPC: A61K 36/58; A61K 31/343; C07D 493/04

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 514/468; 424/761

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Databases: PatBase, Google Patents, Google Web

Search terms: Neem, nimbolide/nimbandiol/2',3'-dihydro nimbolide, 28-deoxonimbolide, 20,21-dihydronimbolide, Azadirachta indica, cancer, prostate/colon/astrocytoma/sarcoma, symptoms, treat, ITGB1, FAK or PTK2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 101972246 B (Yan et al.) 30 May 2012 (30.05.2012) para [0006], [0010], [0082], [0083].	1-3
---	Machine translation attached.	
Y		4-11, 13-16
Y	US 2011/0159121 A1 (McDaniel) 30 June 2011 (30.06.2011) para [0274], [0279].	5, 8
Y	Cui et al. 'LIMONIDS FROM AZADIRACHTA EXCELSA', Phytochemistry; 1998, vol 47, no 7, pg 1283-1287 (pg 1283, compounds 1, 3); (pg 1286, Table 2).	11-14
Y	US 2011/0046121 A1 (Liang et al.) 24 February 2011 (24.02.2011) para [0015], [0082], [0096]	4, 6-11, 14-16
Y	US 5,370,873 A (Udeinya) 06 December 1994 (06.12.1994) (col 9, ln 19-22), (col 9, ln 52-56).	4, 6-11, 14
Y	Polonsky et al. '21,20-Anhydromelianone and melianone from Simarouba amara (Simaroubaceae); Carbon-13 NMR Spectra Analysis of delta7-Tirucallol-Type Triterpenes' Israel Journal of Chemistry; vol 17, pg 16-19 (Polonsky et al.) 01 December 1977 (01.12.1977) (pg 16, compounds 10-11).	9-10, 12
Y	US 6,060,075 A (Rao et al.) 09 May 2000 (09.05.2000) (col 3, ln 11-15).	9-10, 12

☐ Further documents are listed in the continuation of Box C.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 November 2014 (11.11.2014)

Date of mailing of the international search report

04 DEC 2014

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