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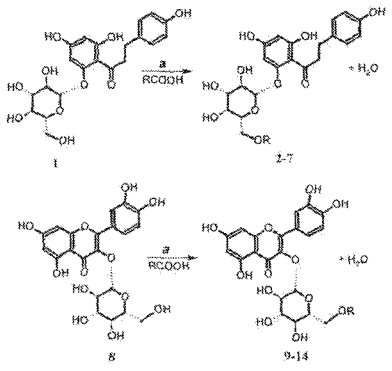
FIG. 1



a) Acetone, 3 °A molecular sieves, Novozyme 435®, 45 °C, Stirring, 24 h; R = Oleic, Stearic, Linoleic, Linolenic, Eicosapentaenoic (EPA), and Docosahexaenoic acids or their esters

Scheme 1

(57) Abstract: The present specification is directed to an acylated flavonoid derivative derived from natural or synthetic flavonoids and fatty acids. The acylated flavonoid derivatives can be part of a pharmaceutical composition. Method of decreasing proliferation and viability of cancer cells in a subject include administering the disclosed pharmaceutical composition.

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ACYLATED DERIVATIVES OF PHLORIDZIN AND ISOQUERCETRIN AS ANTICANCER THERAPEUTICS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of US Provisional Application Serial No. 61/863,790 filed on 8 August 2013, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

[0002] The invention relates generally to the discovery and synthesis of novel derivatives of phloridzin and isoquercetrin that display strong anticarcinogenic effects *in vitro*. Pharmaceutical compositions, methods, and kits for decreasing proliferation and viability of cancer cells and treating cancer in a subject include use of these novel derivatives are also disclosed.

BACKGROUND

[0003] It was estimated that over 508,000 women died in 2011 due to breast cancer worldwide. Triple negative breast cancer (TNBC) represents nearly 15% of all invasive breast cancers and TNBC cells do not express estrogen receptor (ER), progesterone receptor (PGR) and human epidermal growth factor receptor 2 (HER2). Therefore, treatment of TNBC is challenging because neither hormone therapy nor HER2 target therapy is effective in the treatment of TNBC. Among all the types of breast cancers, TNBC and HER2-enriched breast cancers are reported to have poor prognosis when compared to hormone receptor positive breast cancers.

[0004] Flavonoids are polyphenolic plant secondary metabolites which have been shown to have strong antioxidant, antiproliferative and other biological activities beneficial to human health by both epidemiological and *in vitro* studies. For example, oral administration of cranberry concentrate reduced the growth of N-butyl-N-(4-hydroxybutyl)-nitrosamine-(OH-BBN) induced urinary bladder tumor growth in female Fisher-344 rats. Growth of subcutaneously transplanted human non-small-cell lung cancer (NSCLC) (A549) cells in $Nrf2^{-/-}$ mice reduced significantly when luteolin was orally administered either in the presence or absence of cisplatin. Human prostate (PC-3) tumor bearing BALB/cA male nude mice showed significant reduction in tumor volume and weight when tumors were treated with luteolin and quercetin intraperitoneally. Intraperitoneal administration of luteolin

inhibited the growth of subcutaneously implanted Lewis lung carcinoma (LLC) cells in C57BL/6 mice as well. Galangin showed inhibitory effects on metastasis of human melanoma cells (B16F10) *in vitro* and *in vivo*.

[0005] Some flavonoids have shown antiangiogenic and antimetastatic properties in *in vivo* models as well. For example, oral administration of xanthohumol, a flavonoid from *Humulus lupulus* L. inhibited angiogenesis of kaposi's sarcoma cell (KS-IMM) tumor bearing nude mice in a dose-dependent manner. It also showed antiangiogenic properties *in vivo* in C57/b16N male mice.

[0006] Isoquercetrin or quercetin 3-*O*-glucoside has been studied for its anticancerous and toxicological properties in various transformed cell lines making it a candidate for cancer therapeutics. Specifically, dietary supplementation of quercetin significantly suppressed growth of orthotopically transplanted MIA PaCa-2, human pancreatic cells in nude mice. However, poor bioavailability limits its biological effects *in vivo* due to low membrane permeability and hence, its application as a therapeutic agent is limited.

[0007] Phloridzin, like quercetin 3-*O*-glucoside, is a naturally occurring flavonoid, however, it does not possess strong anti-cancer properties.

[0008] Low cellular uptake and reduced stability are major drawbacks of naturally occurring flavonoid glycosides, which restrict their successful applications in treating diseases. This eventually leads to poor bioavailability. Therefore, many attempts have been made to improve the bioavailability of flavonoids while protecting or improving their biological properties. The most commonly used enzymatic modification of flavonoids is the acylation with saturated or mono/poly-unsaturated fatty acids catalyzed by immobilized lipase from *Candida Antarctica*. The acylation of flavonoid glycosides such as, naringin (naringenin-7-*O*-rhamnoglucoside) and isoquercetrin has been done using carboxylic acids (palmitic, cinnamic and phenylpropionic (PPA) acids and hydroxylated derivatives of PPA) as acyl donors. This resulted in major mono-acylated products but also with minor mono-acylated products.

[0009] Antiproliferative properties of flavonoids have prompted researchers to develop alternative natural medicines to substitute synthetic chemotherapeutic agents which apparently are known to have many side effects. Improved anti-cancer therapeutics that display minimum toxicity to normal cells and few or no side effects are needed in a number of cancers (e.g., breast cancer, liver cancer, leukemias, etc.).

SUMMARY

[0010] Described herein are compositions, methods and kits for treating cancers such as leukemias, breast cancer and liver cancer.

[0011] In an aspect there is disclosed an acylated flavonoid derived from a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.

[0012] There is also disclosed a pharmaceutical composition comprising a pharmaceutically acceptable carrier; and an acylated flavonoid derived from a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.

[0013] In another aspect, there is disclosed a method of decreasing proliferation and viability of cancer cells in a patient in need thereof, comprising administering a therapeutically effective amount of a a pharmaceutical composition comprising a pharmaceutically acceptable carrier; and an acylated flavonoid derived from a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.

[0014] Moreover, there is also disclosed a method of treating cancer in a patient in need thereof, comprising administering a therapeutically effective amount of a a pharmaceutical composition comprising a pharmaceutically acceptable carrier; and an acylated flavonoid derived from a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.

[0015] Further, in an aspect is disclosed a kit for treating cancer in a subject, the kit comprising the disclosed pharmaceutical composition in a therapeutically effective amount; instructions for use; and packaging.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 is a schematic illustration of acylation of phloridzin and isoquercetrin using enzymes.

[0017] FIG. 2a-c is a table illustrating structures and percent yield of acylated fatty acid derivatives of phloridzin and isoquercetrin.

[0018] FIG. 3 is a schematic illustration of sonochemical acylation of phloridzin and isoquercetrin using lipases.

[0019] FIG. 4a-h is a table of structures and percent yields of acylated fatty acid derivatives of phloridzin and isoquercetrin, as well as reaction times.

[0020] FIG. 5a-e is a series of bar graphs showing the metabolic inhibition of MDA-MB-231 cells by PZ, DHA, and PZ-DHA at 3 hours (Fig. 5A), 6 hours (Fig. 5B), 12 hours (Fig. 5C), 18 hours (Fig. 5D) and 24 hours of treatment.

[0021] FIG. 6 is a graph showing that low PZ-DHA concentrations selectively inhibits MDA-MB-231 triple negative breast cancer cells.

[0022] FIG. 7A is an illustration of the caliper measurements of tumors on various days and tumor volume. FIG. 7B is an illustration of the weight of excised tumors after day 15.

[0023] FIG. 8 is a series of bar graphs showing the inhibition of phosphatase enzyme activity of MDA-MB-231 cells by PZ, DHA, and PZ-DHA at 3 hours (Fig. 8A), 6 hours (Fig. 8B), 12 hours (Fig. 8C), 18 hours (Fig. 8D) and 24 hours of treatment.

[0024] FIG. 9 is an illustration of the mean luminescence measured in three independent experiments of MDA-MB-231 cells treated with various test compounds.

[0025] FIG. 10 is a table of EC50 values of three human cancer cell lines in relation to dose and exposure time of six fatty acid esters of phloridzin in comparison to parent flavonoid and fatty acids and two prescribed drugs.

[0026] FIG. 11 A-B is a series of graphs showing survival of HepG2 cells (hepatocarcinoma) after incubation with six fatty acid esters of phloridzin in comparison to parent flavonoid and fatty acids and the prescribed drug sorafenib (Nexavar®) at various doses and exposure times. EC50 values were calculated using Graphpad Prism 6 software.

[0027] FIG. 12A-B is a series of graphs showing survival of MDA-MB-231 cells (breast cancer) after incubation with six fatty acid esters of phloridzin in comparison to parent

flavonoid and fatty acids and the prescribed drug doxorubicin hydrochloride at various doses and exposure times. EC50 values were calculated using Graphpad Prism 6 software.

[0028] FIG. 13A-B is a series of graphs showing survival of THP-1 (leukemia) cells after incubation with six fatty acid esters of phloridzin in comparison to parent flavonoid and fatty acids and the prescribed drug doxorubicin hydrochloride at various doses and exposure times. EC50 values were calculated using Graphpad Prism 6 software.

[0029] FIG. 14A-B is a pair of graphs showing antiproliferative effects of long chain fatty acid esters on HepG2 cells. The figure describes the percentage of viable HepG2 cells after treatment with long chain fatty acid esters of Q3G and chemotherapy drugs for 6 (A) and 24 hours (B).

[0030] FIG. 15A-B is a pair of graphs showing the effect of long chain fatty acid esters of Q3G on cytotoxicity in HepG2 cells. The figure describes the percentage of LDH release from HepG2 cells after treatment with long chain fatty acid esters of Q3G and chemotherapy drugs (Sorafenib and Cisplatin) for 6 and 24 hours.

[0031] FIG. 16 is a graph showing the effect of long chain fatty acid esters of Q3G on viability of normal rat hepatocytes. The figure describes the percentage of viable rat hepatocytes cells after treatment with long chain fatty acid esters of Q3G. Cells (1×10^4 cells per well; 96-well plate) were treated with 100 μM off long chain fatty acid esters of Q3G, precursor compounds (quercetin and Q3G) and chemotherapy drug (Sorafenib) for 24 hours. After treatment viable cell percentage was determined by MTS assay as described in materials and methods. Results are expressed relative to control (24-h incubation without test compounds).

[0032] FIG. 17 is a table listing the effects of OA-Q3G treatment on gene level expression in HepG2 cells.

DETAILED DESCRIPTION

[0033] The compositions, methods and kits include acylated flavonoids, such as phloridzin and isoquercetrin derivatives, in amounts effective for decreasing proliferation and viability of cancer cells in a subject. The compositions are administered to a subject (e.g., human) having cancer in an amount effective for treating cancer in the subject.

[0034] Unless otherwise defined, all technical terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

[0035] As used herein, “protein” and “polypeptide” are used synonymously to mean any peptide-linked chain of amino acids, regardless of length or post-translational modification, e.g., glycosylation or phosphorylation.

[0036] By the term “gene” is meant a nucleic acid molecule that codes for a particular protein, or in certain cases, a functional or structural RNA molecule.

[0037] As used herein, a "nucleic acid” or a "nucleic acid molecule" means a chain of two or more nucleotides such as RNA (ribonucleic acid) and DNA (deoxyribonucleic acid).

[0038] The terms "patient," "subject" and "individual" are used interchangeably herein, and mean a vertebrate animal, typically mammalian (e.g., human, rodent, non-human primates, canine, bovine, ovine, equine, feline, etc.), subject to be treated and/or to obtain a biological sample from.

[0039] As used herein, the terms "regulating", "regulation", “modulating” or “modulation” refer to the ability of an agent to either inhibit or enhance or maintain activity and/or function of a molecule. For example, an inhibitor of DNA topoisomerase II would down-regulate, decrease, reduce, suppress, or inactivate at least partially the activity and/or function of DNA topoisomerase II. As used herein, "inhibiting" means slowing or stopping the growth of. Up-regulation refers to a relative increase in function and/or activity.

[0040] The terms “isoquercetrin” and “quercetin 3-*O*-glucoside,” referring to the flavonoid compound, are used interchangeably herein, and encompass naturally occurring as well as synthesized compounds.

[0041] The terms “phloridzin” and “phloritin 2’-*O*-glucoside,” referring to the flavonoid compound, are used interchangeably herein, and encompass naturally occurring, such as polyphenol compounds found in apples, as well as synthesized compounds.

[0042] The phrases "isolated" or biologically pure" refer to material, which is substantially or essentially free from components which normally accompany it as found in its native state.

[0043] By "agent" is meant a polypeptide, peptide, nucleic acid molecule, small molecule, or mimetic.

[0044] By "analog" is meant an agent having structural or functional homology to a reference agent.

[0045] As used herein, the term “derivative” means a compound derived or obtained from another and containing essential elements of the parent substance.

[0046] By "modifies" is meant alters. An agent that modifies a cell, substrate, or cellular environment produces a biochemical alteration in a component (e.g., polypeptide, nucleotide, or molecular component) of the cell, substrate, or cellular environment.

[0047] As used herein, the terms “diagnostic,” “diagnose” and “diagnosed” mean identifying the presence or nature of a pathologic condition (e.g., cancer).

[0048] The term “sample” is used herein in its broadest sense. A sample including polynucleotides, polypeptides, peptides, antibodies and the like may include a bodily fluid, a soluble fraction of a cell preparation or media in which cells were grown, a biopsy, genomic DNA, RNA or cDNA, a cell, a tissue, skin, hair and the like. Examples of samples include saliva, serum, blood, biopsies, urine and plasma.

[0049] As used herein, the term “treatment” is defined as the application or administration of a therapeutic agent to a patient, or application or administration of the therapeutic agent to an isolated tissue or cell line from a patient, who has a disease, a symptom of disease or a predisposition toward a disease, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease, the symptoms of disease, or the predisposition toward disease. Treatment can include, for example, reducing the number of cancer cells in a subject, eliminating cancer cells in a subject, reducing cancer cell viability and proliferation, eliminating a cancerous tumor or reducing the size of a cancerous tumor, etc.

[0050] As used herein, the phrase "safe and effective amount" refers to the quantity of a component, which is sufficient to yield a desired therapeutic response without undue adverse side effects (such as toxicity, irritation, or allergic response) commensurate with a reasonable benefit/risk ratio when used in the manner of this invention. By "therapeutically effective amount" is meant an amount of a composition of the present invention effective to yield the desired therapeutic response. For example, an amount effective to decrease proliferation and viability of cancer cells (e.g., to induce apoptosis of cancer cells) in a subject having cancer. As another example, an amount effective to delay the growth of or to cause a cancer (e.g., breast cancer, liver cancer, leukemia) in a subject to shrink or prevent metastasis of the cancer in the subject. The specific safe and effective amount or therapeutically effective amount will vary with such factors as the particular condition being treated, the physical condition of the patient, the type of mammal or animal 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.

[0051] Flavonoids are polyphenolic compounds found in nature which generally consist of two aromatic rings, connected through a three-carbon bridge that may be part of a six-membered heterocyclic pyran ring. These compounds can exhibit their antiproliferative properties on different cell lines and the activity can be dose- and time-dependent. They exert their anticancer properties by involving in reactive oxygen species (ROS) production, signal transduction pathways governing cell proliferation and growth, apoptosis, and angiogenesis. Flavonoids induce both intracellular and extracellular ROS production.

[0052] Flavonoids for use in the present invention can include natural and synthetic compounds. Non-limiting examples of flavonoids include anthoxanthins, flavanones, flavanonols, and flavans (catechins). The anthoxanthins include flavones, such as luteolin, apigenin, and tangeritin; and flavonols (3-hydroxyflavones), such as quercetin, kaempferol, myricetin, fisetin, galangin, isorhamnetin, pachypodol, rhamnazin, pyranoflavonols, and furanflavonols. Flavanones include hesperetin, naringenin, eirodictyol, and homoeriodictyol. Flavanonols include taxifolin (dihydroquercetin), and dihydrokaempferol. Flavans can include flavan-4-ol; flavan-3,4-diols; and flavan-3-ols, such as catechin, gallocatechin, catechin-3-gallate, gallocatechin-3-gallate, epicatechin, theaflavin, thearubigin, proanthocyanidins. Other non-limiting examples of flavonoids useful in the present invention include phloridzin, luteolin, isoquercetrin, and quercetin. Phloridzin (phloretin 2'-*O*-glucoside) and isoquercetrin (quercetin 3-*O*-glucoside) are two flavonoids compounds found in fruits, especially in apples.

[0053] In an aspect, acylation of flavonoids with a primary aliphatic hydroxyl group on the sugar moiety can occur to form acylated flavonoid derivatives. The acylating agents used in forming the acylated flavonoid derivatives include short, medium, and long chain fatty acids, including long chain saturated or poly-unsaturated fatty acids. Non-limiting examples of fatty acids useful in the acylation of the flavonoids of the invention include, stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic. The acylated flavonoid derivative can be an ester. For example, the acylated derivatives include, but are not limited to: Stearic acid ester of phloridzin, Oleic acid ester of phloridzin, Linoleic acid ester of phloridzin, alpha-linolenic acid ester of phloridzin, Docosahexaenoic acid ester of phloridzin, Eicosapentaenoic acid ester of phloridzin, Stearic acid ester of isoquercetrin, Oleic acid ester of isoquercetrin, Linoleic acid ester of isoquercetrin, alpha-linolenic acid ester of isoquercetrin, Docosahexaenoic acid ester of isoquercetrin, and Eicosapentaenoic acid ester of isoquercetrin. However, any suitable acylated flavonoid derivative can be created using

known flavonoids and acylating agents using known acylating mechanisms. For example, acylated phloridzin and isoquercetrin derivatives can be synthesized by any suitable method, e.g., according to the methods described in Ziaullah et al., Biorganic and Medicinal Chemistry vol. 21:684-692, 2013; and Ziaullah et al., Tetrahedron Letters vol. 54:1933-1937, 2013. These synthesis methods are described in Example 1 below.

[0054] Methods involving conventional molecular biology techniques are described herein. Such techniques are generally known in the art and are described in detail in methodology treatises such as Molecular Cloning: A Laboratory Manual, 3rd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2001; and Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates).

[0055] In an aspect, any suitable acylated flavonoid derivatives that exhibit anticarcinogenic effects *in vitro* can be used. For example, the acylated flavonoid derivative can be used for decreasing cancer cell viability and proliferation and treating cancer, for example, breast cancer, liver cancer, and acute monocytic leukemia cells, in a subject. The acylated flavonoid derivative can induce apoptosis of the cancer cells. Moreover, the acylated flavonoid derivatives of the present invention can activate caspase-3-family member activation and induce s-phase cell cycle arrest in cancer cells. The derivatives can decrease proliferation and viability specifically of cancer cells compared to noncancerous cells.

[0056] A subject (patient) having cancer may have a tumor. Such a tumor may be a solid or hematological tumor, benign or malignant (metastic or nonmetastatic), such as, for example, breast, liver, prostate, cervical, ovarian, colon, brain, pancreatic, bladder esophagus, gut, head and neck, kidney, melanoma, stomach, testes, thyroid, uterine and lung cancers, leukemias and lymphomas, such as acute myelogenous leukemia, acute or chronic lymphocytic leukemia, Hodgkin's and non-Hodgkin lymphoma, and myelomas. Persons of skill in the art will be able to determine by routine experimentation the types of tumors that are amenable to treatment.

[0057] Pharmaceutical compositions comprising the acylated flavonoid derivatives, such as the acylated phloridzin and acylated isoquercetrin derivatives, described herein can be used for decreasing proliferation and cancer cell viability; inducing apoptosis of the cancer cells; and treating cancer in a subject.

[0058] In an aspect, the disclosed pharmaceutical composition can comprise a therapeutically effective amount of an acylated flavonoid derivative, such as an acylated

phloridzin or isoquercetrin derivative, comprising a long chain saturated or poly-unsaturated fatty acid (e.g., one or more of stearic acid, oleic acid, linoleic acid, alpha-linolenic acid, docosahexaenoic acid, and eicosapentaenoic acid), and optionally a pharmaceutically acceptable carrier.

[0059] The pharmaceutical composition can further comprise at least one additional anti-cancer drug (e.g., a second anti-cancer drug). Non-limiting examples of additional anti-cancer drugs, such as a chemotherapy agent or an antiangiogenic agent, include sorafenib, doxorubicin, methotrexate, vinblastine, vincristine, cladribine, fluorouracil, cytarabine, anthracyclines, cisplatin, cyclophosphamide, fludarabine, gemcitabine, aromatase inhibitors, irinotecan, navelbine, oxaliplatin, taxol, docetaxel, bevacizumab, pegaptanib, and ranibizumab, and combinations thereof. In an aspect, a combination therapy including a standard chemotherapy drug and an acylated flavonoid, such as phloridzin or isoquercetrin, derivative may be particularly therapeutic.

[0060] The pharmaceutical compositions described herein can be administered to a mammal (e.g., rodent, human, non-human primates, canine, bovine, ovine, equine, feline, etc.) in an effective amount, that is, an amount capable of producing a desirable result in a treated subject (e.g., inhibiting growth of cancer cells, inducing apoptosis of cancer cells in the subject). Toxicity and therapeutic efficacy of the compositions utilized in methods of the invention can be determined by standard pharmaceutical procedures. As is well known in the medical and veterinary arts, dosage for any one animal depends on many factors, including the subject's size, body surface area, body weight, age, the particular composition to be administered, time and route of administration, general health, the clinical symptoms of the cancer and other drugs being administered concurrently. A pharmaceutical composition as described herein is typically administered at a dosage that can at least one of induce apoptosis of cancer cells, or reduces or eliminates cancer cell growth or proliferation. As an example, in the human cancer cell line experiments described herein, the amount of acylated phloridzin derivatives used to demonstrate antiproliferative activity was from about 0.1 μM to about 200 μM, for example from about 10 μM to about 150 μM, and from about 50 μM to about 100 μM; and the amount of acylated isoquercetrin derivatives used to demonstrate antiprolierative and cytotoxic activity was from about 0.1 μM to about 200 μM, for example from about 10 μM to about 150 μM, and from about 50 μM to about 100 μM.

[0061] Described herein are methods of decreasing proliferation and viability of cancer cells and treating cancer (e.g., liver cancer, breast cancer, leukemia, etc.) and/or disorders or symptoms thereof in a subject. The methods include administering to the subject (e.g., a

mammal such as a human) a therapeutically effective amount of a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of an acylated flavonoid derivative, such as an acylated phloridzin or isoquercetrin derivative, to decrease proliferation and viability of cancer cells in the subject. In the method, an amount of acylated phloridzin or isoquercetrin derivative sufficient to induce death (e.g., apoptosis) of cancer cells in the subject can be typically administered. In the methods, the acylated flavonoid derivative, such as acylated phloridzin or isoquercetrin derivative, may activate caspase-3 family member activation and induce s-phase cell cycle arrest in the cancer cells. Typically, the acylated phloridzin or isoquercetrin derivative decreases proliferation and viability specifically of cancer cells compared to noncancerous cells (i.e., the acylated phloridzin or isoquercetrin derivatives described herein display specificity for cancer cells and are not cytotoxic to noncancerous cells).

[0062] In some embodiments, the pharmaceutical composition can be administered to a subject who is resistant to commonly used standard chemotherapy or other anti-cancer drugs. For example, in the case of liver cancer, a subject who is resistant to Sorafenib may respond to treatment with the disclosed acylated flavonoid derivatives, such as an acylated phloridzin or isoquercetrin derivative described herein. In the methods, the composition can further comprise or be co-administered with a therapeutically effective amount of at least one additional anti-cancer drugs or standard chemotherapy.

[0063] The therapeutic methods of the invention (which include prophylactic treatment) in general include administration of a therapeutically effective amount of the compositions described herein to a subject in need thereof, including a mammal, particularly a human. Such treatment will be suitably administered to subjects, particularly humans, suffering from, having, susceptible to, or at risk for cancer or a disorder or symptom thereof. Determination of those subjects "at risk" can be made by any objective or subjective determination by a diagnostic test or opinion of a subject or health care provider (e.g., genetic test, enzyme or protein marker, marker (as defined herein), family history, and the like).

[0064] The administration of the disclosed pharmaceutical composition comprising an acylated phloridzin or isoquercetrin derivative for the treatment of cancer (e.g., breast cancer, liver cancer, leukemia, etc.) may be by any suitable means that results in a concentration of the therapeutic that, (e.g., in some instances when combined with other components), is effective in ameliorating, reducing, or stabilizing a cancer. The acylated phloridzin or isoquercetrin derivative may be contained in any appropriate amount in any suitable carrier substance, and is generally present in an amount of 1-95% ,for example from about 5 -90%,

from about 10-85%, and as a further example from about 15-80% by weight of the total weight of the composition.

[0065] The composition may be provided in a dosage form that is suitable for local or systemic administration (e.g., parenteral, subcutaneously, intravenously, intramuscularly, or intraperitoneally). For therapeutic uses, the compositions or agents identified using the methods disclosed herein may be administered systemically, for example, formulated in a pharmaceutically-acceptable buffer such as physiological saline. Exemplary routes of administration include, for example, subcutaneous, intravenous, intraperitoneally, intramuscular, or intradermal injections that provide continuous, sustained levels of the drug in the patient. The pharmaceutical compositions may be formulated according to conventional pharmaceutical practice (see, e.g., Remington: The Science and Practice of Pharmacy (20th ed.), ed. A. R. Gennaro, Lippincott Williams & Wilkins, 2000 and Encyclopedia of Pharmaceutical Technology, eds. J. Swarbrick and J. C. Boylan, 1988-1999, Marcel Dekker, New York).

[0066] Compositions as described herein may be administered parenterally by injection, infusion or implantation (subcutaneous, intravenous, intramuscular, intraperitoneal, or the like) in dosage forms, formulations, or via suitable delivery devices or implants containing conventional, non-toxic pharmaceutically acceptable carriers and adjuvants. The formulation and preparation of such compositions are well known to those skilled in the art of pharmaceutical formulation. Formulations can be found in Remington: The Science and Practice of Pharmacy, *supra*.

[0067] Compositions for parenteral use may be provided in unit dosage forms (e.g., in single-dose ampoules), or in vials containing several doses and in which a suitable preservative may be added (see below). The composition may be in the form of a solution, a suspension, an emulsion, an infusion device, or a delivery device for implantation, or it may be presented as a dry powder to be reconstituted with water or another suitable vehicle before use. Apart from the acylated flavonoid that reduces or ameliorates a cancer, the composition may include suitable parenterally acceptable carriers and/or excipients. The acylated flavonoid may be incorporated into microspheres, microcapsules, nanoparticles, liposomes, or the like for controlled release. Furthermore, the pharmaceutical composition may include suspending, solubilizing, stabilizing, pH-adjusting agents, tonicity adjusting agents, and/or dispersing agents.

[0068] As indicated above, the pharmaceutical compositions described herein may be in a form suitable for sterile injection. To prepare such a composition, the acylated flavonoid

derivative is dissolved or suspended in a parenterally acceptable liquid vehicle. Among acceptable vehicles and solvents that may be employed are water, water adjusted to a suitable pH by addition of an appropriate amount of hydrochloric acid, sodium hydroxide or a suitable buffer, 1,3-butanediol, Ringer's solution, and isotonic sodium chloride solution and dextrose solution. The aqueous formulation may also contain one or more preservatives (e.g., methyl, ethyl or n-propyl p-hydroxybenzoate). In cases where one of the compounds is only sparingly or slightly soluble in water, a dissolution enhancing or solubilizing agent can be added, or the solvent may include 10-60% w/w of propylene glycol or the like.

[0069] Materials for use in the preparation of microspheres and/or microcapsules are, e.g., biodegradable/bioerodible polymers such as polygalactin, poly-(isobutyl cyanoacrylate), poly(2-hydroxyethyl-L-glutam- nine) and, poly(lactic acid). Biocompatible carriers that may be used when formulating a controlled release parenteral formulation are carbohydrates (e.g., dextrans), proteins (e.g., albumin), lipoproteins, or antibodies. Materials for use in implants can be non-biodegradable (e.g., polydimethyl siloxane) or biodegradable (e.g., poly(caprolactone), poly(lactic acid), poly(glycolic acid) or poly(ortho esters) or combinations thereof).

[0070] Formulations for oral use include tablets containing the active ingredient(s) (e.g., acylated phloridzin or isoquercetrin derivative) in a mixture with non-toxic pharmaceutically acceptable excipients. Such formulations are known to the skilled artisan. Excipients may include but are not limited to, for example, inert diluents or fillers (e.g., sucrose, sorbitol, sugar, mannitol, microcrystalline cellulose, starches including potato starch, calcium carbonate, sodium chloride, lactose, calcium phosphate, calcium sulfate, or sodium phosphate); granulating and disintegrating agents (e.g., cellulose derivatives including microcrystalline cellulose, starches including potato starch, croscarmellose sodium, alginates, or alginic acid); binding agents (e.g., sucrose, glucose, sorbitol, acacia, alginic acid, sodium alginate, gelatin, starch, pregelatinized starch, microcrystalline cellulose, magnesium aluminum silicate, carboxymethylcellulose sodium, methylcellulose, hydroxypropyl methylcellulose, ethylcellulose, polyvinylpyrrolidone, or polyethylene glycol); and lubricating agents, glidants, and antiadhesives (e.g., magnesium stearate, zinc stearate, stearic acid, silicas, hydrogenated vegetable oils, or talc). Other pharmaceutically acceptable excipients can be colorants, flavoring agents, plasticizers, humectants, buffering agents, and the like.

[0071] The tablets may be uncoated or they may be coated by known techniques, optionally to delay disintegration and absorption in the gastrointestinal tract and thereby

providing a sustained action over a longer period. The coating may be adapted to release the active drug in a predetermined pattern (e.g., in order to achieve a controlled release formulation) or it may be adapted not to release the active drug until after passage of the stomach (enteric coating). The coating may be a sugar coating, a film coating (e.g., based on hydroxypropyl methylcellulose, methylcellulose, methyl hydroxyethylcellulose, hydroxypropylcellulose, carboxymethylcellulose, acrylate copolymers, polyethylene glycols and/or polyvinylpyrrolidone), or an enteric coating (e.g., based on methacrylic acid copolymer, cellulose acetate phthalate, hydroxypropyl methylcellulose phthalate, hydroxypropyl methylcellulose acetate succinate, polyvinyl acetate phthalate, shellac, and/or ethylcellulose). Furthermore, a time delay material, such as, e.g., glyceryl monostearate or glyceryl distearate may be employed.

[0072] Nasal and other mucosal spray formulations (e.g. inhalable forms) can include purified aqueous solutions of the active compounds with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal or other mucous membranes. Alternatively, they can be in the form of finely divided solid powders suspended in a gas carrier. Such formulations may be delivered by any suitable means or method, e.g., by nebulizer, atomizer, metered dose inhaler, or the like.

[0073] A pharmaceutical composition of the present invention can have immediate release, sustained release, delayed-onset release or any other release profile known to one skilled in the art. Pharmaceutical compositions as described herein may be formulated to release the acylated flavonoid derivative substantially immediately upon administration or at any predetermined time or time period after administration. The latter types of compositions are generally known as controlled release formulations, which include formulations that create a substantially constant concentration of the drug within the body over an extended period of time; formulations that after a predetermined lag time create a substantially constant concentration of the drug within the body over an extended period of time; formulations that sustain action during a predetermined time period by maintaining a relatively, constant, effective level in the body with concomitant minimization of undesirable side effects associated with fluctuations in the plasma level of the active substance (sawtooth kinetic pattern); formulations that localize action by, e.g., spatial placement of a controlled release composition adjacent to or in the central nervous system or cerebrospinal fluid formulations that allow for convenient dosing, such that doses are administered, for example, once every one or two weeks; and formulations that target the site of a pathology. For some applications,

controlled release formulations obviate the need for frequent dosing to sustain the enzyme activity at a therapeutic level.

[0074] Optionally, a composition as described herein may be administered in combination with any other anti-cancer therapy; such methods are known to the skilled artisan and described in Remington: The Science and Practice of Pharmacy, *supra.* In one example, an effective amount of an acylated phloridzin derivative or acylated isoquercetrin derivative as described herein is administered in combination with radiation therapy. Combinations are expected to be advantageously synergistic. Therapeutic combinations that inhibit cancer (e.g., leukemia, liver cancer, breast cancer, etc.) cell growth and/or induce apoptosis of cancer cells are identified as useful in the methods described herein.

[0075] In one embodiment, the invention provides a method of monitoring treatment progress. The method includes the step of determining a level of changes in cancer cell viability parameters and/or cell cycle arrest and/or induction of apoptosis as diagnostic markers or diagnostic measurement (e.g., screen, assay) in a subject suffering from or susceptible to cancer (e.g., liver cancer, breast cancer, leukemia, etc.) or a disorder or symptom thereof in which the subject has been administered a therapeutic amount of a composition as described herein. The level of marker determined in the method can be compared to known levels of marker in either healthy normal controls or in other afflicted patients to establish the subject's disease status. In preferred embodiments, a second level of marker in the subject is determined at a time point later than the determination of the first level, and the two levels are compared to monitor the course of disease or the efficacy of the therapy. In certain preferred embodiments, a pre-treatment level of marker in the subject is determined prior to beginning treatment according to the methods described herein; this pre-treatment level of marker can then be compared to the level of marker in the subject after the treatment commences, to determine the efficacy of the treatment.

[0076] Described herein are kits for treating cancer (e.g., liver cancer, leukemia, breast cancer, etc.) in a subject. A typical kit includes a pharmaceutical composition comprising an acylated flavonoid, such as an acylated phloridzin derivative as described herein or an acylated isoquercetrin derivative as described herein, in a therapeutically effective amount for decreasing proliferation and viability of cancer cells (e.g., inducing apoptosis of cancer cells) in the subject, packaging, and instructions for use. In a kit, the composition may further comprise a pharmaceutically acceptable carrier in unit dosage form. In some embodiments, the kit can comprise a sterile container which contains the disclosed pharmaceutical composition; such containers can be boxes, ampules, bottles, vials, tubes, bags, pouches,

blister-packs, or other suitable container forms known in the art. Such containers can be made of plastic, glass, laminated paper, metal foil, or other materials suitable for holding medicaments.

[0077] Although compositions, kits, and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable compositions, kits, and methods are described below. All publications, patent applications, and patents mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. The particular embodiments discussed below are illustrative only and not intended to be limiting.

EXAMPLES

[0078] The present invention is further illustrated by the following specific examples. The examples are provided for illustration only and should not be construed as limiting the scope of the invention in any way.

[0079] Phloridzin (phloretin 2'-*0*-glucoside) and isoquercetrin (quercetin 3-*0*-glucoside) are two flavonoid compounds found in fruits, especially in apples. In this discovery, it was found that the novel derivatives of the above two compounds have strong anticarcinogenic effects *in vitro* using human liver cancer cell lines. Twelve novel compounds (acylated derivatives of phloridzin and isoquercetrin with six different long chain saturated [stearic acid]-, mono[oleic acid]-, and poly-unsaturated [linoleicacid, alpha-linolenic acid, docosahexaenoic acid, eicosapentaenoic acid] fatty acids) were synthesized by regioselective enzymatic acylation. The antiproliferative effects of these twelve fatty esters of phloridzin and isoquercetrin in comparison with the parent compounds (phloridzin and isoquercetrin and their aglycones phloretin and quercetin, respectively), the free fatty acids, and a standard hepatocellular carcinoma drug, sorafenib (Nexavar®) in human hepatocellular carcinoma cells, HepG2 were investigated. In comparison with precursor compounds and free fatty acids, novel phloridzin and isoquercetrin fatty esters were up to 90-fold more effective against the carcinoma cells. The potency of these novel compounds is comparable to the potency of sorafenib (Nexavar®), or even better. To further explore the potential mechanisms of antiproliferation, the activities of these compounds on DNA topoisomerases IIα activity, cell cycle and apoptosis were determined. Phloridzin fatty esters inhibited DNA topoisomerases IIα activity that might induce GO/GI phase arrest, and apoptosis via activation of caspase-3in human hepatocellular carcinoma cells. These results suggest that phloridzin fatty esters have potential chemopreventive

effects mediated through inhibition of DNA topoisomerases IIα followed by cell cycle arrest and apoptosis.

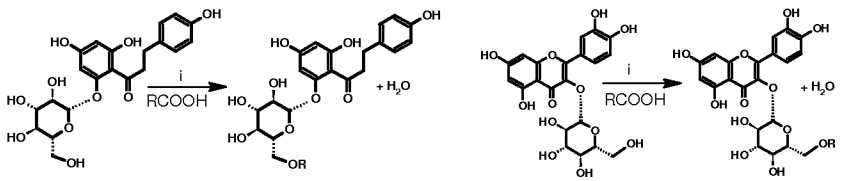
[0080] The compounds described herein, however, are synthesized using two chemical families of natural origin and are known to have no side effects because they are food components (flavonoid and fatty acids). These compounds are more effective when compared to a standard hepatocellular carcinoma drug such as sorafenib (Nexavar®).

[0081] The compounds were synthesized in gram quantities under laboratory conditions. A standard operating procedure was developed for synthesis. Structural conformations were examined using NMR. Methods of synthesizing these novel compounds are described in Ziaullah et al., Biorganic and Medicinal Chemistry. 21:684-692, 2013. The efficiency of the synthesis has been enhanced using microwave-assisted catalysis as described in Ziaullah et al., Tetrahedron Letters 54:1933-1937, 2013. The findings described herein are based on antiproliferative properties studies conducted using a cancer cell line (human hepatocarcinoma, HepG2) and toxicity studies completed using normal liver and lung cell lines.

Example 1 – Methods of Synthesizing Acylated Flavonoids, such as Phloridzin and Isoquercetrin Derivatives

[0082] Biocatalytic Synthesis, Structural Elucidation, Antioxidant Capacity and Tyrosinase Inhibition Activity of Long Chain Fatty Acid Acylated Derivatives of Phloridzin and Isoquercitrin:

[0083] Described herein is the regioselective enzymatic acylation of two series of acylated derivatives of phloridzin and isoquercitrin with six different long chain saturated, mono- and poly-unsaturated fatty acids. The biocatalytic synthesis was optimized to achieve 81 to 98% yields, using immobilized lipase B, from *Candida antarctica* (Novozym 435®), in acetone at 45 °C. The synthesized esters were analyzed by ^{1}H NMR, ^{13}C NMR spectroscopy and evaluated for their antioxidant capacity and tyrosinase inhibition, using *in vitro* assays.

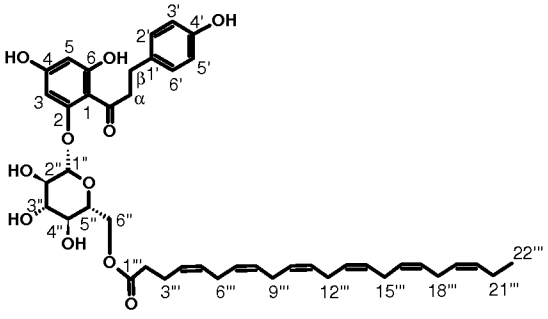


i) Acetone, 3 °A molecular sieves, Novozyme 435®, 45 °C, Stirring, 24 h; R = Oleic, Stearic, Linoleic, Linolenic, Eicosapentaenoic (EPA), Docosahexaenoic Acids (DHA) or their corresponding esters.

[0084] Procedure for the Synthesis of Saturated/Unsaturated Fatty Acid Esters of Phloridzin and Isoquercitrin (2-7 and 9-14): To flame dried 3 °A molecular sieves, in round bottom flask, was added phloridzin (0.500 g; 1.15 mmol), stearic acid (1.62 g, 5.72 mmol), Novozyme 435® (1.30 g). It was followed by the addition of dry acetone (5 ml) and the mixture was stirred and heated at 45 °C for 12-24 h. The progress of reaction was monitored by thin layer chromatography (TLC), followed by staining with anisaldehyde spray reagent and then heating at 110 °C. After completion of reaction, it was filtered, evaporated and passed through column chromatography (acetone: toluene; 35:75 to 50:50) to get the pure stearic acid ester (2) of phloridzin. All other reactions followed the same procedure and produced esters 3-7 and 9-14, with yields in the range of 81-98%. The pure compounds were then analyzed by IR, ^{1}HNMR and ^{13}C NMR spectroscopy. All other compounds were prepared by the same method.

[0085] Acylation of phloridzin and isoquercitrin with long chain fatty acids were carried out at 45-50 °C in extra dry acetone along with constant stirring. Briefly, defined quantities of flavonoids and fatty acids were dissolved in acetone. Enzymatic reactions were initiated by the addition of lipase (Novozyme 435®; with an activity of 10,000 propyl laurate units). As the water content (<200 ppm) is a very important parameter in enzymatic catalysis in organic media, it was assumed that the activity of the enzyme could be improved by reducing the hydration level of the different components in the reaction medium. In the present investigation, the highest efficiency was reached by drying the enzyme for 18-20 h over P_2O_5 before use; besides, using flame dried molecular sieves (3 °A) to remove any *in situ* generated water in the reaction mixture (Scheme 1 and 2 shown in Figures 1 and 2a-c).

[0086] Numbering of the esters of phloridzin (7) and isoquercetrin (14):



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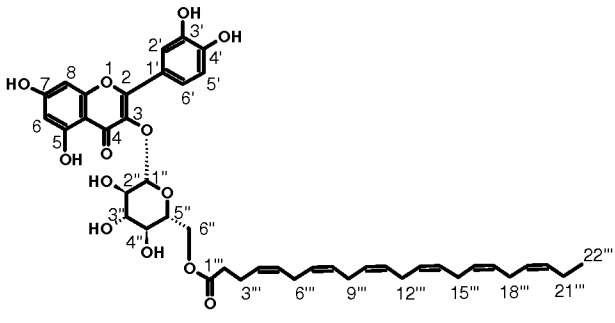

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[0087] Sonochemical Enzyme-Catalyzed Regioselective Acylation of Flavonoid Glycosides: Described herein is a highly efficient and alternative method of sonication-

assisted lipase catalyzed acylation of quercetin-3-O-glucoside and phloretin-2'-glucoside, using Novozyme 435®, with a range of fatty acids. In this study, sonication-assisted irradiation coupled with stirring was found to be more efficient and economical than conventional reaction conditions. Sonication-assisted acylation accelerated the reactions and reduced the time required by 4-5 fold.

[0088] Esterification of phloridzin and isoquercitrin with 20 different fatty acids of various chain lengths (C4-C18) were carried out at 40-45 °C in extra dry acetone along with ultrasonication or sonication coupled with stirring. The biocatalytic reactions were started by the addition of lipase (Novozyme 435®; purchased from Sigma-Aldrich (Mississauga, ON, Canada) into a solution of flavonoid glycosides and fatty acids in acetone, in the presence of flame dried molecular sieves (3 °A) (Figures 3 and 4a-h). In the conventional method, the reactions were carried out according to the procedure described above.

[0089] General procedure for the biocatalytic esterification reaction of phloridzin and isoquercitrin (2-11 and 13-21): To flame dried 3 °A molecular sieves in an amber colour vial, was added phloridzin (0.100 g; 0.23 mmol), butyric acid (0.06 ml, 0.69 mmol), Novozyme 435® (0.300 g). It was followed by the addition of dry acetone (5 ml). The reaction was carried out in an ultrasonic bath of 20 kHz/1000 Watts (model 750D, VWR, West Chester, PA, USA) and exposed for 3.5-5 h under ultrasound irradiation alone (for 15-20 min each with 10 min interval in between) and for 2-3.5 h (for 15-20 min each with 10 min interval in between) under ultrasound irradiation coupled with stirring at 40-45 °C (Figure 4a-h).

[0090] The progress of reaction was monitored by thin layer chromatography, followed by staining with anisaldehyde spray reagent and then heating at 110 °C. After completion of reaction, it was filtered, evaporated and chromatographed (acetone: toluene; 35:75 to 50:50) to get the pure butyric acid ester (2) of phloridzin. All other reactions followed the same procedure and produced esters 2-8 and 9-15, with yields in the range of 81-97%. The pure compounds were then analyzed by IR and NMR spectroscopy.

[0091] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl butyrate (2): Yield: 81%; light yellow liquid; R_f: 0.53 (Acetone:Toluene; 5:5: few drops of AcOH); IR (KBr) cm^{-1}: 3386, 2955, 2836, 2529, 2241, 2043, 1715, 1632, 1589, 1511, 1453, 1265, 1118, 1029, 916, 736, 653; ^1H NMR (MeOD, 300 MHz): δ 13.47 (s, 1H, ArOH), 10.56 (br s, 1H, ArOH), δ 7.06 (d, 2H, J = 8.5 Hz, H-2, H-6), 6.71 (d, 2H, J = 8.5 Hz, H-3, H-5), 6.15 (d, 1H, J = 2.1 Hz, H-3'), 6.00 (d, 1H, J = 2.1 Hz, 1H, H-5'), 5.02 (br d, 1H, J = 7.5 Hz, H-1"), 4.40 (dd, 1H, J = 11.8 Hz, J = 2.1

Hz, H-6a"), 4.27 (dd, 1H, *J* = 11.8 Hz, 6.2 Hz, H-6b"), 3.70-3.63 (br m, 1H, *J* = 7.7 Hz, H-4"), 3.51-3.31 (m, 6H, 2xH_{α} H-2", H-3", H-5", OH), 2.88 (br t, 2H, *J* = 7.6 Hz, 2xH_{β}), 2.29 (br t, 2H, *J* = 7.4 Hz, 2xH-2'"), 1.56 (m, 2H, 2xH-3'"), 0.88 (br t, 3H, *J* = 7.4 Hz, CH_3); ^{13}C NMR (MeOD, 75 MHz): δ 205.19 (CO), 173.92 (OCO), 166.14 (C-4'), 164.48 (C-6'), 160.77 (C-2'), 154.98 (C-4), 132.46 (C-1), 129.02 (C-2, C-6), 114.73 (C-3, C-5), 105.59 (C-1'), 100.60 (C-1"), 97.07 (C-3'), 94.48 (C-5'), 76.89 (C-3"), 74.24 (C-5"), 73.28 (C-2"), 70.11 (C-4"), 62.95 (C-6"), 45.57 (C_{α}), 35.46 (C-2'"), 29.53 (C_{β}), 18.03 (C-3'"), 12.56 (C-4'").

[0092] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl octanoate (3): Yield: 84%; light yellow liquid; R_f: 0.53 (Acetone:Toluene; 5:5: few drops of AcOH); IR (KBr) cm^{-1}: 3387, 2951, 2835, 2519, 2241, 2044, 1715, 1637, 1593, 1516, 1451, 1266, 1112, 1025, 913, 739, 647; ^{1}H NMR (DMSO-d^6, 300 MHz): δ 13.47 (s, 1H, ArOH), 10.56 (br s, 1H, ArOH), 7.03 (d, 2H, *J* = 8.3 Hz, H-2, H-6), 6.64 (d, 2H, *J* = 8.3 Hz, H-3, H-5), 6.10 (d, 1H, *J* = 1.6 Hz, H-3'), 5.95 (d, 1H, *J* = 1.6 Hz, 1H, H-5'), 5.36 (br.s, 1H, OH), 5.29 (br s, 1H, OH), 5.25 (br s, 1H, OH), 4.99 (d, 1H, *J* = 6.9 Hz, H-1"), 4.32 (br d, 1H, *J* = 10.8 Hz, H-6a"), 4.12 (dd, 1H, *J* = 10.8 Hz, 6.8 Hz, H-6b"), 3.62 (br t, 1H, *J* = 7.7 Hz, H-4"), 3.49-3.17 (m, 6H, 2xH_{α} H-2", H-3", H-5", OH), 2.78 (br t, 2H, *J* = 7.3 Hz, 2xH_{β}), 2.27 (br t, 2H, *J* = 7.4 Hz, 2xH-2"), 1.52-1.42 (m, 2H, 2xH-3"), 1.24-1.16 (m, 8H, 4xCH_2), 0.81 (br t, 3H, *J* = 6.3 Hz, CH_3); ^{13}C NMR (DMSO-d^6, 75 MHz): δ 205.12 (CO), 173.30 (OCO), 165.83 (C-4'), 164.92 (C-6'), 161.06 (C-2'), 155.75 (C-4), 131.94 (C-1), 129.60 (C-2, C-6), 115.41 (C-3, C-5), 105.60 (C-1'), 101.00 (C-1"), 97.36 (C-3'), 94.94 (C-5'), 76.85 (C-3"), 74.34 (C-5"), 73.55 (C-2"), 70.24 (C-4"), 63.49 (C-6"), 45.47 (C_{α}), 33.86 (C-2'"), 31.58 (C_{β}), 29.52, 28.85, (C-4'", C-5'", C-6'"), 24.85 (C-3'"), 22.50 (C-7'"), 14.38 (C-8'").

[0093] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl dodecanoate (4): Yield: 97%; light yellow solid; R_f: 0.53 (Acetone:Toluene; 5:5: few drops of AcOH); IR (KBr) cm^{-1}: 3382, 2943, 2837, 2525, 2246, 2048, 1714, 1635, 1596, 1517, 1458, 1264, 1119, 1027, 736, 658; ^{1}H NMR (DMSO-d^6, 300 MHz): δ 13.49 (s, 1H, ArOH), 10.52 (br s, 1H, ArOH), 7.03 (d, 2H, *J* = 8.4 Hz, H-2, H-6), 6.65 (d, 2H, *J* = 8.4 Hz, H-3, H-5), 6.10 (d, 1H, *J* = 2.1 Hz, H-3'), 5.95 (d, 1H, *J* = 2.1 Hz, 1H, H-5'), 5.36 (br.s, 1H, OH), 5.29 (br s, 1H, OH), 5.26 (br s, 1H, OH), 4.98 (d, 1H, *J* = 7.2 Hz, H-1"), 4.32 (br d, 1H, *J* = 10.2 Hz, H-6a"), 4.12 (dd, 1H, *J* = 10.2 Hz, 6.8 Hz, H-6b"), 3.61 (br t, 1H, *J* = 7.65 Hz, H-4"), 3.48-3.19 (m, 6H, 2xH_{α} H-2", H-3", H-5", OH), 2.78 (br t, 2H, *J* = 7.3 Hz, 2xH_{β}), 2.26 (br t, 2H, *J* = 7.3 Hz, 2xH-2"), 1.52-1.41 (m, 2H, 2xH-

3''), 1.25-1.13 (m, 16H, 8xCH_2), 0.84 (br t, 3H, *J* = 6.4 Hz, CH_3); ^{13}C NMR (DMSO-d^6, 75 MHz): δ 205.10 (CO), 173.29 (OCO), 165.89 (C-4'), 164.94 (C-6'), 161.09 (C-2'), 155.75 (C-4), 131.93 (C-1), 129.59 (C-2, C-6), 115.41 (C-3, C-5), 105.57 (C-1'), 101.04 (C-1''), 97.38 (C-3'), 94.95 (C-5'), 76.86 (C-3''), 74.34 (C-5''), 73.55 (C-2''), 70.25 (C-4''), 63.52 (C-6''), 45.47 (C_α), 33.86 (C-2'''), 31.79 (C_β), 29.50, 29.39, 29.38, 29.21, 29,14, 28.90 (C-4''', C-5''', C-6''', C-7''', C-8''', C-9''', C-10'''), 24.85 (C-3'''), 22.59 (C-11'''), 14.42 (C-12''').

[0094] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl palmitate (5): Yield: 97%; light yellow solid; R_f: 0.53 (Acetone:Toluene; 5:5: few drops of AcOH); IR (KBr) cm^{-1}: 3388, 2944, 2836, 2527, 2244, 2048, 1716, 1634, 1593, 1513, 1454, 1266, 1117, 1024, 918, 738, 659; ^{1}H NMR (DMSO-d^6, 300 MHz): δ 13.49 (s, 1H, ArOH), 10.53 (br s, 1H, ArOH), 7.04 (d, 2H, *J* = 8.3 Hz, H-2, H-6), 6.65 (d, 2H, *J* = 8.3 Hz, H-3, H-5), 6.11 (br s, 1H, H-3'), 5.95 (br s, 1H, H-5'), 5.38 (br.s, 1H, OH), 5.37 (br s, 1H, OH), 5.26 (br s, 1H, OH), 4.99 (d, 1H, *J* = 6.81 Hz, H-1''), 4.34 (br d, 1H, *J* = 11.5 Hz, H-6a''), 4.12 (dd, 1H, *J* = 11.5 Hz, 6.7 Hz, H-6b''), 3.65 (br t, 1H, *J* = 8.3 Hz, H-4''), 3.64-3.18 (m, 6H, 2xH_α, H-2'', H-3'', H-5'', OH), 2.79 (br t, 2H, *J* = 7.2 Hz, 2xH_β), 2.27 (br t, 2H, *J* = 7.3 Hz, 2xH-2''), 1.47 (m, 2H, 2xH-3''), 1.27-1.16 (m, 24H, 12(CH_2)), 0.85 (br t, 3H, *J* = 6.8 Hz, CH_3); ^{13}C NMR (DMSO-d^6, 75 MHz): δ 205.09 (CO), 173.29 (OCO), 165.87 (C-4'), 164.92 (C-6'), 161.09 (C-2'), 155.75 (C-4), 131.92 (C-1), 129.58 (C-2, C-6), 115.40 (C-3, C-5), 105.57 (C-1'), 101.03 (C-1''), 97.36 (C-3'), 94.94 (C-5'), 76.85 (C-3''), 74.33 (C-5''), 73.55 (C-2''), 70.25 (C-4''), 63.52 (C-6''), 45.46 (C_α), 33.86 (C-2'''), 31.79 (C_β), 29.54, 29.48, 29.38, 29.21, 29,14, 28.90 (C-4''', C-5''', C-6''', C-7''', C-8''', C-9''', C-10''', C-11''', C-12''', C-13''', C-14'''), 24.85 (C-3'''), 22.59 (C-15'''), 14.42 (C-16''').

[0095] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl stearate (6): Yield: 96%; light yellow solid; R_f: 0.53 (Acetone:Toluene; 4:6: few drops of AcOH); IR (KBr) cm^{-1}: 3383, 2944, 2832, 2523, 2247, 2043, 1719, 1631, 1591, 1512, 1450, 1269, 1113, 1028, 913, 737, 652; ^{1}H NMR (DMSO-d^6, 300 MHz): δ 13.55 (s, 1H, ArOH), 9.18 (br s, 1H, ArOH), 7.06 (d, 2H, *J* = 8.4 Hz, H-2, H-6), 6.67 (d, 2H, *J* = 8.4 Hz, H-3, H-5), 6.13 (d, 1H, 1.8 Hz, H-3'), 5.98 (d, 1H, *J* = 1.8 Hz, H-5'), 5.46 (br.s, 1H, OH), 5.37 (br s, 2H, 2OH), 5.04 (d, 1H, H-1''), 4.35 (br d, 1H, *J* = 11.6 Hz, H-6a''), 4.14 (dd, 1H, *J* = 11.6 Hz, 6.9 Hz, H-6b''), 3.65 (br t, *J* = 7.2 Hz, H-4''), 3.46-3.20 (m, 6H, 2xH_α, H-2'', H-3'', H-5'', OH), 2.81 (br t, 2H, *J* = 7.5 Hz, 2xH_β), 2.30 (t, 2H, *J* = 7.2 Hz, 2xH-2''), 1.49 (m, 2H, 2xH-3''), 1.25-1.19 (m, 28H, 2(CH_2)), 0.87 (br t, 3H, *J* = 6.9 Hz, CH_3); ^{13}C NMR (DMSO-d^6, 75 MHz): δ 205.34 (CO), 173.44 (OCO), 166.05 (C-

4'), 165.13 (C-6'), 161.30 (C-2'), 156.03 (C-4), 132.25 (C-1), 129.72 (C-2, C-6), 115.74 (C-3, C-5), 106.18 (C-1'), 101.55 (C-1"), 97.79 (C-3'), 95.44 (C-5'), 77.30 (C-3"), 74.77 (C-5"), 73.94 (C-2"), 70.68 (C-4"), 63.81 (C-6"), 45.61 (C_{α}), 34.17 (C-2'"), 31.96 (C_{β}), 29.84, 29.34, 29.13 (C-4'", C-5'", C-6'", C-7'", C-8'", C-9'", C-10'", C-11'", C-12'", C-13'", C-14'", C-15'", C-16'"), 25.08 (C-3'"), 22.73 (C-17'"), 14.53 (C-18'").

[0096] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl (*Z*)-9-octadecenoate (7): Yield: 93%; light brownish yellow spongy solid; R_f: 0.56 (Acetone:Toluene; 4:6: few drops of AcOH); IR (KBr) cm^{-1}: 3383, 2927, 2855, 2253, 1714, 1627, 1599, 1514, 1455, 1264, 1203, 1078, 908, 734, 650; ^1H NMR (DMSO-d^6, 300 MHz): δ 13.61 (s, 1H, OH), 10.66 (br s, 1H, OH), 9.25 (br s, 1H, OH), 7.09 (d, 2H, *J* = 8.7 Hz, H-2, H-6), 6.72 (d, 2H, *J* = 8.7 Hz, H-3, H-5), 6.17 (d, 1H, 1.8 Hz, H-3'), 6.01 (s, 1H, H-5'), 5.48-5.29 (m, 4H, H-9'", H-10'"), 5.04 (d, 1H, *J* = 6.9 Hz, H-1"), 4.38 (br d, 1H, *J* = 11.4 Hz, H-6a"), 4.18 (dd, 1H, *J* = 11.4 Hz, 6.6 Hz, H-6b"), 3.67 (br t, 1H, *J* = 7.8 Hz, H-4"), 3.55-3.23 (m, 6H, 2xH_{α}, H-2", H-3", H-5", OH), 2.84 (br t, 2H, *J* = 6.9 Hz, 2xH_{β}), 2.32 (br t, 2H, *J* = 6.9 Hz, 2xH-2"), 2.01-1.98 (m, 4H, 2xH-8", 2xH-11"), 1.54-1.50 (m, 2H, 2xH-3"), 1.27-1.20 (m, 20H, 10(CH_2)), 0.87 (br t, 3H, *J* = 6.9 Hz, CH_3); ^{13}C NMR (DMSO-d^6, 75 MHz): δ 205.37 (CO), 173.41 (OCO), 166.13 (C-4'), 165.15 (C-6'), 161.34 (C-2'), 156.06 (C-4), 132.30 (C-1), 130.30 (C-9", C-10"), 129.73 (C-2, C-6), 115.78 (C-3, C-5), 106.25 (C-1'), 101.63 (C-1"), 97.86 (C-3'), 95.50 (C-5'), 77.37 (C-3"), 74.83 (C-5"), 73.98 (C-2"), 70.74 (C-4"), 63.85 (C-6"), 45.63 (C_{α}), 34.19 (C-2"), 31.97 (C_{β}), 29.83 (C-7'", C-12'"), 29.54 (C-15'"), 29.33 (C-4'", C-5'", C-14'"), 29.20 (C-6'", C-13'", C-16'"), 27.33 (C-8'", C-11'"), 25.10 (C-3'"), 22.74 (C-17'"), 14.50 (C-18'").

[0097] (6-{3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenoxy}-3,4,5-trihydroxytetrahydro-2*H*-pyran-2-yl)methyl (9*Z*,12*Z*)-9,12-octadecadienoate (8): Yield: 89%; light brownish yellow spongy solid; R_f: 0.57 (Acetone:Toluene; 4:6: few drops of AcOH); IR (KBr) cm^{-1}: 3396, 2928, 2856, 2253, 1711, 1628, 1599, 1515, 1454, 1378, 1263, 1204, 1173, 1080, 907, 733, 650; ^1H NMR (DMSO-d^6, 300 MHz): δ 10.67 (br s, 1H, OH), 9.23 (br s, 1H, OH), 7.08 (d, 2H, *J* = 8.1 Hz, H-2, H-6), 6.70 (d, 2H, *J* = 8.1 Hz, H-3, H-5), 6.16 (d, 1H, *J* = 1.2 Hz, H-3'), 6.01(d, 1H, *J* = 1.2 Hz, H-5'), 5.47 (br s, 1H, OH), 5.40-5.27 (m, 5H, H-9'", H-10'", H-12'", H-13'", OH), 5.04 (d, 1H, *J* = 6.9 Hz, H-1"), 4.37 (br d, 1H, *J* = 11.4 Hz, H-6a"), 4.18 (dd, 1H, *J* = 11.4 Hz, 6.6 Hz, H-6b"), 3.66 (br t, 1H, *J* = 7.8 Hz, H-4"), 3.57 (br s, 2xOH), 3.48-3.22 (m, 5H, 2xH_{α}, H-2", H-3", H-5"), 2.83 (br t, 2H, *J* = 6.9 Hz, 2xH_{β}), 2.74 (br t, 2H, *J* = 5.4 Hz, 2xH-11'"), 2.30 (br t, 2H, *J* = 7.5 Hz, 2xH-2'"), 2.15-1.99 (m, 4H, 2xH-

8'''', 2xH-14''''), 1.53-1.48 (m, 2H, 2xH-3''''), 1.37-1.18 (m, 14H, 7(CH_2)), 0.86 (br t, 3H, *J* = 6.6 Hz, CH_3); ^{13}C NMR (DMSO-d^6, 75 MHz): δ 205.42 (CO), 173.45 (OCO), 166.20 (C-4'), 165.16 (C-6'), 161.38 (C-2'), 156.09 (C-4), 132.36 (C-1), 130.50 (C-9'''', C-13''''), 129.75 (C-2, C-6), 128.48 (C-10'''', C-12''''), 115.84 (C-3, C-5), 106.33 (C-1'), 101.68 (C-1''), 97.94 (C-3'), 95.56 (C-5'), 77.43 (C-3''), 74.89 (C-5''), 74.03 (C-2''), 70.80 (C-4''), 63.88 (C-6''), 45.66 (C_α), 34.23 (C-2''''), 31.64 (C_β), 29.95 (C-7'''',), 29.73 (C-15''''), 29.44 (C-4''''), 29.23 (C-5'''', C-6'''', C-11''''), 27.39 (C-8'''', C-14''''), 25.99 (C-16''''), 25.13 (C-3''''), 22.65 (C-17''''), 14.47 (C-18'''').

[0098] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4Hchromen-3-yl]oxy}-4,5-dihydroxytet-rahydro-2*H*-pyran-2-yl)methyl butyrate (9): Yield: 83 %; yellowish solid; R_f: 0.42 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3453, 3276, 3072, 3003, 2927, 2859, 2543, 2247, 2131, 1989, 1834, 1763, 1659, 1450, 1363, 1307, 1203, 1169, 1053, 823, 737, 627; ^{1}H NMR (DMSO-d$_6$, 300 MHz): δ 12.65 (br.s, 1H, ArOH), 7.54-7.50 (m, 2H, H-2', H-6'), 6.84 (br d, 1H, *J* = 9.0 Hz, H-5'), 6.40 (d, 1H, *J* = 1.95 Hz, H-8), 6.22 (d, 1H, *J* = 1.95 Hz, H-6), 5.45 (d, 1H, *J* = 7.35 Hz, H-1''), 5.29-4.99 (m, 3H, 3OH), 4.15 (br.d, 1H, *J* = 10.0 Hz, H-6a''), 3.97-3.91 (dd, 1H, *J* = 10.0 Hz, *J* = 6.87 Hz, H-6b''), 3.55-3.09 (m, 6H, H-2'', H-3'', H-4'', H-5'', 2OH), 1.95 (dd, 2H, *J* = 7.53 Hz, *J* = 3.48 Hz, 2xH-2''''), 1.27-1.17 (m, 3H, 2xH-3'''', OH), 0.64 (br. t, 3H, *J* = 7.33 Hz, CH_3); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ 177.80 (CO), 172.75 (OCO), 164.72 (C-7), 161.71 (C-5), 156.84, 156.78 (C-2, C-9), 148.98 (C-4'), 145.31 (C-3'), 133.39 (C-3), 121.94 (C-1'), 121.49 (C-6'), 116.53 (C-5'), 115.58 (C-2'), 104.27 (C-10), 101.01 (C-1''), 99.14 (C-6), 93.95 (C-8), 76.71 (C-3''), 74.57 (C-5''), 74.36 (C-2''), 70.51 (C-4''), 63.34 (C-6''), 35.66 (C-2''''), 18.25 (C-3''''), 13.62 (C-4'''').

[0099] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4Hchromen-3-yl]oxy}-4,5-dihydroxytet-rahydro-2*H*-pyran-2-yl)methyl octanoate (10): Yield: 81 %; yellowish solid; R_f: 0.42 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3458, 3277, 3078, 3009, 2925, 2856, 2549, 2246, 2135, 1987, 1833, 1768, 1653, 1459, 1368, 1309, 1206, 1165, 1058, 828, 736, 627; ^{1}H NMR (DMSO-d$_6$, 300 MHz): δ 12.65 (br.s, 1H, ArOH), 7.54-7.50 (m, 2H, H-2', H-6'), 6.83 (br d, 1H, *J* = 9.0 Hz, H-5'), 6.39 (d, 1H, *J* = 1.94 Hz, H-8), 6.19 (d, 1H, *J* = 1.94 Hz, H-6), 5.45 (br. dd, 1H, *J* = 7.35 Hz, H-1''), 5.20 (br. s, 2H, 2OH), 4.17 (br.d, 1H, *J* = 10.0 Hz, H-6a''), 3.99-3.92 (dd, 1H, *J* = 10.0 Hz, *J* = 7.05 Hz, H-6b''), 3.35-3.08 (m, 6H, H-2'', H-3'', H-4'', H-5'', 2OH), 1.99-1.90 (m, 2H, 2xH-2''''), 1.27-1.01 (m, 12H, 5xCH_2, 2OH), 0.82 (t, 3H, *J* = 6.84 Hz, CH_3); ^{13}C NMR (DMSO-d$_6$, 75 MHz): δ 177.80 (CO), 172.88 (OCO), 164.62 (C-7), 161.70 (C-5), 156.84, 156.77 (C-2, C-9), 148.94 (C-4'), 145.26

(C-3'), 133.40 (C-3), 123.03 (C-1'), 122.95 (C-6'), 116.59 (C-5'), 114.49 (C-2'), 104.30 (C-10), 102.18 (C-1"), 99.91 (C-6), 94.96 (C-8), 77.68 (C-3"), 75.33 (C-5"), 73.39 (C-2"), 71.50 (C-4"), 63.31 (C-6"), 33.65 (C-2'"), 31.96 (C-14"), 30.40, 28.74, 26.97 (C-4'", C-5'", C-6'"), 24.67 (C-3'"), 22.51 (C-7'"), 13.57 (C-8'").

[00100] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-4,5-dihy-droxy-tetrahydro-2*H*-pyran-2-yl)methyl dodecanoate (11): Yield: 92 %; yellowish solid; R_f: 0.42 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3453, 3276, 3072, 3003, 2927, 2859, 2543, 2247, 2131, 1989, 1834, 1763, 1659, 1450, 1363, 1307, 1203, 1169, 1053, 823, 737, 627; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.62 (s, 1H, ArOH), 10.88 (br s, 1H, ArOH), 7.53-7.50 (m, 2H, H-2', H-6'), 6.82 (br d, 1H, *J* = 8.79 Hz, H-5'), 6.39 (d, 1H, *J* = 2.04 Hz, H-8), 6.19 (d, 1H, *J* = 2.04 Hz, H-6), 5.46-5.41 (m, 2H, H-1", OH), 5.22 (br. s, 2H, 2OH), 4.17 (br.d, 1H, *J* = 10.0 Hz, H-6a"), 3.99-3.93 (dd, 1H, *J* = 10.0 Hz, *J* = 7.01 Hz, H-6b"), 3.35-3.08 (m, 5H, H-2", H-3", H-4", H-5", OH), 1.99-1.93 (m, 2H, 2xH-2"), 1.27-0.95 (m, 19H, 9xCH_2, OH), 0.83 (t, 3H, *J* = 6.45 Hz, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 177.82 (CO), 172.90 (OCO), 164.64 (C-7), 161.74 (C-5), 156.84, 156.76 (C-2, C-9), 148.95 (C-4'), 145.28 (C-3'), 133.44 (C-3), 121.89 (C-1'), 121.51 (C-6'), 116.57 (C-5'), 115.54 (C-2'), 104.29 (C-10), 101.11 (C-1"), 99.11 (C-6), 93.89 (C-8), 76.74 (C-3"), 74.60 (C-5"), 74.37 (C-2"), 70.54 (C-4"), 63.36 (C-6"), 33.66 (C-2"), 31.80 (C-10"), 29.50, 29.35, 29.23, 29.01, 28.78 (C-4", C-5", C-6", C-7", C-8", C-9"), 24.68 (C-3"), 22.59 (C-11"), 14.42 (C-12").

[00101] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4*H*-chromen-3-yl]oxy}-4,5-dihydroxytet-rahydro-2*H*-pyran-2-yl)methyl palmitate (12): Yield: 93 %; yellowish solid; R_f: 0.42 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3455, 3273, 3077, 3007, 2925, 2854, 2546, 2244, 2136, 1981, 1837, 1765, 1657, 1457, 1364, 1306, 1203, 1165, 1053, 828, 737, 625; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 12.62 (s, 1H, ArOH), 10.86 (br s, 1H, ArOH), 7.36-7.30 (m, 2H, H-2', H-6'), 6.83 (br d, 1H, *J* = 8.64 Hz, H-5'), 6.39 (d, 1H, *J* = 2.01 Hz, H-8), 6.19 (d, 1H, *J* = 2.01 Hz, H-6), 5.46-5.40 (m, 2H, H-1", OH), 5.22 (br. s, 2H, 2OH), 4.17 (br.d, 1H, *J* = 10.1 Hz, H-6a"), 3.99-3.93 (dd, 1H, *J* = 10.1 Hz, *J* = 7.02 Hz, H-6b"), 3.33-3.06 (m, 5H, H-2", H-3", H-4", H-5", OH), 1.99-1.93 (m, 2H, 2xH-2'"), 1.25-0.94 (m, 19H, 13xCH_2, OH), 0.83 (t, 3H, *J* = 6.36 Hz, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 177.83 (CO), 172.89 (OCO), 164.65 (C-7), 161.75 (C-5), 156.84, 156.77 (C-2, C-9), 148.95 (C-4'), 145.29 (C-3'), 133.45 (C-3), 121.88 (C-1'), 121.51 (C-6'), 116.58 (C-5'), 115.54 (C-2'), 104.29 (C-10), 101.13 (C-1"), 99.10 (C-6), 93.88 (C-8), 76.75 (C-3"), 74.61 (C-5"), 74.38 (C-2"), 70.53 (C-4"), 63.37 (C-6"), 33.66 (C-2'"), 31.79 (C-10'"), 29.57, 29.49, 29.36, 29.23,

29.03, 28.80 (C-4''', C-5''', C-6''', C-7''', C-8''', C-9''', C-10''', C-11''', C-12''', C-13'''), 24.69 (C-3'''), 22.59 (C-15'''), 14.40 (C-16''').

[00102] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4Hchromen-3-yl]oxy}-4,5-dihydroxytet-rahydro-2*H*-pyran-2-yl)methyl stearate (13): Yield: 96 %; greenish solid; R_f: 0.42 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3449, 3271, 3070, 3007, 2926, 2854, 2559, 2250, 2124, 1997, 1830, 1767, 1655, 1454, 1366, 1308, 1203, 1172, 1057, 931, 822, 759, 731, 623; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 10.63-8.98 (br.s, 2H, ArOH), 7.56 (br.s, 2H, H-2', H-6'), 6.86 (br s, 1H, H-5'), 6.41 (s, 1H, H-8), 6.22 (s, 1H, H-6), 5.47-5.26 (m, 3H, H-1", 2OH), 4.20 (br.d, 1H, *J* = 10.5 Hz, H-6a"), 4.00 (br s, 1H, H-6b"), 3.55-3.20 (m, 5H, H-2", H-3", H-4", H-5", OH), 2.00 (br s, 2H, H-2", OH), 1.24-1.09 (m, 31H, 15xCH_2, OH), 0.83 (br. s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 178.14 (CO), 173.09 (OCO), 165.09 (C-7), 162.09 (C-5), 157.16 (C-2, C-9), 149.25 (C-4'), 145.59 (C-3'), 133.97 (C-3), 122.19 (C-1'), 121.95 (C-6'), 116.98 (C-5'), 115.90 (C-2'), 104.66 (C-10), 101.73 (C-1"), 99.47 (C-6), 94.21 (C-8), 77.26 (C-3"), 75.01 (C-5"), 74.79 (C-2"), 71.01 (C-4"), 63.78 (C-6"), 34.00 (C-2'''), 31.99 (C-16'''), 29.73 29.37, 29.23, 29.06 (C-4''', C-5''', C-6''', C-7''', C-8''', C-9''', C-10''', C-11''', C-12''', C-13''', C-14''', C-15'''), 24.95 (C-3'''), 22.74 (C-17'''), 14.51 (C-18''').

[00103] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4Hchromen-3-yl]oxy}-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-yl)methyl (*Z*)-9-octadecenoate (14): Yield: 93 %; greenish yellow spongy solid; R_f: 0.58 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3373, 3065, 2947, 2836, 2497, 2251, 2121, 2042, 1898, 1763, 1640, 1596, 1560, 1459, 1448, 1229, 1165, 1026, 942, 739; ^{1}H NMR (DMSO-d_6, 300 MHz): δ 11.45-9.01 (br.s, 2H, ArOH), 7.53 (d, 2H, *J* = 9.0 Hz, H-2', H-6'), 6.85 (d, 1H, *J* = 9.0 Hz, H-5'), 6.41 (d, 1H, *J* = 1.8 Hz, H-8), 6.22 (d, 1H, *J* = 1.8 Hz, H-6), 5.48-5.25 (m, 5H, H-9", H-10", H-1", 2OH), 4.20 (br.d, 1H, *J* = 11.7 Hz, H-6a"), 3.99 (dd, 1H, *J* = 11.7 Hz, *J* = 7.2 Hz, H-6b"), 3.45-3.13 (m, 6H, H-2", H-3", H-4", H-5", 2xOH), 2.06-1.97 (m, 6H, 2xH-2''', 2xH-8''', 2xH-11'''), 1.27-1.09 (m, 23H, 11xCH_2), 0.85 (br t, 3H, *J* = 6.6 Hz, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 178.12 (CO), 173.05 (OCO), 164.99 (C-7), 162.06 (C-5), 157.12 (C-2, C-9), 149.21 (C-4'), 145.56 (C-3'), 133.92 (C-3), 130.29 (C-9", C-10"), 122.16 (C-1'), 121.93 (C-6'), 116.95 (C-5'), 115.88 (C-2'), 104.65 (C-10), 101.65 (C-1"), 99.42 (C-6), 94.18 (C-8), 77.23 (C-3"), 74.98 (C-5"), 74.76 (C-2"), 70.98 (C-4"), 63.75 (C-6"), 33.96 (C-2'''), 31.92 (C-16'''), 29.79 (2C), 29.49, 29.29 (3C), 29.13, 29.03 (C-4''', C-5''', C-6''', C-7''', C-12''', C-13''', C-14''', C-15'''), 27.31 (C-8''', C-11'''), 27.90 (C-3'''), 22.69 (C-17'''), 14.49 (C-18''').

[00104] (6-{[2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4Hchromen-3-yl]oxy}-3,4,5-trihydroxy-tetrahydro-2*H*-pyran-2-yl)methyl (9*Z*,12*Z*)-9,12-octadecadienoate (15): Yield: 91%; greenish yellow spongy solid; R_f: 0.59 (Acetone:Toluene; 1:1: few drops of AcOH); IR (KBr) cm^{-1}: 3346, 2946, 2835, 2492, 2181, 2043, 1897, 1762, 1643, 1591, 1466, 1447, 1230, 1027, 941, 737, 700; ^1H NMR (DMSO-d_6, 300 MHz): δ 11.46-8.97 (br.s, 2H, ArOH), 7.58 (br s, 2H, H-2', H-6'), 6.88 (br d, 1H, *J* = 6.9 Hz, H-5'), 6.43 (s, 1H, H-8), 6.24 (s, 1H, H-6), 5.50-5.37 (m, 7H, H-9''', H-10''', H-12''', H-13''', H-1", 2OH), 4.22 (br.d, 1H, *J* = 10.5 Hz, H-6a"), 4.02 (dd, 1H, *J* = 10.5 Hz, *J* = 7.2 Hz, H-6b"), 3.55-3.20 (m, 6H, H-2", H-3", H-4", H-5", 2xOH), 2.78 (br s, 2H, 2xH-11'''), 2.16-2.04 (m, 6H, 2xH-2''', 2xH-8''', 2xH-14'''), 1.29-1.11 (m, 17H, 8xCH_2, OH), 0.88 (br s, 3H, CH_3); ^{13}C NMR (DMSO-d_6, 75 MHz): δ 178.12 (CO), 173.05 (OCO), 165.01 (C-7), 162.06 (C-5), 157.13 (C-2, C-9), 149.22 (C-4'), 145.58 (C-3'), 133.91 (C-3), 130.49 (C-9''', C-10'''), 128.45 (C-12''', C-13'''),122.17 (C-1'), 121.93 (C-6'), 116.96 (C-5'), 115.89 (C-2'), 104.65 (C-10), 101.64 (C-1"), 99.43 (C-6), 94.19 (C-8), 77.22 (C-3"), 74.99 (C-5"), 74.77 (C-2"), 70.98 (C-4"), 63.75 (C-6"), 33.96 (C-2'''), 31.57 (C-16'''), 29.66 (C-15'''), 29.38 (C-7'''), 29.16 (C-5'''), 29.03 C-4''', C-6'''), 27.34 (C-8''', C-14'''), 25.94 (C-11'''), 24.91 (C-3'''), 22.59 (C-17'''), 14.48 (C-18''').

Example 2 – Anti-proliferative Activity of Phloridzin Fatty Acid Esters In Three Different Human Cancer Cell Lines

[00105] The study of anti-proliferative effects of stearic-, oleic-, linoleic, α-linolenic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) esters of phloridzin was carried out using human hepatocellular carcinoma (HepG2), breast carcinoma (MDA-MB-231) and acute monocytic leukemia (THP-1) cell lines with concentrations ranging from 0.1 μM to 100 μM and incubation periods of 3, 6, 12, 18, and 24 hours (h).

[00106] Cell lines and Culture Conditions: MDA-MB-231 is an adherent, epithelial like cell line derived from the metastatic site (pleural effusion) of human breast (mammary gland) tissue of 51 year old female Caucasian (MDA-MB-231: ATCC catalogue number HTB 26, Manassas, VA, USA). It is an estrogen independent cell line which serves as a useful *in vitro* model for human breast adenocarcinoma studies and express epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α). The base medium for MDA-MB-231 cell line was Dulbecco's Modified Eagle's Medium (Cat no. D5796: Sigma-Aldrich Canada Ltd., Oakville, ON) and was supplemented with 10% heat-inactivated (56 °C for 30 min) fetal bovine serum (Cat no. 12483: Life Technologies Inc. Burlington, ON), 5 mM N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid (7.4 pH: Cat no. 15630-080: Life Technologies Inc. Burlington, ON), 2 mM L-glutamine (Cat no. 25030-081: Life Technologies Inc. Burlington, ON), 100 U/mL penicillin, and 100 µg/mL streptomycin (Cat no. 15140-122: Life Technologies Inc. Burlington, ON). Cells were maintained at 37 °C in a humidified incubator supplied with 5 % CO_2. Tissue culture flasks (T-75) with 9-10 mL of media were used for regular culturing. Sub-culturing was performed in 1:1 ratio every 4 days at 90% confluency. Medium was changed 2 days after splitting. Unless otherwise stated, 96-well and 6-well plates were seeded with 5×10^3 and 5×10^4 cells/well, respectively. All the centrifugations were performed at 500 gravity (g) for 5 min, except for acid phosphatase assay. Tryphan blue (0.1% v/v) in 1 × PBS was used to test cell viability. Cells were stained with tryphan blue and dead cells turned blue when viewed under the microscope. Live cells extruded the dye as their cell membrane integrity was not damaged. Cells used for all the *in vitro* and *in vivo* assays were 95-100% viable on tryphan blue staining.

[00107] Cell Proliferation Assay:

[00108] The reduction in metabolic activity induced by phloretin (PT), doxorubicin (DOX) and docetaxel (DOC) was evaluated using MTS assay. The assay was performed using commercially available MTS assay kit (Promega, Madison, WI, USA). MDA-MB-231 cells (100 µL) were seeded in 96-well flat-bottom cell culture plates at a density of 5×10^3 cells per well and were incubated overnight to facilitate cell adhesion. Adhered cells were treated with PT, phloridzin (PZ), docosahexaenoic acid (DHA), phloridzin docosahexaenoate (PZ-DHA), DOX and DOC (10, 50, 100, 150 and 200 µmol/L) and incubated for 3, 6, 12, 18 and 24 hr at 37 °C. Vehicle controls were used for all the treatment concentrations separately and untreated control (cells treated with cDMEM without any treatment) was also used. Treatment blank (cDMEM with treatment) and vehicle control (cDMEM with vehicle) were used to compensate the background absorbance. After the incubation of cells with treatments, 20 µL of combined MTS/PMS reagent (final concentrations of 333 µg/mL MTS and 25 µM PMS (Sigma Aldrich, Oakville, ON)) was added to all the experimental wells in the plate and incubated for 3 hours at 37 °C. To determine the percentage inhibition of metabolic activity, the absorbance of the colored product was measured at 490 nm using a micro-plate reader (BMG-LABTECH) (Ortenberg, Germany). Percentage relative metabolic activity was calculated as shown in the following expression, where A_T: absorbance of test compound treated cells; A_{TB}: absorbance of treatment blanks; A_C: absorbance of vehicle treated cells; A_{CB}: absorbance of vehicle blanks.

$$\% \textbf{ Relative metabolic activity } = \frac{(A_T - A_{TB})}{(A_C - A_{CB})} \times 100$$

Data expressed above as mean ± SEM (n=12) are averaged results of three independent experiments conducted in quadruplicates. Differences among means were compared using Tukey's test. Values with different letters (A-D: Time-dependency, a-e: Dose-dependency) are significantly different at $\alpha = 0.05$ ($P<0.05$). As measured by the MTS assay, PT, DHA and PZ-DHA but not PZ showed a time- and dose-dependent inhibition in the metabolic activity (Figure 5). PT-induced metabolic activity inhibition was both time- and dose-dependent. Dose dependency was observed for PT through 3-24 hr. DOX and DOC were used as positive controls and both the positive controls showed tome- and dose-dependency. Selectivity of antiproliferative properties of PZ-DHA on inhibiting growth of MDA-MB-231 triple negative breast cancer cells with minimum inhibitory effects on normal healthy epithelial cells was measured in MTS assay in terms of reduction of percentage metabolic activity. One hundred and 200 µM concentrations killed both MDA-MB-231cells and HMEpiCs equally but interestingly, PZ-DHA-induced cytotoxicity was selective toward MDA-MB-231 breast cancer cells at 50 µM concentration causing almost no toxicity to HMEpiCs (Figure 6).

[00109] Mice Model: Six to eight weeks old NOD-SCID mice were acclimatized for 1 week. MDA-MB-231 cells for injection were mouse-pathogen tested and thawed from liquid nitrogen freshly. They were grown in T-75 and only cells in 3-4 flasks were harvested at a time. To maintain cells at its maximum viability, cells were maintained at ice cold temperature throughout the procedure preparing them for injection. Injections were prepared in 1 mL syringes (BD PrecisionGlideTM, Franklin Lakes, NJ, USA) and not more than 4 injections were prepared at a time. Cells (5×10^5) were subcutaneously injected into right hind flank in 100 µL plain DMEM (without supplements) using 26G needles (BD PrecisionGlideTM, Franklin Lakes, NJ, USA) and body weights were recorded. Two weeks after xenografting, tumor sizes and body weights were started to record every other day till the last day (day 15) of the experiment. Tumor volume was calculated according to the expression, ((L×P^2))/2 where, L: longest tumor diameter and P: diameter perpendicular to the longest diameter. Once the tumor volume reached 100 mm^3 in size (recorded as day one), mice were randomly assigned into three treatment groups and vehicle control group and intratumoral injections were started to achieve estimated 200 µM drug concentration in the tumor. Altogether, five intratumoral injections of each test compound (PT: 0.27 mg/kg;

DHA: 0.327 mg/kg and PZ-DHA: 0.75 mg/kg) or vehicle control (0.6 % v/v DMSO in saline) were given every other day (day 1, 3, 5, 7 and 9) for nine days. Mice were monitored for one more week (day 11, 13 and 15) and their tumor sizes and body weights were recorded. Tumor bearing mice were euthanized by isofluorane inhalation followed by CO_2 inhalation at day-15 (at the end of experimental period) or earlier if they show any signs of tumor ulceration or distress. Euthanized mice were photographed and the tumors were excised. Excised tumors were weighed, photographed and fixed in 10% v/v acetate buffered formalin (0.2 L, 37% formaldehyde, 1.8 L, distilled water and 46.1 g sodium acetate.$3H_2O$) (Fisher Scientific, ON, Canada).

[00110] *In vivo* tumor suppression was shown in a NOD-SCID mice model xenografted with MDA-MB-231 cells to the right hind flank. After five intratumoral injections mice were monitored for 1 more wk and at the end of the experimental period tumor volumes were recorded as vehicle: 392.2651± 27.58, PT: 263.69± 21.32, DHA: 213.19 ± 12.11 and PZ-DHA: 195.58± 11.91 mm^3 (Figure 7B). All the test compound-treated tumors showed statistically significant reduction in the tumor volumes. (PT: $p<0.01$, DHA and PZ-DHA: $p<0.001$). Weights of excised tumors were noted at the day 15 as vehicle: 238.8±18.6, PT: 170.4±20.5, DHA: 153.9±19.8 and PZ-DHA: 144.5±10.9 mg (Figure 7B). Though PT-induced *in vivo* tumor suppression was significant at $p < 0.01$ in terms of tumor volume, the difference in tumor weight was not statistically significant compared to the vehicle control. Mice were euthanized and their tumors were photographed.

[00111] $\%\ \textbf{Relative phosphatase activity} = \frac{(A_T - A_B)}{(A_C - A_B)} \times 100$Metabolic Activity:

Acid phosphatase assay measures the metabolic activity of live cells in terms of cytosolic acid phosphatase activity by hydrolyzing the phosphatase substrate, p-nitrophenyl phosphatase at acidic pH levels. The hydrolyzed product, p-nitrophenol produces the yellow-colored end product, p-nitrophenolate under alkaline conditions which could be read at 405 nm in a microplate reader and the absorbance is directly proportional to viable cell count. In alkaline pH, the acid phosphatase activity is ceased marking the end point of reaction. Acid phosphatase assay was performed as described in Yang et al., (1996) with minor modifications. MDA-MB-231 cells (in 100 µL cDMEM) were seeded in 96-well flat-bottom cell culture plates at a density of 5×10^3 cells per well and were incubated overnight to promote cell adhesion. Adhered cells were treated with PT, PZ, DHA, PZ-DHA, DOX and DOC (10, 50, 100, 150 and 200 µmol/L) and incubated for 3, 6, 12, 18 and 24 hr at 37 °C. Vehicle controls were used for all the treatment concentrations separately and untreated

control (cells treated with cDMEM without any treatment) was also included. At the end of the incubation period the plates were centrifuged at 405 × g for 10 minutes and supernatant was discarded. Cell monolayers were then washed with 200 µL 1 × sterile PBS. Assay buffer (100 µL) (0.1 M sodium acetate; pH 5.5, 0.1% v/v Triton X-100 and 4 mg/mL phosphatase substrate) was added to each well and incubated for 2 hr at 37 °C. Ten microliter of 1N NaOH was added to each well to cease the reaction and to develop the color of the end product. The absorbance of the colored product was measured at 405 nm using a micro-plate reader (BMG-LABTECH) (Ortenberg, Germany) and percentage relative phosphatase activity was calculated as given in the following equation where A_T: absorbance of test compound treated cells; A_C: absorbance of vehicle treated cells; A_B: absorbance of blanks. Blank (100 µL of assay buffer with 10 µL of 1N NaOH) was used to compensate the background absorbance.

$$\% \textbf{ Relative phosphatase activity } = \frac{(A_T - A_B)}{(A_C - A_B)} \times 100$$

Data expressed above as mean ± SEM (n=12) are averaged results of three independent experiments conducted in quadruplicates. Differences among means were compared using Tukey's test. Values with different letters (A-D: Time-dependency, a-e: Dose-dependency) are significantly different at $\alpha = 0.05$ ($P<0.05$).

[00112] Cytosolic acid phosphatase activity of MDA-MB-231 cells was inhibited by all the test compounds as in MTS assay except for PT and PZ. Both PT and PZ did not inhibit phosphatase activity significantly. However, the inhibitory effects of DHA and PZ-DHA on the phosphatase activity were more prominent than that of metabolic activity measured in MTS assay. Dose-dependent phosphatase activity inhibition showed by DHA was started at a relatively early time point (12 hr) (Figure 8). PZ-DHA showed a unique consistency in inhibiting phosphatase activity in a time- and dose-dependent manner throughout the experimental period (Figure 8). The ester, PZ-DHA showed dose dependency starting at an early time point of treatment (3 hr). At and above 50 µM concentration of PZ-DHA, effect of time-dependency was observed through 3-24 hr treatment duration.

[00113] Confirmation of Reduction in Metabolic Activity: 7-AAD (7-Aminoactinomycin) assay was carried out to confirm the reduction in metabolic activity measured in MTS and acid phosphatase assays was due to the death of cells and not false positive results given by cell growth inhibition. For 7-AAD staining, MDA-MB-231 cells were plated at a density of 1 × 10^5 cells per well in 6-well plates. The cells were incubated overnight at 37 °C to support

cell adhesion. Adhered cells were treated with 50 and 100 µmol/L of PT, PZ, DHA, PZ-DHA, vehicle or medium and incubated for 24 hr at 37 °C. At the end of incubation period, cells were harvested using TrypLE express and combined with its respective media. Cells were centrifuged at 500 × g and resuspended in 1 × PBS. Cells were incubated with 5 µL of 7-AAD viability staining solution (ebioscience Inc. San Diego, CA, USA) at room temperature. Flow cytometric analysis was performed using a FACS Calibur instrument (BD Bioscience, Mississauga, ON) on detector, FL3. Cells (1×10^4) were counted per sample and both live and dead cells were included in counts. Loss of cell membrane integrity at the late apoptosis/necrosis could be measured by 7-AAD which could penetrate into the cells once membrane become permeable. Upon 7-AAD staining a promising cell death was detected on FL3 after incubating cells at 50 µM and 100 µM concentrations of the test compounds for 24 hr. The percentage of dead cells noticed in PT, DHA, PZ-DHA but not PZ treated cultures was notably different from both vehicle and medium treated cells. However, PT-, DHA- and PZ-DHA- induced cell death observed at 100 µM and 50 µM were comparable.

[00114] Determination of Apoptosis: Apoptosis, programmed cell death is characterized by a series of morphological and biochemical changes taking place in a cell and DNA fragmentation is considered a hallmark of apoptosis. TUNEL staining was carried out using commercially available In *Situ* Cell Death Detection Kit, POD (Roche applied Science, Laval, QC, Canada) according to the manufacturer's instructions. MDA-MB-231 cells were seeded in 6-well plates containing sterile cover slips, at a density of 4×10^5 cells per well and incubated overnight at 37 ◦C to induce cell adhesion. Adhered cells were incubated with 100 µmol/L of PT, PZ, DHA, PZ-DHA, vehicle or medium and incubated for 3, 6, 12, 18, and 24 hours at 37 °C. DOX, DOC (100 µmol/L) and 1 µmol/L staurosporine were used as the positive control to induce DNA fragmentation. $MgCl_2$ (2 mM) in 1 × PBS was used as the washing buffer in all the steps. At the end of incubation cells attached on cover slips were washed with washing buffer and fixed in freshly prepared 4% w/v paraformaldehyde (PFA) in 1 × PBS for 1 hr at room temperature. Cells rinsed with washing buffer and incubated in blocking solution (freshly prepared 3% v/v H_2O_2 in methanol) at room temperature for 10 min. Then, cells were rinsed with washing buffer and incubated in permeabilization solution (0.1% v/v Triton X-100 in 1 × PBS) for 8 min at -20 ◦C. Cells were again thoroughly washed with washing buffer and area around the sample was dried for staining. The TUNEL reaction mixture (50 µL) (50 µL, enzyme solution and 450 µL label solution) was placed carefully and spread homogeneously on the sample. Cells were incubated for 1 hr at 37 °C in humidified atmosphere in the dark. At the end of incubation, cells were washed with washing buffer,

dried and mounted on a glass slide for viewing under the fluorescence microscope at the excitation wavelength in the range of 450-500 nm and detected in the range of 515-565 nm. Cell morphology of MDA-MB-231 cells was started to change within 3 hr of PZ-DHA treatment. Very few labeled DNA fragments were seen in PT-treated cells and PZ-DHA-treated cells showed extensive labeling of DNA fragments. However, DHA-treated cells did not show any TUNEL stained DNA fragments even though caspase 3/7 activation was detected. Similarly, PZ-treated cells also did not show positive results on TUNEL staining.

[00115] Amplex Red Assay: The Amplex Red assay was carried out in a cell-free environment with H_2O_2 as the positive control and with comparison to 100 µM of EGCG in phenol red-free cDMEM. This assay was performed to assess whether test compounds (PT, PZ, DHA and PZ-DHA) reacts with sodium bicarbonate in cell culture medium to produce hydrogen peroxide (H_2O_2). Amplex Red assay was performed in a 96-well flat-bottom plate in quadruplicates and the final concentration of PT, PZ, DHA, PZ-DHA and positive control was kept at 100 µmol/L. Standard curve of H_2O_2 was generated using a standard series (0.1, 0.056, 0.032, 0.018, 0.01 and 0.006 mM) of H_2O_2. In the dark, 100 µL/well of master mix (at a final concentration of 25 µM Amplex red and 0.005 U/mL HRP in phenol red-free cDMEM) was added on top of the treatments and incubated for 2 and 24 hr at 37 °C. Absorbance was measured at 570 nm on an Expert 96-well microplate reader (Admiral Place, Guelph, ON) at 2 and 24 hr post-treatment. as a preliminary step to check the involvement of ROS and contribution of HCO_3^- containing medium toward production of H_2O_2 was measured and the negative results demonstrated by the Amplex red assay indicated that none of PT, PZ, DHA and PZ-DHA are involved in H_2O_2 production in the absence of MDA-MB-231 cells. Involvement of ROS in PT, DHA and PZ-DHA-induced cell death was investigated by incorporating N-acetylcysteine (NAC) in Annexin-V-FLUOS/PI staining. NAC is an antioxidant that scavengse the ROS produced in the cells which could be involved in the induction of cell apoptosis. The cell death caused by test compounds was measured in Annexin-V-FLUOS/PI staining at 6 hr of post treatment. Apoptosis and late apoptosis/necrosis was detected by analyzing cells at FL1 and FL2 for Annexin-V-FLUOS and PI, respectively. Incorporation of NAC in the culture medium did not alter the cytotoxicity induced by the test compounds. Hence, NAC failed to protect breast cancer cells from cell death induced by the test compounds. When the anticipated background indicated by DMSO vehicle control treated cells is considered, late apoptosis/necrosis noticed in PZ-treated cells was minimal, while all the other test compounds showed more substantial cell death on Annexin-V-FLUOS/PI staining.

[00116] Assay of Caspase-3 Activity: Activation of caspase 3 and 7 enzymes were tested using caspase-Glo® 3/7 assay kit (Promega, Madison, WI, USA). Caspase (cysteine-dependent aspartate-directed protease) enzymes are the central components of apoptosis and caspase 3 is the most frequently activated protease in mammalian cell apoptosis. Because caspase 7 is comparable to caspase 3 in *in vitro* substrate preference (toward substrate Ac-DEVD-pNA) this assay measures activation of both caspase 3 and 7. The assay was performed in triplicates according to the manufacturer's instructions. MDA-MB-231 cells (in 100 µL cDMEM) were seeded in 96-well, flat-bottom white-walled cell culture plates at a density of 5×10^3 cells per well and were incubated overnight to activate cell adhesion. Adhered cells were treated with 100 µmol/L of PT, PZ, DHA or PZ-DHA and incubated for 6 and 12 hr at 37 °C. Doxorubicin, docetaxel (100 µmol/L) and staurosporine (1 µmol/L) were used as positive controls. Vehicle controls and medium controls were also included in triplicates. At the end of the incubation period, contents of Caspase-Glo® 3/7 buffer vial was transferred into Caspase-Glo® 3/7 substrate and mixed by swirling. The 96-well plates incubated at 37 °C were equilibrated to room temperature before adding Caspase-Glo® 3/7 assay buffer mixture. Assay mixture (100 µL) was added to each well taking extreme care to avoid cross contamination and incubated for 2 hr at 37 °C. Luminescence was measured on a microplate reader (BMG-LABTECH) (Ortenberg, Germany) using blank (cDMEM with DMSO and assay buffer mixture in the absence of cells) to compensate the background luminescence. With extending the exposure of cells to the test compounds or positive controls to 12 hr, PZ, DHA and PZ-DHA indicated a significant activation of caspase 3/7 with compared to the control. PZ-induced caspase 3/7 activation was comparable to as that of doxorubicin and docetaxel and PZ-DHA induced activation measured was statistically equivalent to staurespорine. The highest caspase 3/7 activation was observed with DHA and this was also statistically equivalent to staurosporine, but not to PZ-DHA (Figure 9).

[00117] In general, phloridzin fatty acid esters exhibited a dose- and time- dependent decrease in viability of all three cancer cell lines. The anti-proliferative effect of the esters and two commercial drugs that were used for comparison were prominent at 12 hr after incubation and concentration above 100 µM (Figures 10-13B). When compared to the newly synthesized esters, the parent compounds (phloridzin or six fatty acids) seemed to be ineffective at all concentrations and time points.

[00118] In HepG2 cells, there was a significant decrease in cell survival in response to phloridzin stearic acid esters (EC50 53.3 µM at 3 h), oleic acid ester (EC50 64.6 µM in

3 h), linoleic acid ester (EC50 43.3 μM at 3 h), alpha linolenic acid ester (EC50 76.5 μM at 3h), DHA ester (EC50 76.9 μM at 3 h) and EPA ester (EC50 39.4 μM at 3 h) which further decreased by time to 37.8, 31.5, 29.2, 53.1, 51.9 and 26.8 μM, respectively at 24 h. Whereas the parent compounds phloridzin, stearic acid, oleic acid, linoleic acid, alpha linolenic acid and EPA were ineffective. DHA fatty acid showed less potency (EC50 of 119.9 μM at 24h) which is 2.3-fold higher than phloridzin DHA ester. Phloretin (the aglycone of phloridzin) showed a median effective concentration of 97.20 μM at 24h. The standard liver cancer drug sorafenib (Nexavar®) showed a similar effect to phloridzin esters with EC50 of 67.5 μM at 3h, which further decrease to 10.7 μM at 24h. In MDA-MB-231 and THP-1cell lines, phloridzin esters, parent compounds, phloretin showed similar effects in cell survival as HepG2 cells. In THP-1 cells, phloretin was effective with a lower EC50 of 17.3 μM compared to HepG2 (EC50 97.20 μM) or MDA- MB-231 (EC50 104.7 μM). Doxorubicin hydrochloride, a standard drug for breast cancer and acute monocytic leukemia (a type of acute myeloid leukemia) also showed a similar effect in cell viability as of phloridzin esters. Data obtained revealed that there was a time- and dose-dependent decrease in cell viability by phloridzin fatty acid esters or standard drugs. Phloridzin fatty acid esters exhibited much stronger cytotoxic activity than its parent compound in various cell lines, with EC50 values ranging from 6.2 to 63.8 μM after 24h exposure. In conclusion, phloridzin fatty acid esters function as a broad spectrum antiproliferative agent in human cancer cells and are more effective than their natural occurring parent compounds from which they were derived.

Example 3 – Antiproliferative Properties of Long Chain Acylated Esters of Quercetin-3-*O*-Glucoside In HepG2 Cells

[00119] Despite their strong role in human health, poor bioavailability of flavonoids limits their biological effects *in vivo*. Enzymatically catalyzed acylation of fatty acids to flavonoids is one of the proposed ways of increasing cellular permeability and hence, biological activities. In this study six long chain fatty acid esters of quercetin-3-*O*-glucoside (Q3G) acylated enzymatically were used for determining their antiproliferative action in comparison to precursor compounds (quercetin, Q3G and six fatty acids namely, stearic acid, oliec acid, linolec acid, alpha-linolenic acid, eicosapentaenoic and docosahexanoic acids) using HepG2 cells *in vitro*. Long chain fatty acid esters of Q3G showed significant inhibition of cell proliferation (approximately 85% to 90%) in HepG2 cells compared to the precursor compounds and two prescribed drugs (sorafenib and cisplatin) for liver cancer chemotherapy (P-value < 0.05) after 6 hrs and 24 hrs of treatment. The cell death due to these novel

compounds was associated with cell cycle arrest and apoptosis based on DNA fragmentation, cell cycle analysis by flow cytometer, fluorescent microscopy and elevated caspase-3 activity. Additionally, the long chain fatty acid esters of Q3G exhibited strong DNA topoisomerase II inhibition. Among the six fatty acid esters of Q3G, oleic acid ester displayed the greatest antiproliferation action and a high potential as a cancer therapeutic.

[00120] Materials and chemicals: Quercetin, quercetin-3-*O*-glucoside, Cisplatin, propidium iodide, fatty acids namely, oleic, stearic, linoleic, linolenic, eicosapentaenoic, docosahexanoic acids and two-well chambered cover slides were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Sorafenib (Nexovar®) was purchased from Cayman Chemical (Michigan, USA). Cell Titer 96TM Aqueous One solution cell proliferation (MTS) assay and CytoTox 96® nonradioactive cytotoxicity (LDH) Assay kits were purchased from Promega (Madison, WI). ApoTarget™ Quick apoptotic DNA ladder detection kit from Invitrogen (Burlington, ON). Caspase 3 colorimetric assay kit was purchased from BioVision, Inc. (California, USA). All cell culture vessels and plates were purchased from BD Biosciences (San Jose, CA). BCA protein purification kit was purchased from Thermo Scientific (Burlington, ON).

[00121] Synthesis of long chain fatty acid esters of Q3G: The compounds for the study were synthesized as previously described by Ziaullah et al., (2012). Briefly, in a round bottom flask, defined quantities of Q3G and individual fatty acids were dissolved in acetone. Enzymatic reactions were initiated by the addition of lipase (Novozyme 435®); with an activity of 10,000 propyl laurate units). The mixture was stirred and heated at 45 °C for 12–24 h. and was monitored by thin layer chromatography (TLC), followed by staining with anisaldehyde spray reagent and then heating at 110 °C. After completion of reaction, it was filtered, evaporated and column chromatography (acetone/toluene; 35:75 to 50:50) was performed to get the pure fatty acid esters of Q3G. The pure compounds were then analyzed by IR, ^{1}H NMR and ^{13}C NMR spectroscopy to confirm the structures.

[00122] HepG2 cell culture system: HepG2 cells were obtained from American Type Culture Collection (ATCC, 8065) and maintained according to ATCC's instructions. Briefly, the cells were cultured in Eagle's Minimum Essential Growth Medium (EMEM) with 2 mM L-glutamine and 10% Fetal Bovine Serum (FBS) at 37°C and 5% CO2. T-75 tissue culture flasks with 12-15 ml of media were used for regular culturing. Sub-culturing was performed in 1:4 or 1:5 ratio every 3 to 4 days when cells reached a confluency of 70-80%. Cells were counted under Nikon Eclipse TS 100 phase contrast microscope using haemocytometer and

then transferred to fresh flasks.

[00123] Measurement of cell proliferation: Commercially available Cell Titer 96TM Aqueous One solution cell proliferation (MTS) assay was employed for the assay. Manufacturer's instructions were followed for performing assay. Briefly, HepG2 cells in the exponential growth phase were collected and seeded in 96-well microplate in density of 2 x 10^4 cells per well, final volume being 200 µl/well with the help of a multi-channel pipette. The microplates were placed in culture incubator in standard conditions (37°C with 5% CO2) and cultured for 24 hrs. After incubation, 100 µM of long chain fatty acid esters of Q3G and control samples in fresh media were added to each well in triplicates. The DMSO concentration for 100 µM of test compounds in all assays was less than 1%. The plates were then incubated for different time intervals (6 and 24 hrs) in culture incubator (37° C, 5% CO2, 90% humidity). According to manual instructions, 20 µl of MTS was added to each well (5 g/L in PBS) and again incubated for 1-4 hrs. Absorbance was recorded directly at 490 nm using Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

[00124] There was a significant decrease (approximately 85% to 90%) in cell proliferation ($P < 0.05$) in comparison to the precursor compounds quercetin and Q3G, free fatty acids and prescribed drugs Sorafenib and Cisplatin. Within 6 hours of incubation, all long chain fatty acid esters of Q3G except stearic acid ester of Q3G treated cells showed a drastic reduction in cell viability as compared to the respective controls (Figure 14A). After 24 hours of incubation, viability of HepG2 cells further decreased significantly in Q3G fatty acid esters treated cells as compared to the precursor compounds and control drugs treated cells (Figure 14B). Also, at low concentrations (mainly, 10 and 50 µM) of long chain fatty acid esters of Q3G, longer incubations of 48, 72 and 96 hours were necessary to obtain a significant reduction in cell viability.

[00125] Measurement of cell cytotoxicity: Commercially available CytoTox 96® Non-Radioactive Cytotoxicity (LDH) Assay kits from Promega was utilised for this assay. The manufacturer's instructions were followed for the assay. Briefly, HepG2 (5000 cells/well) were plated in 96-well microplates, the final volume per well was kept at 100 µl. The controls included (i) Assay medium without cells, (ii) low control with cells and assay medium to see spontaneous or normal LDH activity and (iii) high control with cells treated with Triton X-100 to achieve maximum LDH release which served as positive control. The microplates were placed in culture incubator in standard conditions (37° C with 5% CO_2) and cultured for 24 hrs. After incubation, 100 µM of the long chain fatty acid esters of Q3G and control

samples in fresh media were added to each well in triplicates. The plates were incubated for different time intervals (mainly, 6 and 24 hrs) in culture incubator (37° C, 5% CO_2, 90% humidity). After treatment, the 96-well microplate was centrifuged and supernatant was transferred to a fresh 96-well microplate and subjected to LDH assay. Absorbance was taken at 490-492 nm using Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

[00126] There was a significant increase in LDH enzyme release ($P < 0.05$) in comparison to the precursor compounds quercetin and Q3G and control drugs, Sorafenib and Cisplatin. The LDH release data was consistent with the MTS data. Within 6 hours of incubation, all long chain fatty acid esters of Q3G except steric acid ester of Q3G treated cells showed a strong increase in LDH release as compared to the respective controls (Figure 15A). On the other hand after 24 hours of incubation, LDH release was seen relatively lower than 6 hours incubation data (Figure 15B). This may be explained through the fact that LDH has a half-life of 8-9 hrs (Lisa et al., 2004 and Riss et al., 2004) and because the compounds cause a significant amount (over 85%) of cell death within 6 hours, by 24 hrs LDH gets degraded in the medium and hence 24 hours incubation readings were seen comparatively lesser than 6 hours readings. Nevertheless, the LDH release which signifies the cytotoxic action of the long chain fatty acid esters of Q3G was significantly greater than the precursor compounds and control cancer drug, Cisplatin ($P < 0.05$) . Oleic acid ester of Q3G emerged as the most effective compound showing the greatest antiproliferative action (over 95%), while all other long chain fatty acid esters of Q3G except steric acid ester showed relatively lesser antiproliferative action (~ over 85%).

[00127] To see the cytotoxicity effect of the long chain fatty acid esters of Q3G on normal hepatocytes, 100 µM long chain fatty acid esters of Q3G esters were incubated with normal rat hepatocytes cells and MTS assay was performed as described earlier. The results showed a significantly higher viability in long chain fatty acid esters of Q3G treated normal rat hepatocytes compared to the long chain fatty acid esters of Q3G treated transformed HepG2 cells. Additionally, the long chain fatty acid esters of Q3G showed a significant lower cytotoxicity than control drug Sorafenib (Figure 16).

[00128] DNA fragmentation: This assay was performed by utilizing commercially available ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit. The manufacturer's instructions were followed for the assay. Briefly, HepG2 cells (5×10^5 cells/well) were grown in 12 well culture plate (75-80%) confluency and then treated with 100 µM of test compounds for 24 and 48 hrs. Cells were collected and total DNA was isolated from each

sample. Extracted DNA pellet was dissolved in 30 µl of DNA suspension buffer (provided with the kit) and resolved on 1.2% agarose gel containing GelRed™ stain (used 1X) in 1X TAE buffer (pH 8.5, 20 mM Tris-acetic acid, 2 mM EDTA) in BioRad mini-gel electrophoresis kit. The DNA gel was then moved to UV sample tray for examining the bands visualised and photographed by BioRad's Gel Doc™ EZ system (Mississauga, ON).

[00129] As expected, the long chain fatty acid esters of Q3G treated cells showed consistent DNA damage and fragmentation within 24 hours as seen on the gel image. The intensity of the damage and fragmentation increased at 48 hours incubation.

[00130] Caspase Assay: The caspases activation was quantified by utilizing Caspase-3/CPP32 Colorimetric Assay Kit. The assay was performed according to the manufacturer's instruction. Briefly, HepG2 cells (1×10^6 cells/well) were plated in six-well tissue culture plate. After treatment with the test compounds for 24 hrs, the cells were lysed with lysis buffer provided by the manufacturer and centrifuged at 13000 rpm. After collecting the supernatant, the protein was quantified using BCA protein quantification kit and 250 µg of protein per treatment sample was used for the assay. Reaction buffer (50 µl) was added to each treatment well of microplate reader followed by addition of 5 µl DEVD-pNA (Asp-Glu-Val-Asp p-nitroanilide) caspase substrate. The microplate was incubated at 37° C for 1-2 hour. The absorbance of the samples was read at 405 nm in Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany). After subtracting background readings from all the samples (induced and uninduced), fold-increase in CPP32 activity was determined by comparing these results with the level of the uninduced control.

[00131] After 24 hours of treatment of HepG2 cells with 100 µM of long chain fatty acid esters of Q3G, a significant increase in the caspase-3 family was observed as compared to the control untreated cells. This relative change in caspase-3 activity data was consistent with the MTS and LDH release assays. Consistent with above results oleic ester of Q3G showed greatest caspase activity among the other esters of Q3G.

[00132] Topoisomerase Assay: Commercially available topoisomerase II drug screening kit (TopoGEN, Inc., Columbus, Ohio, USA) was utilized and assay was performed as explained by Patra et al., (2011). Briefly, substrate supercoiled pHot1 DNA (0.25 µg) was incubated with four units (2 µl) of human DNA topoisomerase II, test compounds (2 µl) and assay buffer (4 µl) in 37° C for 30 minutes. The reaction was terminated by the addition of 10% sodium dodecyl sulphate (2 µl) followed by digestion with proteinase K (50 µg/µl) at 37° C for 15 minutes. After incubation DNA was run on 1% agarose gel in BioRad gel

electrophoresis system at 70V for 1-2 hours. The gel was stained with GelRed™ stain for two hours and destained for 15 minutes with TAE buffer followed by gel imaging via BioRad's Gel Doc™ EZ system. Supercoiled DNA and linear strand DNA were incorporated in the gel as markers for DNA topology and DNA topoisomerase II poison (inhibitor). Additionally, a positive control drug VP16 (provided with the kit) a known DNA toposiomerase II inhibitor (poison) was also incorporated in the gel for reference. The presence of a single linear band in the positive control reaction confirmed the inhibitory effect of the VP16 on DNA topoisomearse II activity. The inhibitory activity was calculated as relative activity of topoisomerase enzyme (in this case, intensity of the supercoiled band and presence/absence of single linear band) in the presence of test compounds in comparison to that in the negative control solution (DMSO control).

[00133] The assay was performed to test whether long chain fatty acid esters of Q3G act as a poison and increase the DNA cleavage via topoisomearse II. The esters did not stabilize topoisomerase II cleavage complexes and failed to exhibit the formation of single linear DNA, and increased the supercoiled DNA intensity, whereas, positive control drug VP 16 increased the formation of linear DNA. This result shows that long chain fatty acid esters of Q3G do not act as human topoisomearse II poison but as a catalytic inhibitor by inhibiting the DNA relaxation activity. The intensity of the supercoiled bands in comparison to the negative control (DMSO) is very high clearly suggesting that due to DNA topoisomearse II inhibition, the supercoiled DNA did not get relaxed

[00134] Fluorescence microscopy: GFP-Certified™ Apoptosis/Necrosis Detection Kit was purchased from Enzo Life Sciences Inc. (Farmingdale, NY) for the detection of adherent apoptotic cells. Briefly, 2 x 10^5 HepG2 cells were seeded on two-well chambered cover slides (Sigma-Aldrich) and grown to about 75% confluency; this was followed by treatment with 100 μM long chain fatty acid esters of Q3G for 24 hours. Adherent cells were stained according to the manufacturer's instructions by dual detection reagent (containing annexin V coupled with PI). The dual-labeled cells were visualized by fluorescence microscopy with a Leica DMBL (20x/.040) fluorescent microscope (Houston, TX) incorporated with Nikon Cool Pix 4500 Digital camera (Mississauga, Ontario). Cells with bound annexin-V show green staining in the plasma membrane. Cells that have lost membrane integrity show red staining (PI) throughout the cytoplasm with an impression of green staining on the plasma membrane. Cells with green staining were scored as apoptotic, cells with both green and red staining were scored as late apoptotic, whereas those with only red staining were considered

necrotic.

[00135] The untreated cells did not show any staining, suggesting that these cells did not undergo significant apoptosis or necrosis. Long chain fatty acid esters of Q3G treated cells displayed some staining for both annexin V and PI which signifies late apoptotic cells and for PI only which signifies necrotic cells and significant staining for annexin V only which signifies apoptotic cells except for stearic acid ester of Q3G which did not show apparent staining. This data suggests that the inhibitory effects of the long chain fatty acid esters of Q3G may be due to the apoptosis leading to late apoptosis.

[00136] Cell cycle analysis: HepG2 cells were plated in six well culture plate (1×10^6 cells/well). After 24 hours of incubation at 37° C, 5% CO2, the cells were treated with 100 µM long chain fatty acid esters of Q3G for another 24 hours. Briefly, cells were trypsinized and centrifuged at 1200 rpm at 4 ° C for 10 minutes followed by another PBS wash. The pellet was resuspended in 0.3 ml of PBS. The cells were then fixed by adding 0.7 ml of ice cold ethanol for 2 hours. After fixation, the cells were centrifuged again at 1200 rpm at 4 ° C for 10 minutes and cell pellet was re-suspended in 0.25 ml of PBS with the addition of 5 µl of 10 mg/ml Rnase A (the final concentration being 0.2-0.5 mg/ml) and incubation at 37°C for 1 hour. After incubation 10 µl of 1 mg/ml PI solution (the final concentration being 10 µg/ml) was added to the cell suspension and kept in the dark at 4°C until analysis. The cells were then analyzed for cell cycle using flowcytometer FACS Caliber (BD Biosciences, San Jose, California) with an excitation wavelength at 488 nm and emission at 670 nm. DNA content was determined by MotFit LT™ software, version 4.0 (Topsham, ME), which provided histograms to evaluate cell cycle distribution.

[00137] Consistent with their growth inhibitory effects, long chain fatty acid esters of Q3G increased the population in the S phase with a corresponding decrease of cells in the G1 phase after 24 hours of treatment, implying that the DNA synthesis was retarded. In addition to increasing the population of cells in the S phase from 11.1% (control) to 35.4% (Oleic acid ester of Q3G), long chain fatty acid esters of Q3G also appeared to increase the cell population in the G2-M phase, implying the cell mitosis stage was inhibited for the cells that managed to move from S phase to G2-M phase. These data support the potent inhibitory effect of long chain fatty acid esters of Q3G on DNA synthesis and possibly in parts, cell mitosis.

[00138] Statistical Analysis: Data was analyzed using Minitab 16 statistical software. The assays were replicated three times using a completely randomized design (CRD) model. Data

was analysed using one way ANOVA. All treatments were checked for normality and constant variation check before running ANOVA. Tukey's test was performed for achieving significant difference between different treatment compounds. Significance level in all assays was taken at $P < 0.05$. All data were expressed as mean ± SD with at least three independent experiments.

[00139] Observation under the phase contrast microscope after 6 hours showed a dramatic decrease in cell number of the cells treated with long chain fatty acid esters of Q3G as compared to the control cells with no treatment. Interestingly, it revealed that the long chain fatty acid esters of Q3G treated cells were undergoing an excessive morphology change in comparison to the precursor compounds, control drugs and control with no treatment. This was accompanied by a very low cell number. Cell membrane shrinkage, blebbing, clustering and lysis were clearly visible. Similarly, severe changes in cell number and morphology were observed after 24 hours of treatment as well. However, consistent with above results, stearic acid ester of Q3G failed to show any major effect on cell number and morphology.

[00140] In summary, this data clearly suggests that acylation of Q3G with long chain fatty acid esters enhances its antiproliferative activity *in vitro*. The long chain fatty acid esters of Q3G also showed significantly lower cytotoxic effect on normal hepatocytes as compared to the transformed HepG2 suggesting their specific action on cancer cells. These data suggested the apoptotic action of the novel long chain fatty acid esters of Q3G on HepG2 cells. This study provides preliminary data for these novel compounds' high antiproliferative action on cancer cells (HepG2) and very low cytotoxic action on normal cells (rat hepatocytes) as compared to the precursor compounds and currently used chemotherapy drugs at same concentration.

[00141] The novel synthesized long chain fatty acid esters of Q3G can inhibit liver cancer cell proliferation (HepG2) through induction of apoptosis by the activation of caspase-3 family followed by necrosis, through cell cycle changes, and possibly through inhibition of DNA topoisomerase II activity. Interestingly, as hypothesized, long chain fatty acid esters of Q3G exhibited much stronger anti-proliferative property than precursor compounds (quercetin, Q3G and free fatty acids) and two prescribed chemotherapy drugs, Sorafenib and Cisplatin. The long chain fatty acid esters of Q3G inhibited proliferation of HepG2 cells within 6 h of incubation in comparison to quercetin, Q3G, free fatty acids and chemo drugs at the similar concentration (100 μM). The cell proliferation was shown to further reduce by 24 h of incubation. Also, at lower concentrations of 10, 30 and 50 μM the esters, 48 to 72 h incubations were necessary to obtain a significant reduction in cell viability (data not

included). Based on the effects on cell viability and morphology, the data suggested that the test compounds caused cytotoxicty to the HepG2 cells resulting in the cell membrane shrinkage and eventually breakage. This result was further assessed by the membrane integrity test via LDH release assay which showed that there was clear membrane breakage when compared with untreated control cells. Interestingly, the strong inhibition of cell proliferation by the fatty acid esters of Q3G when compared to the precursor compounds alone and the chemotherapy drugs is noteworthy.

[00142] Interestingly, oleic acid ester of Q3G appeared to show the strongest antiproliferative action whereas, stearic acid ester of Q3G showed the least growth inhibitory action among all the tested esters of Q3G. The experimental results support the assumption that there is a structure-activity relationship due to the fact that stearic acid is the only saturated fatty acid among the six fatty acids used for acylation of Q3G. Once the stearic acid is attached to the Q3G skeleton, the change in the orientation may not be favourable for membrane interaction thereby, getting less absorbed by cells and subsequently showing less activity. Overall, this data suggested the potential of long chain fatty acid esters of Q3G as strong antiproliferative agents. Interestingly, the precursor compounds (quercetin and Q3G) that have been shown to display strong antiproliferative action by previous studies are in fact concentration and time dependent. In this study it was shown that the long chain fatty acid esters of Q3G display the growth inhibitory effect on HepG2 cells within 6 h of treatment (Figure 1 and 3). This data revealed that acylation of Q3G with unsaturated long chain fatty acid esters enhances its antiproliferative activity *in vitro*.

[00143] The long chain fatty acid esters of Q3G also showed significantly lower cytotoxic effect to normal hepatocytes as compared to the transformed HepG2 suggesting their specific action on HepG2 cells. Additionally, fluorescence microscopy showed cell membrane breakage suggesting symptoms of late apoptosis and necrosis. To distinguish between apoptosis and necrosis, cells were analyzed after staining with Annexin V and PI through fluorescence microscopy. After 24 h of treatment of HepG2 cells with long chain fatty acid esters of Q3G showed that, some treated cells were positive for PI and some for both Annexin V and PI, suggesting that the cells at this time present necrotic and also apoptotic features presumably late apoptosis.

[00144] To further analyze and confirm the apoptotic effect of the Q3G esters, the caspase-3 activity which is a key enzyme in apoptotic signaling was examined. Consistent with the fluorescent data, a significant activation of caspase-3 was observed in long chain fatty acid esters of Q3G treated cells in comparison to the un-induced control and precursor compounds

after 24 h of incubation. Additionally, this data was supported by the DNA fragmentation analysis. After 24 h and 48 h of treatment of HepG2 cells with long chain fatty acid esters of Q3G, the fragmented DNA like pattern was observed which is a basic hallmark of apoptosis. These data suggested the apoptotic action of the long chain fatty acid esters of Q3G on HepG2 cells.

[00145] Cell cycle analysis showed that long chain fatty acid esters of Q3G blocked the HepG2 cells in the S phase, this effect being related with the possible inhibition of DNA synthesis. Previous studies have shown that quercetin can induce cell cycle arrest in the S phase as well as in the G1/S phase or G2/M phase depending on the cancer cell type. Apparently, a considerable increase in cell population in G2/M phase was also seen in the long chain fatty acid esters of Q3G treated HepG2 cells. This suggests that the cells that were in fact able to cross S phase got arrested in G2/M phase. This data is thus in accordance with the previous studies that the cell cycle alterations are cell type and treatment dependent.

[00146] As expected, the data showed that long chain fatty acid esters of Q3G are behaving as strong topoisomearse II inhibitors. Stearic acid ester of Q3G showed a significant reduction in topoisomerase II activity. This shows that the compound is an active and potent topoisomerase II inhibitor. A reduction in DNA topoisomerase II activity observed with a reduced ability to relax the supercoiled DNA, indicates that the reduced cell proliferation may be, in parts, a result of DNA topoisomearse II inhibition which may have generated double strand breaks in DNA. It is also possible that DNA topoisomearse II-mediated DNA damage activated the cell cycle checkpoint causing growth arrest which in turn triggered and activated apoptotic signalling through caspase-3 and hence caused cell death which agrees with the literature

Example 4 – Molecular Targets of Oleic Acid Ester of Quercetin-3-*O*-Glucoside In Human Hepatocellular Carcinoma Cells

[00147] In this study, possible molecular targets of oleic acid ester of Q3G (OA-Q3G) were investigated in HepG2 cells. Three different approaches, namely, RT Profiler PCR array, sandwich ELISA and ELISA array were utilized for this investigation. Results from the cancer drug target RT-PCR array suggested that antiproliferative effects of OA-Q3G *in vitro* could be related to enhanced gene expression of pro-apoptotic genes BCL2 (2-fold), RHOB (3.3fold), IRF5 (6.3-fold); down-regulation of cell cycle genes CDK1 (2-fold), CDK2 (2fold), CDK8 (12.2-fold), growth factor-receptor EGFR (12.2-fold), protein kinases AURKB (2-fold), PRKCA (2-fold) and DNA topoisomerase II (2.7fold). In addition, OA-Q3G treatment

considerably down-regulated gene expression of certain genes which are found over-expressed in cancer cells such as HIF1A (2.4-fold), NFKB1 (2.2-fold), HDAC6 (25.9-fold) and BIRC5 (11.2-fold). Furthermore, results from ELISA and ELISA array revealed a significant increase in the protein expression of pro-apoptotic genes phospho-p53, phospho-Bad, cleaved-caspase-3, cleaved-parp, ERK1/2, and decrease in protein expression of anti-apoptotic and cell survival genes AKT, PRAS40 and p70S6kinase in HepG2 cells upon treatment with OA-Q3G. In conclusion, this study provides possible molecular targets of OA-Q3G in HepG2 cells through the attenuated regulation of genes involved in cell cycle, survival and apoptosis.

[00148] HepG2 cell culture system: HepG2 cells were obtained from American Type Culture Collection (ATCC, 8065) and maintained according to ATCC's instructions. Briefly, the cells were cultured in Eagle's Minimum Essential Growth Medium (EMEM) with 2 mM L-glutamine and 10% Fetal Bovine Serum (FBS) at 37°C and 5% CO_2. T-75 tissue culture flasks with 12-15 ml of the media were used for regular culturing. Cells were counted under Nikon Eclipse TS 100 phase contrast microscope (Mississauga, ON) using haemocytometer and then transferred to fresh flasks.

[00149] Cancer-related genes expression profiling: To identify possible molecular targets for antiproliferation action of OA-Q3G, Human Cancer Drug Target RT2 Profiler™ PCR Array (SABioscience, Frederick, MD) was used. Assay was performed according to manufacturer's instructions. Briefly, HepG2 cells were treated with OA-Q3G for 24 h. Total RNA was isolated using Aurum™ Total RNA Mini Kit (Bio-Rad, Mississauga, ON) according to the manufacturer's protocol. RNA concentration was measured with NanoDrop 1000 Spectrophotometer (Thermo Scientific, Ottawa, ON), RNA quality was evaluated by electrophoresis. cDNA was synthesized from 500 µg total RNA using RT2 First Strand cDNA Synthesis kit (Qiagen, Toronto, ON). Real-time PCR was performed using CFX96TM Real-Time PCR detection System (Bio-Rad, Mississauga, ON) according to manufacturer's instructions. Obtained data were analyzed with Excel-based PCR Array Data Analysis Software (SABioscience, Frederick, MD). This system profiles the expression of 84 actively sought targets for anticancer therapeutics and drug development which includes genes dysregulated during carcinogenesis, including those involved in key cellular growth pathways such as apoptosis, DNA damage repair, epigenetics, and growth factor and other signaling pathways.

[00150] The relative expression and modulation of each gene in up- and down-regulation form is shown in Figure 17. Results showed that 22 genes showed more than a 2-

fold change in expression (up- or down-regulated). Figure 17 shows only the genes with a significant and greater than 2-fold change in expression. Treatment of HepG2 cells with OA-Q3G influenced the expression of some key signaling genes involved in cancer cell survival and cell death pathways:

1. Cell cycle and survival: down-regulation of cell cycle proteins, CDK1 (-2-fold), CDK2 (-2-fold), CDK (-12.2-fold), CDC25A (-2-fold), topoisomerase II TOP2A (-2.7-fold), growth factors and receptors, EGFR (-12.2-fold), GRB2 (-2-fold) receptor tyrosine kinase AKT2 (-2.1-fold), protein kinases, AURKB (-2-fold), PRKCA (-2-fold) and histone deacetylases HDAC2 (-2-fold), HDAC6 (-25.9-fold).

2. Cell death and apoptosis: up-regulation of pro-apoptotic genes BCL2 (+2.2-fold), cathepsins CTSB (+2.2-fold), IRF5 (+6.3-fold), RHOB (+3.3-fold) and down-regulation of anti-apoptotic genes BIRC5 (-11.2-fold), HIF1A (-2.4-fold), NFKB1 (-2.2-fold) and HRAS (-2-fold).

[00151] The obtained data suggested that the antiproliferative effect of OA-Q3G could be due to change in expression of cell cycle genes and apoptotic genes in HepG2 cells.

[00152] Multi-Target Sandwich ELISA analysis: To investigate the effect of OA-Q3G treatment on intracellular apoptotic markers, PathScan® Apoptosis Multi-target Sandwich ELISA kit (Cell Signaling Technology, Danvers, MA) was used. The assay is a solid phase is a solid phase sandwich enzymelinked immunosorbent assay (ELISA) that combines the reagents necessary to detect endogenous levels of p53 protein, phospho-p53 protein (Ser15), Bad, phospho-Bad (Ser112), Cleaved Caspase-3 (Asp175) and Cleaved PARP (Asp214), which are key signaling proteins of cell survival and apoptosis pathways. The assay was performed according to manufacturer's instructions. Briefly, HepG2 cells were treated with OA-Q3G for 0, 6 and 24 h in fresh media. Cells were harvested under non-denaturing conditions by adding 1X cell lysis buffer (provided with the kit) supplemented with 1X protease inhibitor (Sigma-Aldrich, Mississauga, ON) and kept on ice for 10 min. Lysates were then microcentrifuged for 10 min at 4 °C and supernatents were transferred in fresh tubes and stored at -80 °C in single-use aliquotes. The assay was performed using 500 µg/ml protein quantified by BCA Protein Assay (Thermo Scientific, Ottawa, ON) according to the manufacturer's instructions. To appropriate wells of ELISA microplate, 100 µl of each cell lysate was added and incubated the plate overnight at 4 °C. After incubation, wells were washed four times with 1X wash buffer (provided with kit). After washing, 100 µl of detection antibody (provided with the kit) for each protein in respective wells and incubated the plate for 1 h at 37 °C followed by washing with wash buffer. After washing, 100 µl of

HRP-linked secondary antibody was added to corresponding wells and incubated the plate for 45 min at 37 °C followed by another wash procedure. After washing, 100 µl TMB substrate was added to each well and plate was incubated for 30 min at 37 °C followed by addition of stop solution. Protein activity was measured by reading absorbance at 450 nm in Fluostar Optima micro plate reader (BMG Labtech, Ortenberg, Germany).

[00153] Results from sandwich ELISA showed that total p-53 and phosphorylated p-53 amounts significantly increased at 6 h of incubation and comparatively decreased after 24 h of incubation. Similar results were seen by densitometry analysis from ELISA array which showed a significant 2-fold increase in levels of phospho p-53 within 6 h of treatment with OA-Q3G.

[00154] Multi-target ELISA Array analysis: To indentify intracellular protein level targets of OA-Q3G in HepG2 cells, PathScan® Intracellular Signaling Array kit (Cell Signaling Technology, Danvers, MA) was used. It is a slide-based antibody array founded upon the sandwich immunoassay principle. The array kit allows for the simultaneous detection of 18 important and well-characterized signaling molecules when phosphorylated or cleaved. The glass slides are nitrocellulose-coated spotted with target-specific capture antibodies. The assay was performed according to the manufacturer's instructions. Briefly, cell lysate were prepared and quantified similar to sandwich ELISA explained above. Protein (500 µg/ml) were incubated on the slide overnight at 4 °C followed by addition of a biotinylated detection antibody cocktail (provided with kit). Streptavidin-conjugated HRP and LumiGLO® Reagent (provided with the kit) was then used to visualise the bound detection antibody by chemiluminesence. The bands were developed using Carestream® Kodak film (Sigma-Aldrich, Mississauga, ON) and were quantified using Image J 1.47 software according to NIH guidelines.

[00155] Results showed an up-regulation in pro-apoptotic proteins ERK1/2, Bad, p-53, SAPK/JNK, PARP and caspase-3 active products expression and down-regulation of cell survival and growth proteins AKT, p70 S6 kinase, GSK 3β, Stat3 and PRAS40 within 6 h of treatment with OA-Q3G. On the other hand, results from 24 h treatment showed a decrease in the expression of ERK1/2, Stat1, HSP27 and p53 active products and an increase in the expression of p38, SAPK/JNK, PARP and caspase-3 active products. Results from this analysis suggest a strong role of OA-Q3G in active regulation of cell survival and cell death factors.

[00156] To evaluate the effect of p-53 and phospho-p53 expression on its downstream effectors, the expression of CDK1, Bcl-2 and Bad was studied. Results from the RT Profiler

PCR array analysis showed a 2-fold downregulation in the expression of CDK1 and an up-regulation in the expression of pro-apoptotic Bcl-2 family as compared to untreated control cells within 24 h of treatment with OA-Q3G. Additionally, results from sandwich ELISA showed a significant increase in the expression of pro-apoptotic Bad after 6 and 24 h of treatment with OA-Q3G.

[00157] Because activation of pro-apoptotic Bcl-2 family initiates the caspase cascade, caspase-3 activation in HepG2 cells upon treatment with OA-Q3G was examined. Results from the sandwich ELISA showed a significant up-regulation of cleaved-caspase-3 expression after 6 and 24 hours of treatment with OA-Q3G. Consistent results were observed by densitometry analysis from ELISA array. This was accompanied by an approximately 2-fold increase in cleaved PARP expression in HepG2 cells within 6 hours and 1.6-fold increase after 24 h of treatment with OA-Q3G. These results suggest that OA-Q3G treatment in HepG2 cells induces p-53 and phopho-p-53 expression resulting in activation of downstream effectors for cell death response.

[00158] In summary, these results suggest that treatment with OA-Q3G induces significant changes in the regulation of gene level expressions of the genes involved in drug metabolism, growth factors and receptors, G-protein signaling, receptor tyrosine kinase, cell cycle, transcription factors and apoptosis. These results further suggest that the treatment with OA-Q3G in HepG2 cells induces changes in protein level expression of various proteins involved in cell survival and cell death signaling pathways including apoptosis. The data suggest that OA-Q3G treatment in HepG2 cells regulate key signaling molecules involved in cell survival pathways.

Example 5 - Long Chain Fatty Acid Acylated Derivatives of Phloridzin Induce DNA Topoisomerases II Inhibition and Apoptosis in Human Liver Cancer Cells

[00159] The anticarcinogenic effect of phloridzin and its novel derivatives using a human liver cancer cell line was examined. Synthesised acylated derivatives of phloridzin with six different long chain saturated, mono-, and poly-unsaturated fatty acids by regioselective enzymatic acylation using *Candida Antarctica* lipase B were used. The antiproliferative effects of these six phloridzin fatty acid esters were investigated in comparison with the parent compounds, phloridzin, aglycone phloretin, the six free fatty acids, and a standard hepatocellular carcinoma drug, sorafenib (Nexavar®) using human hepatocellular carcinoma cells, HepG2, and normal human liver cells. In comparison with phloridzin and free fatty acids, phloridzin fatty acid esters significantly inhibited the growth

of HepG2 cells while similar treatment had a little effect on normal human hepatocytes. The antiproliferative potency of phloridzin fatty acid esters was comparable or greater than the potency of sorafenib. To further explore the potential mechanism(s) of antiproliferation, the activities of these new compounds on DNA topoisomerases IIα activity, cell cycle and apoptosis were determined. Phloridzin fatty acid esters inhibited DNA topoisomerases IIα activity that might induce G0/G1 phase arrest, and apoptosis via activation of caspase-3 in HepG2 cells. These results suggest that phloridzin fatty esters have potential chemopreventive effects mediated through inhibition of DNA topoisomerases IIα followed by cell cycle arrest and apoptosis. Phloridzin fatty acid esters have the anticancer potential against liver cancer cells in vitro and deserve further investigation.

[00160] Test Compounds and chemicals: Fatty acid esters of phloridzin (Pz) viz. stearic acid ester of Pz (Pz-stearic acid), oleic acid ester of Pz (Pz-oleic acid), linoleic acid ester of Pz (Pz-linoleic acid), α-linolenic acid ester of Pz (Pz-α-linolenic acid), DHA ester of Pz (Pz-DHA) and eicosapentaenoic acid ester (EPA) of Pz (Pz-EPA) were synthesised as described in Example 1 above.

[00161] Phloridzin, phloretin, caspase 3 colorimetric assay kit, propidium iodide, fatty acids namely oleic, stearic, linoleic, α-linolenic, eicosapentaenoic and docosahexanoic acids were purchased from Sigma-Aldrich (Mississauga, ON, Canada). Cell Titer 96 Aqueous One solution cell proliferation (MTS) assay, CytoTox 96 non-radioactive cytotoxicity (LDH) assay, and CellTiter-Glo® luminescent assay kits were purchased from Promega (Promega, Madison, WI), Sterile dimethyl sulfoxide (DMSO) (ATCC, Manassas, VA, USA), GFP-certified™ apoptosis/necrosis detection kit for microscopy from Enzo Lifesciences (Brockville, ON, Canada) and ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit from Invitrogen (Burlington, ON) DCFDA-Cellular Reactive Oxygen Species detection assay kit form Abcam, Toronto, ON, Canada; and 5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) from Cayman Chemicals, Burlington, ON, Canada were also used for the study.

[00162] Cell lines and culture conditions: Human hepatocellular carcinoma cell line (HepG2) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured as recommended by the ATCC. HepG2 cells were grown in Eagle's modified minimum essential media (EMEM) supplemented with 10% FBS (FBS; ATCC, Rockville, MD), and 1% penicillin-streptomycin (ATCC, Rockville, MD, USA). THP-1 cells were cultured in RPMI-1640 media supplemented 0.05 mM 2-mercaptoethanol and 10% fetal bovine serum to a final concentration of 10%. MDA-MB-231 breast cancer cells (ATCC

HTB-26™) were obtained from Cedarlane, Berlington, ON, Canada) and were maintained in DMEM medium (Sigma-Aldrich Canada) supplemented with 100 u/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, 5 mM HEPES (pH 7.4) and 10% heat-inactivated fetal bovine serum (Invitrogen, Burlington, ON, Canada).

[00163] Cryopreserved normal human hepatocytes (HP-F), hepatocyte plating medium and hepatocyte maintenance medium were purchased from Zen-Bio, Research Tiangle Park, NC, USA. Normal human hepatocytes plated on 96 well collagen 1 coated cell culture plates (Life Technologies) and maintained in hepatocyte maintenance medium for 24 h to allow for cell recovery and attachment. Rat hepatocytes (RTCP10), thawing media and incubation media were purchased from Life Technologies. Rat hepatocytes were plated in collagen 1 coated 96 well plates (Life Technologies, Burlington, ON, Canada) using thawing media and maintained in incubation medium. All cell types were maintained at 37°C in an incubator under 5% CO_2/95% air atmosphere at constant humidity.

[00164] Cells were counted using a haemocytometer (Bright-Line Hemacytometer, Sigma-Aldrich (Mississauga, ON) and were plated accordi^{ng} to the number of cells for each experiment in 6, 24 or 96 well format for 24 h prior to addition of test samples. All the test samples were solubilised in sterile filtered DMSO (< 0.5% in the culture medium) prior to addition to the culture media. Control cells were also run in parallel and subjected to the same changes in media with < 0.5% DMSO.

[00165] Cell Proliferation assay: Cell viability was determined by using the MTS assay. In brief, HepG2 cells (5×10^3 cells/100 µL/well), MDA-MB-231 (5×10^3 cells/100 µL/well), THP-1 (25×10^3/100 µL/well), normal human and rat hepatocytes (1×10^4 cells/100 µL/well) were plated in triplicate, in a 96 well sterile flat bottom tissue culture plates. After 24 h incubation, phloridzin fatty esters, phloridzin, phloretin, free fatty acids of respective esters or sorafenib were prepared in media and 100 µL of each treatment was added to each well, each treatment in three replications. Thereby, cells were exposed to various concentrations (0.1, 1, 10, 25, 50, 75, 100 µM) of each treatment. Controls consist of cells with media containing DMSO (<0.5%), test blank wells contained the test compound in media with no cells and blank wells contained media with no cells. After additional 3, 6, 12, 18 or 24 h, 20 µL of the MTS reagent in combination with the electron coupling agent, phenazine methosulfate were added to the wells and cells were incubated in a humidified incubator for 3 h. Absorbance at 490 nm (OD490) was measured by using a Flurostar Optima microplate reader (BMG Labtech, Cary, NC, USA) to obtain the number of viable cells relative to the control

population. Percentage of viability of the test compound treated cells are expressed as percentage compared to control (<0.5% DMSO). EC_{50} values (concentration required to reduce cells viability by 50% as compared to control cells) for each test compound was analysed using Graphpad Prism software, La Jolla, CA, USA. The selective index (SI) of the fatty acid esters of phloridzin is defined as the ratio of cytotoxicity (EC_{50} values) on normal HP-F cells to solid cancer HepG2, MDA-MB-231 cells (SI=EC_{50} on HP-F cells/EC_{50} on solid cancer cells). Samples with SI values greater than three were considered to have high selectivity towards cancer cells.

[00166] Data obtained revealed that 100 μM of oleic, linolenic, α-linolenic, EPA and DHA esters of phloridzin induced proliferation inhibition as early as 6 h. A moderate inhibition of cell viability was found after 6 h treatment with 100 μM stearic acid (68.0±5.0%) and sorafenib (67.9±5.3%). The most profound effects of all the saturated, mono- and poly-unsaturated fatty acid esters of phloridzin (100 μM) and commercial drug, sorafneib were achieved at 24 h. The treatment of cells with 10 μM of phloridzin oleic acid ester (Pz-Oleic acid) for 96 h caused a significant ($P < 0.001$) decrease in cell viability (4.2±0.55%). Phloretin showed inhibition at 200 μM from 6 h where as its lower concentration showed no effect. The parent compounds of phloridzin fatty esters viz, phloridzin and free fatty acids showed no inhibition on HepG2 cell proliferation.

[00167] The dose required to reduce cancer cell growth by 50% (EC_{50}) was also calculated. Lower EC_{50} value is indicative of greater inhibitory activity. Phloridzin DHA ester (Pz-DHA) had the lowest EC_{50} values of 30.1 μM followed by phloridzin fatty acid esters with linoleic acid (31.7 μM), EPA (39.4 μM), α-linolenic acid (41.8 μM), oleic acid (44.8), stearic acid (51.6 μM) and sorafneib (52.0 μM). EC_{50} values indicate the antiproliferative activities of fatty acid esters of phloridzin are comparable or even better than that for sorafenib.

[00168] Notably, there were slight (10-16%) but statistically significant ($p < 0.001$) inhibition of the growth of the normal hepatocytes (NHeps) by the linoleic, α-linolenic, phloridzin EPA esters (Pz-EPA) and sorafneib (100 μM at 24 h) compared to the control. Oleic acid, stearic acid and DHA esters of phloridzin have no significant proliferation inhibition of normal cells compared to the control. DHA ester of phloridzin demonstrated to have the highest viability (93.8±2.4%) and least inhibition of normal hepatocyte growth. Thus, the cancer cells were more sensitive to the phloridzin fatty acid esters compared to the normal cells. These results indicate a marked selectivity of the phloridzin fatty acid esters towards the liver cancer cell line compared to the normal cells.

[00169] Cytotoxicity assay: Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon membrane damage in apoptotic/necrotic cells. LDH act^{i}vity was measured using CytoTox 96 Non-Radio$_{ac}$tive Cytotoxicity $_{A}$ssay (Promega, Madison, WI), in which LDH released in culture supernatants is measured with a coupled enzymatic assay, resulting in conversion of a tetrazolium salt into a red formazan product. HepG2 cells (5000/100 µl/well) were seeded and treated with 100 µM of phloridzin fatty acid esters, phloridzin, phloretin, free fatty acids of respective esters or sorafenib prepared in serum free media and incubated (37°C / 5% CO_2) for 6 h. After centrifugation, the supernatant was removed to an assay plate, and the LDH released from the cells into culture medium was measured. The maximal release was obtained after treating control cells with 1% Triton X-100 for 30 minutes at room temperature. The apoptotic/necrotic percentage was expressed using the formula: (sample value/maximal release) × 100%. Previous studies by the supplier had clearly stated that in HepG2 cells, LDH activity maximum concentration is at 1 to 6 h incubations because LDH activity released from cells has a half-life of approximately 9 h (Riss and Moravec, 2004). MTS assay results showed that all the phloridzin fatty acid esters inhibited 70-80% cell proliferation in 6 h, therefore, we analysed LDH activity after 6 h incubation.

[00170] There was a significant increase in LDH activity ($P<0.001$) at 6 h compared to sorafenib, phloridzin and phloretin. Maximum LDH release was showed by phloridzin EPA ester (82.7±1.5%) and least by phloretin (7.9 ±0.5 %) compared to the positive control (Maximum release by 1% Ttriton-10X treated cells and incubated for 30 min).

[00171] Morphologic observation under inverted phase contrast microscope: HepG2 cells were equally seeded in 24-well flat bottom tissue culture treated plates (BD Biosciences), and then treated with 100 µM of phloridzin fatty acid esters, phloridzin, phloretin, sorafenib and DMSO (<0.5%) control. After 24 h of treatment, the morphology of HepG2 cells was observed under an inverted phase contrast microscope (Nikon Eclipse E 100, Nikon, ON) and images were captured at 400X magnification using Infinity digital microscopy camera (Lumenera corporation, Ott^{a}wa, ON, C$_{a}$nada).

[00172] The images showed a distorted membrane structure, shrinkage of the cells and the nucleus as well as condensation of nuclear chromatin into sharply delineated masses. The cell detaches from the plate and its outlines become convoluted and form extensions. These cells showed morphological changes which are characteristic to apoptosis. Phloridzin treated cells have shown no effect and morphology was similar to the control cells.

[00173] Determination of Apoptosis/Necrosis by Fluorescence Microscopy: HepG2 cells were seeded into Nunc Lab-Tek two chamber slide (Sigma-Aldrich, Mississauga, ON, Canada) at a density of 1×10^6 cells/chamber. The attached cells were then treated either with 100 µM phloridzin fatty acid esters, phloridzin, phloretin, sorafenib or DMSO vehicle (as control) for 24 h. The slides were washed with PBS. After removing the chamber, each slide was added with 50 µl of Dual Detection Reagent containing apoptosis detection reagent (Annexin V-EnzoGold) and necrosis detection reagent (7-AAD) in 1X binding buffer. The samples were incubated at room temperature for 15 min in the dark. After staining, the cells were washed with binding buffer and covered with a glass coverslip. The stained cells were observed under a fluorescence Zeiss Axiovert 200 m inverted microscope (Carl Zeiss, ON, Canada) at magnification of ×40 with a filter set for Annexin V-EnzoGold (Ex/Em: 550/570 nm) and 7-AAD (Ex/Em: 546/647 nm).

[00174] Fluorescence imaging was conducted to visually differentiate between apoptosis induced and necrotic cell death. After incubation of HepG2 cells with 100 µM of phloridzin fatty acid esters for 24 h, the number of cells remaining as an adherent monolayer was greatly decreased compared to the control cells. In addition, floating cells showed morphological changes, with characteristics similar to apoptosis or necrosis. The cells were co-incubated with AnnexinV Enzogold (enhanced cyanine), an early marker of phosphatidylserine externalization at the cell membrane and red emitting dye 7-AAD, marker of late apoptosis or necrosis. Upon exposure to phloridzin fatty acid esters most of the HepG2 cells treated with fluorescent dyes stained green for Annexin-V denoting apoptosis and less number of cells stained red for 7-AAD indicating late apoptosis or necrosis. DMSO (<0.5%) control, phloridzin and phloretin treated cells showed less colour indicate less apoptotic death of cells. The results suggested that phloridzin fatty acid esters induced marked apoptotic and less necrotic morphology in HepG2 cells.

[00175] Analysis of apoptosis by DNA Fragmentation: HepG2 (1×10^5) cells were seeded in 24-well culture plates and were allowed to adhere overnight. Following this, cells were treated either with 100 µM phloridzin fatty acid esters, phloridzin, phloretin, sorafenib or DMSO vehicle (as control). The plates were re-incubated for another 24 h. DNA fragmentation was detected using ApoTarget™ Quick Apoptotic DNA Ladder Detection Kit (Invitrogen, Burlington, ON) according to the manufacturer's protocol. The principle involves detecting the internucleosomal DNA fragments formed during apoptosis. Briefly, floating dead cells and trypsinized adherent cells were collected and centrifuged at 1,000 rpm for 10 min. After washing with PBS, the cells were lysed with 35 µl TE lysis buffer (a kit

component). To the lysate, 5 µl of Enzyme A (a kit component) was added and incubated at 37°C for 10 min. Afterwards, 5 µl of Enzyme B (a kit component) was added, gently mixed and incubated at 50°C for 30 min. The DNA was precipitated with the ammonium acetate and absolute ethanol at -20°C. After centrifugation (10 minutes at 12,000 rpm) and air drying, the DNA pellet was dissolved in 30 µl of DNA suspension buffer. For detecting the DNA ladder, the extracted DNA samples were run on a 1.2% agarose gel containing 0.5 µg/ml gel red in Tris-Borate-EDTA (TBE) buffer. After electrophoresis, the gel image was captured using Gel Doc 100 system (Bio-Rad, Mississauga, ON, Canada).

[00176] Treatments with DMSO (<0.5%) control (C) or free fatty acids of respective esters phloridzin or phloretin showed only intact genomic DNA and do not marked any DNA laddering or even smearing effect. The pattern of laddering of DNA from phloridzin fatty acid esters or sorafenib treated cells is a characteristic that is commonly associated with apoptotic process, in which the DNA is cleaved into fragments of 180-200 base pairs (bp) by the endogenous endonucleases.

[00177] Assay of Caspase-3 Activity: The activity of caspase 3 enzyme was measured using caspase 3 colorimetric assay kit purchased from Sigma-Aldrich (Mississauga, ON, Canada). HepG2 cells (2×10^6 cells/well), grown in 6-well plates, were treated either with 100 µM phloridzin fatty acid esters, phloridzin, phloretin, sorafenib or DMSO vehicle (as control). Cells were lysed and the protein content of cell lysate was quantified by the BCA protein assay (Thermo Fisher Scientific Inc., Ottawa, ON, Canada). Caspase-3 activity was measured in the 200 µg of cell lysate using the caspase-specific peptide substrate, DEVD (Asp-Glu-Val-Asp), conjugated to reporter p-nitroanaline (ρ-NA) molecules. Cleavage of this peptide by caspase releases the chromophore which is measured colorimetrically at a wavelength of 405 nm as described in the supplier's protocol.

[00178] Incubation of HepG2 cells with phloridzin fatty esters or sorafenib for 24 h induced a significant ($P<0.001$) increase in caspase 3 activity compared to DMSO treated control at 24 h. These findings clearly demonstrated that phloridzin fatty acid esters induces caspase 3 activation that is required for execution of apoptotic cell death. Phloridzin and phloretin showed enhanced caspase 3 activity but significantly ($P<0.001$) lower than phloridzin fatty acid esters or the drug.

[00179] Cell Cycle Analysis: HepG2 cells were plated at 5×10^5 cells per ml in a six-well plate. After 24 h incubation (37°C, 5% CO_2), the cells were treated with 100 µM phloridzin fatty acid esters, phloridzin, phloretin, sorafenib or DMSO (<0.5%) control prepared in media and incubated for additional 24 h. Following trypsinization, cells were washed and

centrifuged at 2000×g for 10 min and the pellet re-suspended in 0.5 ml PBS. Fixation was completed by adding 1.2 ml of 70% cold ethanol for 2 h. The fixed cells were washed with PBS and centrifuged at 2000 × g for 10 min. After suspending cells in 0.3 ml PBS, 8 µl of DNAase free RNAse (10 mg/ml) was added and incubated for 1 h. After adding, 15 µl of propidium iodide (0.5 mg/ml), cells were incubated in 4°C for 30 minutes. The cells were analyzed for cell cycle using flow cytometer FACS calibur (Beckman Coulter, Fullerton, CA) with an excitation wavelength of 488 nm and emission at 670 nm. DNA content was determined by ModFit software (Verity Software House, Topsham, ME), which provided histograms to evaluate cell cycle distribution.

[00180] Treatment of HepG2 cells with 100 µM of phloridzin esters of saturated, mono- or poly-unsaturated fatty acids resulted in a significantly higher number of cells in the G0/G1 phase (52 to 60%) compared with control (48%). Potency of phloridzin oleic, α-linolenic acid, DHA and EPA esters to induce G0/G1 phase arrest was similar to prescribed commercial drug sorafenib. As, in each case, there was a concomitant reduction in the number of cells in the S and G2M phases. This experiment suggests that phloridzin esters of fatty acids induce G0G1 phase cell cycle arrest in HepG2 cells.

[00181] ATP level assay: Cellular ATP levels were measured with CellTiter-Glo® luminescent assay kit obtained from Promega according to the manufacturer's instructions. HepG2 cells plated on a black walled clear bottom 96-well plate were incubated with 100 µM fatty acid esters of phloridzin, phloridzin, phloretin, sorafenib, free fatty acids or DMSO (<0.5%) control in media. After 24 h, CellTiter-Glo®Reagent equal to the volume of cell culture medium present in each well and mixed contents for 2 min on an orbital shaker to induce cell lysis. Luminescence was recorded on Flurostar Optima microplate reader (BMG Labtech) after incubation at room temperature for 10 min to stabilize luminescent signal. The level of ATP in a sample was presented as percentage compared to untreated control.

[00182] All the test compounds at 100 µM inhibited pHOT DNA relaxation and most of the pHOT DNA remained in supercoiled state. VP-16, an interfacial poison showed linear band and less potent than catalytic inhibition induced by phloridzin fatty acid esters. Inhibition of topo IIα is a good target for anticancer drugs.

[00183] Mitochondrial membrane potential (MMP): HepG2 cells were seeded in a black walled clear bottom 96-well sterile flat bottom tissue culture plates (BD Biosciences, USA) at a density of 5×10^4 cells/well (100 µL) and incubated in a CO_2 incubator for 24 h at 37°C. Cells were treated with 100 µM fatty acid esters of phloridzin, phloridzin, phloretin, sorafenib, free fatty acids or DMSO (<0.5%) control prepared in media and incubated for 24

h. The staining solution JC-1 was prepared with PBS and 5 μM was added to each well. The cells were further incubated in a CO_2 incubator at 37°C for 1 h. After washing the plate with PBS twice, the fluorescence was measured using a Fluostar Optima microplate reader (BMG Labtech) at 535 nm for JC-1 monomers and at 590 nm for JC-1 aggregates

[00184] Human Topoisomerase IIα (topo IIα) Catalytic Activity: The topo IIα catalytic activity was monitored via electrophoresis using topoisomerase II drug screening kit (TopoGEN, Inc., Columbus, Oh, USA). Briefly, 20 μl of reaction mixtures contained 0.5 M Tris-HCl, pH 8.0, 1.50 M NaCl, 100 mM $MgCl_2$, 20 mM ATP, 300 μg BSA/ml and 5 mM dithiothreitol. Supercoiled DNA (pHOT1 DNA), supercoiled provided in the kit was determined to be ideal for this assay because it is small and easy to handle and has a large number of topo IIα recognition elements. After 2 μl (0.25 μg) of pHOT1 DNA was added, followed by the addition of 100 μM phloridzin fatty acid esters, phloridzin, phloretin, sorafenib or DMSO (<0.5%) control in solvent, the reaction was initiated by adding 4 units (2 μl) of human DNA topo IIα and carried out at 37 °C for 30 min. The reaction was terminated by adding 2 μl of 10% sodium dodecyl sulfate (SDS) followed by digestion with 2 μl of proteinase K (50 μg/ml) at 37 °C for 15 min to degrade enzyme. After adding 2 μl of loading buffer (0.25% bromophenol blue and 50% glycerol) was added to the mixture, samples were loaded onto 1% agarose gel. Electrophoresis was conducted at 66 V (2 V/cm) for 5 h in TBE buffer using Biorad Electrophoretic Gel System (Bio-Rad, Hercules, CA). Supercoiled DNA (pHOT1 DNA) and relaxed DNA were included in the electrophoresis run as markers for DNA topology. Gels were then stained in 0.5 μg/ml gel red in TBE for 30 min and destained for 15 min in distilled water prior to digital image acquisition using Gel Doc 100 system (Bio-Rad, Hercules, CA). One unit of topoisomerase II activity was defined as the minimum amount of enzyme required to achieve complete relaxation of 0.5 mg superhelical pHOT1 DNA in 30 min at 37° C. Inhibition of topoisomerase II relaxation activity was investigated by the same procedure using four units of enzyme and 100 μM test compounds. The percent of inhibition was calculated by the following formula:

[00185]
$$\frac{(S - S_0)}{S_{control} - S_0} \times 100\ (\%)$$

[00186] Where $S_{control}$ is the percent of supercoiled DNA in the control lane (without enzyme and test compounds), S_0 is the percent of supercoiled DNA in the lane without test

compounds and S is the percent of supercoiled DNA in the lane with test compounds and enzyme.

[00187] Real Time RT-PCR analysis: Gene expression profiles were obtained from HepG2 cells treated with DHA esters of phloridzin or sorafenib or DMSO treated control cells. Total RNA extraction was performed using Arum Total RNA minikit (Bio-Rad, Hercules, CA, USA). RNA concentration and purity was determined by measuring the absorbance using a NanoDrop (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was assessed by formaldehyde agarose gel electrophoresis. RNA (400 ng) was used to synthesize cDNA using RT^2 First Strand kit (SABiosciences, Frederick, MD, USA). RT^2 RNA QC PCR arrays (SABiosciences, Frederick, MD, USA) was used to assess the quality of cDNA samples before characterization with the human cancer drug targets RT^2 profiler™ PCR array (SABiosciences, Frederick, MD, USA). Gene expression profiles of 84 genes were investigated using the human cancer drug targets RT^2 profiler™ PCR array (PAHS-507ZD) on a Bio-Rad CFX Connect (Bio-Rad, Hercules, CA, USA) using RT^2 real-time SYBR green PCR master mix (SABiosciences, Frederick, MD, USA). The array also has five reference genes (beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase 1 (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and actin beta (ACTB), three reverse transcription controls (RTCs), three positive PCR controls (PPCs), and one genomic DNA control (GDC), making up to 96 assays. After normalization with RPL13A reference gene, gene expression levels were individually assessed by using the threshold cycle (Ct) values using RT^2 profiler PCR array data analysis software (Microsoft Excel-based program of SABiosciences, Mississauga, ON, Canada). It calculates: 1) ΔCt of each gene = Ct of gene of interest - average Ct of chosen reference genes 2) ΔΔCt for each gene across two groups; ΔΔCt = ΔCt (Pz-DHA ester or sorafenib) - ΔCt (control) & 3) fold-change for each gene from control group to phloridzin DHA ester treated group as $2^{(-\Delta\Delta Ct)}$. RT^2 RNA QC PCR data showed no genomic DNA contamination (Ct <35 will indicate least GDC) or presence of impurities in RNA samples based on the Ct value of PPC (Ct should be 20 ± 2 on each array) and showed no inhibition of reverse transcription based on the Ct values of RTC and PPC. Reproducibility was maintained by using three biological replicates from three individual experiments.

[00188] Statistical analyses: EC_{50} values were calculated using Graphpad Prism 6 software (GraphPad Software Inc., San Diego CA). Statistical analysis was performed using Statistical Analysis System (SAS, Version 9.2). One-way ANOVA with Tukey's post hoc comparisons

at $P<0.001$ was used for statistical comparisons. All data are presented as a mean value with its standard deviation indicated (Mean ± SD).

[00189] In conclusion, this study demonstrated that the chemopreventive potential of six saturated, mono- and poly-unsaturated fatty acid esters of phloridzin. Phloridzin-DHA ester has the greatest potential and efficacy as a chemopreventive agent. These esters showed very low cytotoxicity to normal cells revealing their specific action against the cancer cells. The HepG2 cell growth inhibition mechanism of the fatty acid esters of phloridzin is related to inhibition of apoptosis and cell cycle arrest. Novel compounds inhibited topo IIα and triggered DNA damage. DHA ester of phloridzin has the greatest potential and efficacy as a chemopreventive agent. The anti-proliferative properties of the ester seems to be through a mechanism that down regulate key signalling pathways including PI3K/AKT/mTOR.

[00190] Any improvement may be made in part or all of the compositions, kits, and method steps. All references, including publications, patent applications, and patents, cited herein are hereby incorporated by reference. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended to illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. Any statement herein as to the nature or benefits of the invention or of the preferred embodiments is not intended to be limiting, and the appended claims should not be deemed to be limited by such statements. More generally, no language in the specification should be construed as indicating any non-claimed element as being essential to the practice of the invention. This invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contraindicated by context.

What is claimed is:

1. An acylated flavonoid derived from:
    a. a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and
    b. an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.
2. The acylated flavonoid of claim 1, wherein the acylated flavonoid is selected from the group consisting of stearic acid ester of phloridzin, oleic acid ester of phloridzin, linoleic acid ester of phloridzin, alpha-linolenic acid ester of phloridzin, docosahexaenoic acid ester of phloridzin, eicosapentaenoic acid ester of phloridzin, stearic acid ester of isoquercetrin, oleic acid ester of isoquercetrin, linoleic acid ester of isoquercetrin, alpha-linolenic acid ester of isoquercetrin, docosahexaenoic acid ester of isoquercetrin, and eicosapentaenoic acid ester of isoquercetrin.
3. A pharmaceutical composition comprising:
    a. a pharmaceutically acceptable carrier; and
    b. an acylated flavonoid derived from:
        i. a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and
        ii. an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.
4. The pharmaceutical composition of claim 3, wherein the pharmaceutical composition decreases proliferation and viability of cancer cells.
5. The pharmaceutical composition of claim 3, wherein the pharmaceutical composition induces apoptosis of cancer cells.

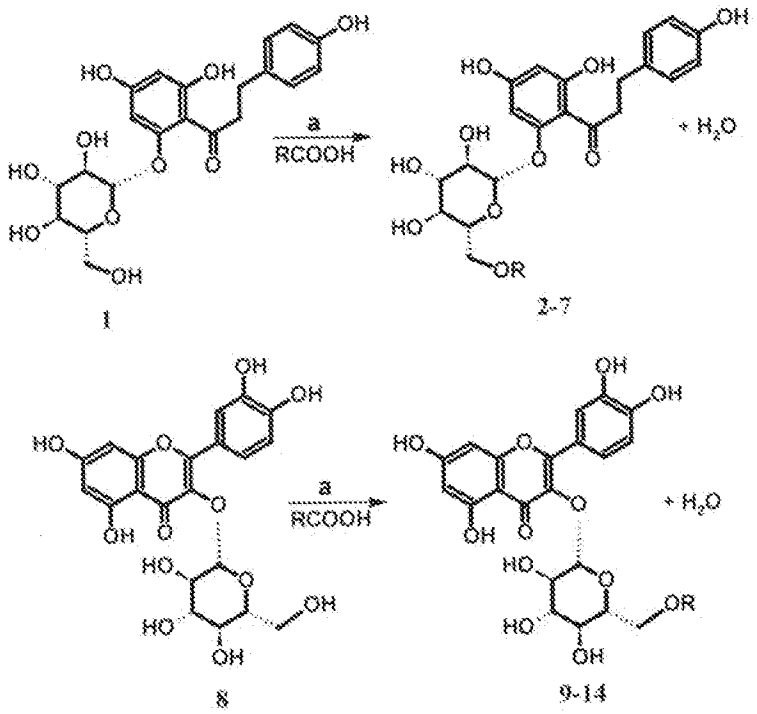
6. The pharmaceutical composition of claim 3, wherein the acylated flavonoid is selected from the group consisting of stearic acid ester of phloridzin, oleic acid ester of phloridzin, linoleic acid ester of phloridzin, alpha-linolenic acid ester of phloridzin, docosahexaenoic acid ester of phloridzin, eicosapentaenoic acid ester of phloridzin, stearic acid ester of isoquercetrin, oleic acid ester of isoquercetrin, linoleic acid ester of isoquercetrin, alpha-linolenic acid ester of isoquercetrin, docosahexaenoic acid ester of isoquercetrin, and eicosapentaenoic acid ester of isoquercetrin.
7. The pharmaceutical composition of claim 3, further comprising at least one additional anti-cancer drug selected from the group consisting of sorafenib, doxorubicin, methotrexate, vinblastine, vincristine, cladribine, fluorouracil, cytarabine, anthracyclines, cisplatin, cyclophosphamide, fludarabine, gemcitabine, aromatase inhibitors, irinotecan, navelbine, oxaliplatin, taxol, docetaxel, bevacizumab, pegaptanib, and ranibizumab.
8. The pharmaceutical composition of claim 3, further comprising at least one excipient selected from the group consisting of inert diluents or fillers, granulating and disintegrating agents, binding agents, lubricating agents, glidants, antiadhesives, colorants, flavoring agents, plasticizers, humectants, and buffering agents.
9. A method of decreasing proliferation and viability of cancer cells in a patient in need thereof, comprising:

   administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising:

   a. a pharmaceutically acceptable carrier; and
   b. an acylated flavonoid derived from:
      i. a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and
      ii. an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.
10. The method of claim 9, wherein the pharmaceutical composition decreases proliferation and viability specifically of cancer cells compared to noncancerous cells.

11. The method of claim 9, wherein the pharmaceutical composition is in an amount effective for inducing apoptosis of the cancer cells.
12. The method of claim 9, wherein the cancer cells are selected from the group consisting of: breast cancer cells, liver cancer cells, and acute monocytic leukemia cells.
13. The method of claim 9, wherein the cancer cells are human cancer cells.
14. The method of claim 9, wherein the pharmaceutical composition activates caspase-3 family member activation and induces s-phase cell cycle arrest in the cancer cells.
15. The method of claim 9, wherein the acylated flavonoid is selected from the group consisting of stearic acid ester of phloridzin, oleic acid ester of phloridzin, linoleic acid ester of phloridzin, alpha-linolenic acid ester of phloridzin, docosahexaenoic acid ester of phloridzin, eicosapentaenoic acid ester of phloridzin, stearic acid ester of isoquercetrin, oleic acid ester of isoquercetrin, linoleic acid ester of isoquercetrin, alpha-linolenic acid ester of isoquercetrin, docosahexaenoic acid ester of isoquercetrin, and eicosapentaenoic acid ester of isoquercetrin.
16. The method of claim 9, wherein the step of administering is by parenteral, subcutaneously, intravenously, intramuscularly, or intraperitoneally.
17. A method of treating cancer in a subject having breast, liver or acute monocytic leukemia cancer, the method comprising:
    administering to the patient a therapeutically effective amount of a pharmaceutical composition comprising:
        a. a pharmaceutically acceptable carrier; and
        b. an acylated flavonoid derived from:
            i. a flavonoid selected from the group consisting of phloridzin, luteolin, isoquercetrin, and quercetin; and
            ii. an acylating agent selected from the group consisting of long chain saturated or poly-unsaturated fatty acids, wherein the fatty acids are selected from the group consisting of stearic, oleic, linoleic, alpha-linolenic, docosahexaenoic, eicosapentaenoic, palmitic, cinnamic, phenylpropionic, butyric, octanoic, and dodecanoic.

18. The method of claim 17, wherein the acylated flavonoid is selected from the group consisting of stearic acid ester of phloridzin, oleic acid ester of phloridzin, linoleic acid ester of phloridzin, alpha-linolenic acid ester of phloridzin, docosahexaenoic acid ester of phloridzin, eicosapentaenoic acid ester of phloridzin, stearic acid ester of isoquercetrin, oleic acid ester of isoquercetrin, linoleic acid ester of isoquercetrin, alpha-linolenic acid ester of isoquercetrin, docosahexaenoic acid ester of isoquercetrin, and eicosapentaenoic acid ester of isoquercetrin.
19. The method of claim 17, wherein the step of administering is by parenteral, subcutaneously, intravenously, intramuscularly, or intraperitoneally.
20. A kit for treating cancer in a subject, the kit comprising:
    (a) the pharmaceutical composition of claim 1 in a therapeutically effective amount;
    (b) instructions for use; and
    (c) packaging.



a) Acetone, 3 °A molecular sieves, Novozyme 435®, 45 °C, Stirring, 24 h; R = Oleic, Stearic, Linoleic, Linolenic, Eicosapentaenoic (EPA), and Docosahexaenoic acids or their esters

Scheme 1

FIG. 1

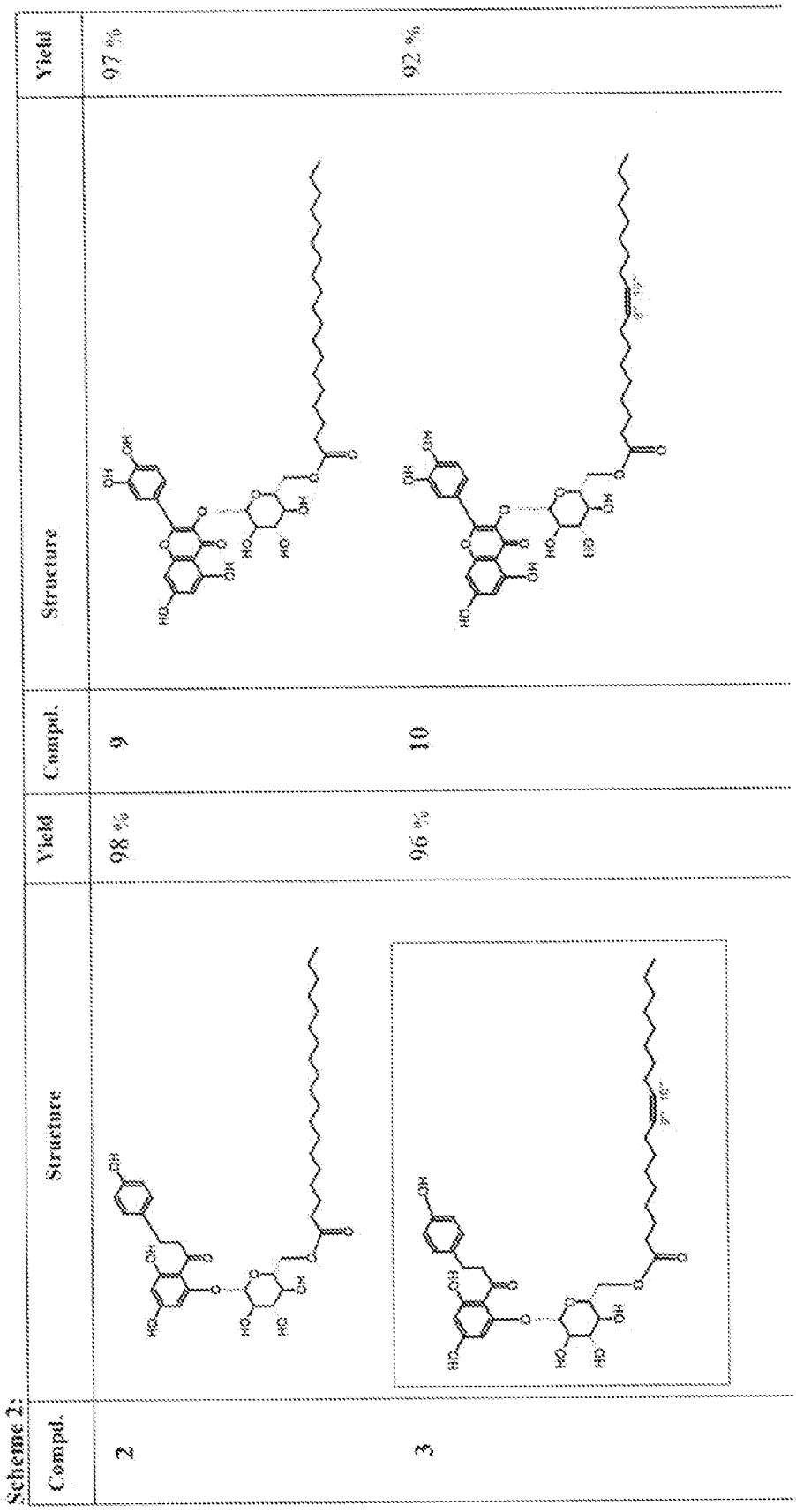


FIG. 2a

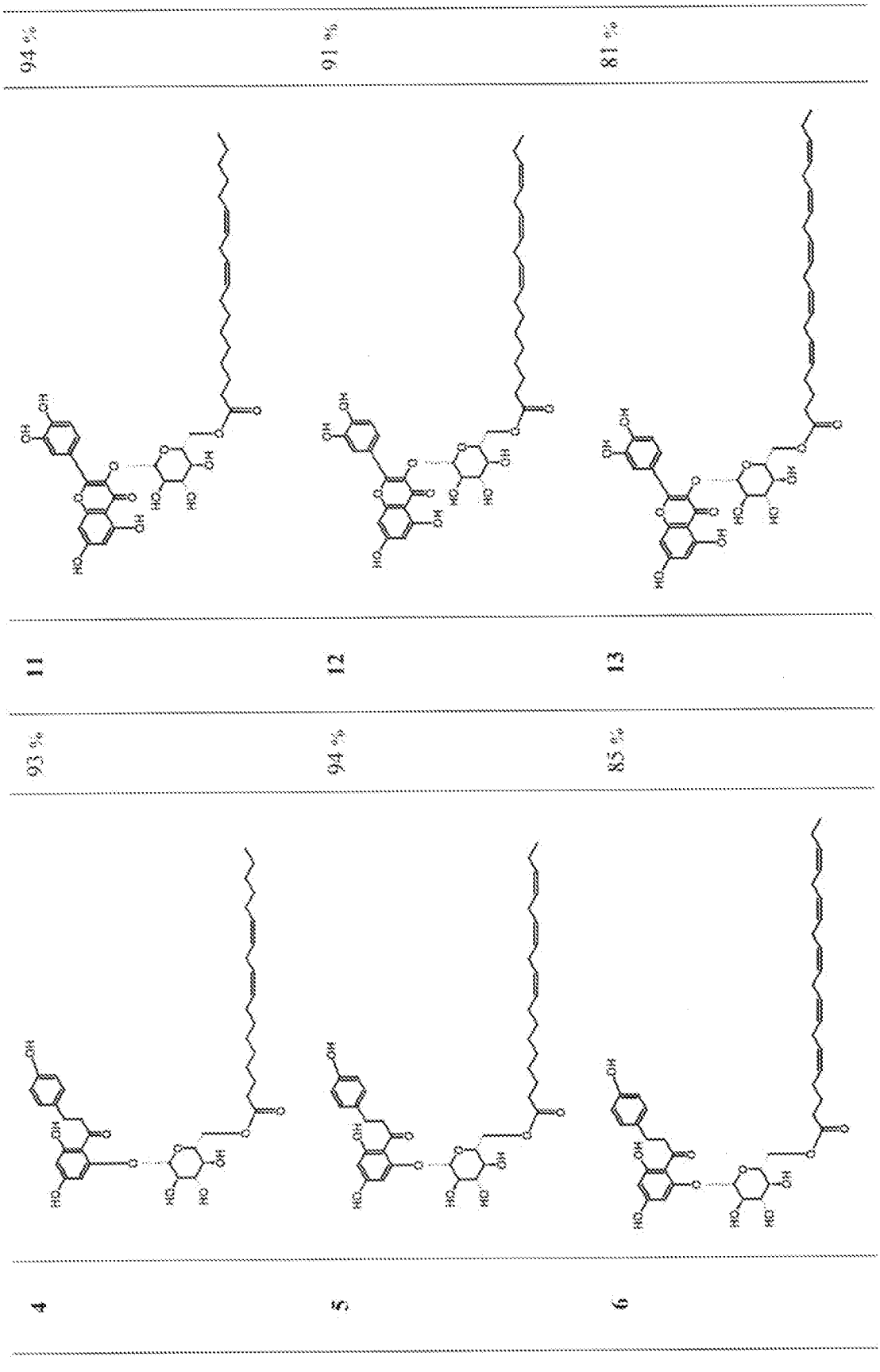


FIG. 2.b

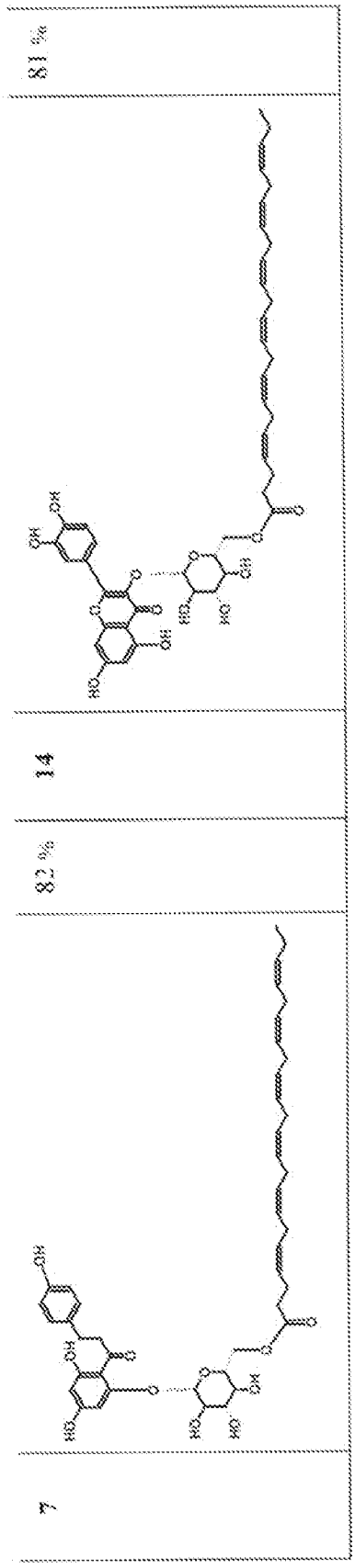
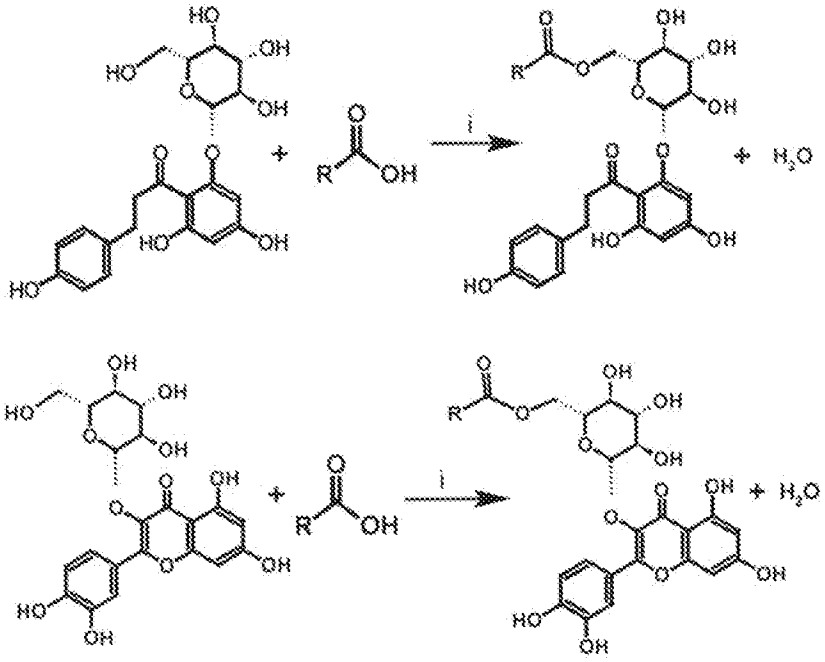


FIG. 2c



i) Immobilized lipase B, from *Candida antarctica* (Novozyme 435®), 3 °A molecular sieves, ultrasonication, Acetone, 40-45 °C, 2-3.5 h or ultrasonication coupled with stirring (3-5 h); R = Butyric, Octanoic, Dodecanoic, Palmitic, Oleic, Stearic, Linoleic, Linolenic, Eicosapentaenoic, Docosahexaenoic Acids or their corresponding esters.

FIG. 3

Flavonoids	Fatty acid	Structure of the Product	Reaction Time			Yield[a]
			Conventional[42]	Ultrasonication	Ultrasonication and Stirring	
Phloridzin	Butyric Acid ($C_4H_8O_2$)	2	24h	3.5h	2h	81%[b]
Phloridzin	Octanoic acid ($C_8H_{16}O_2$)	3	24h	3.5h	2h	84%[b]

FIG. 4a

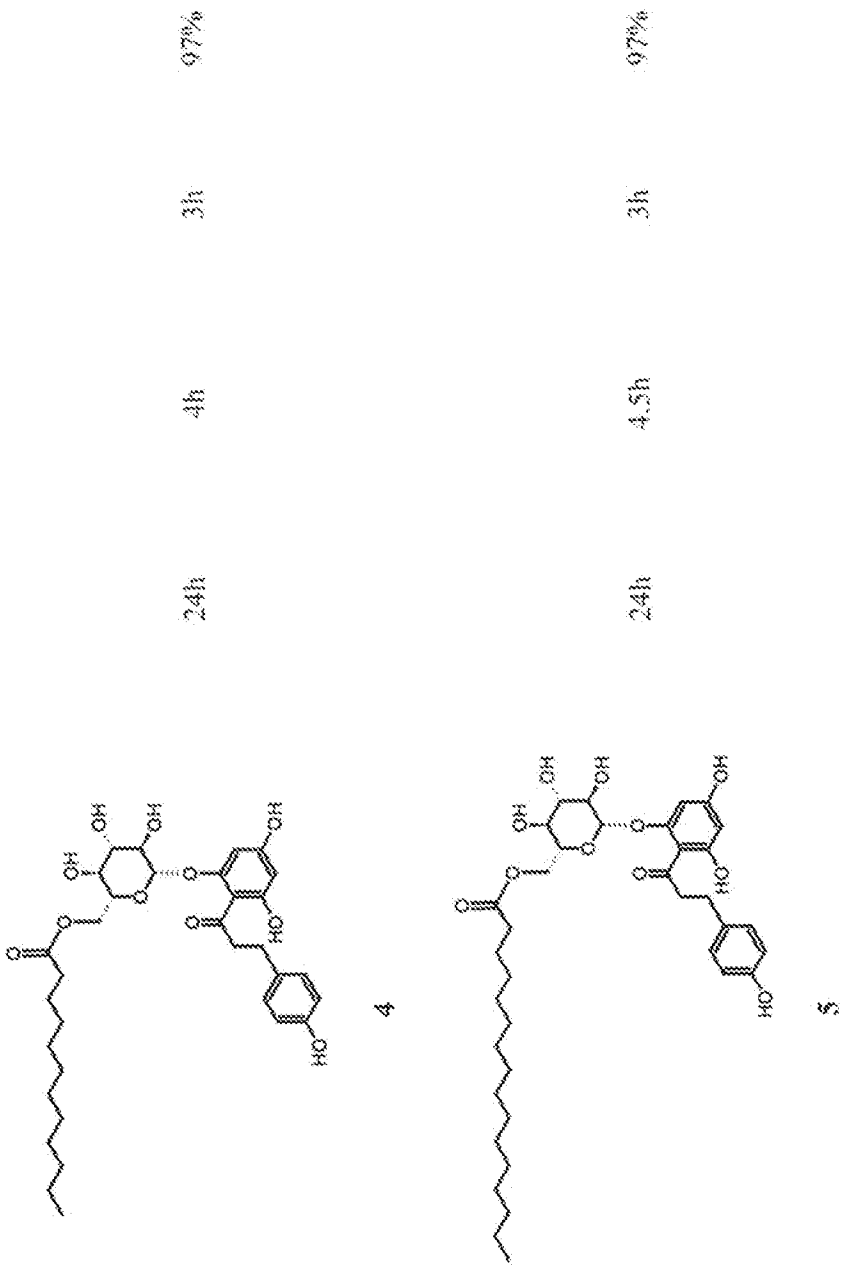
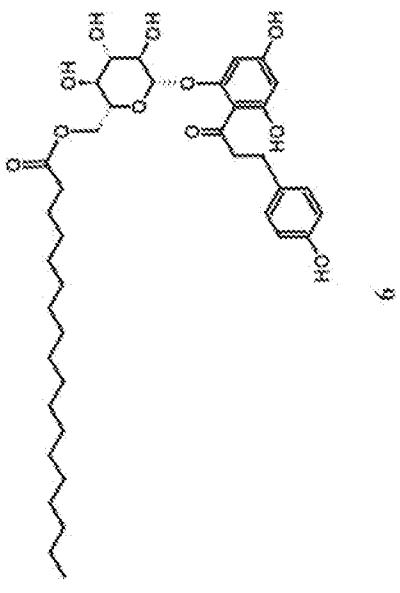

Phloridzin
Dodecanoic acid
($C_{12}H_{24}O_2$)
24h
4h
3h
97%
4
Phloridzin
Palmitic acid
($C_{16}H_{32}O_2$)
24h
4.5h
3h
97%
5

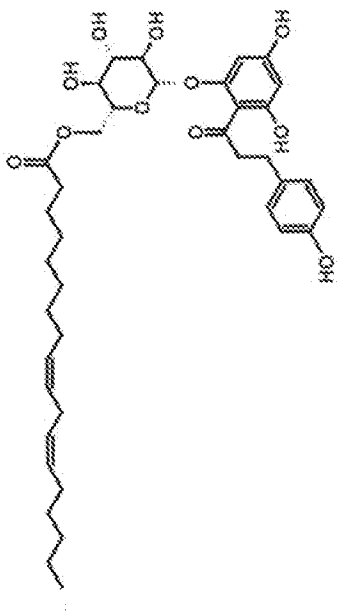
FIG. 4b



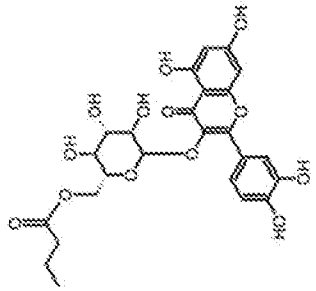
Phloridzin	Stearic acid ($C_{18}H_{36}O_2$)	24h	4.5h	3h	96%
Phloridzin	Oleic acid ($C_{18}H_{34}O_2$)	24h	4h	2.5h	93%

FIG. 4.c

		24h	4h	3h	89%
Phlorizin	Linoleic acid ($C_{18}H_{32}O_2$)				
		24h	3h	2h	83%[b]
Isoquercitrin	Butyric Acid ($C_4H_8O_2$)				



8



9

FIG. 4d

Octanoic acid ($C_8H_{16}O_2$)		24h	3h	2.5h	81%[b]
Isoquercitrin		10				

FIG. 4e

Isoquercitrin	Dodecanoic acid ($C_{12}H_{24}O_2$)	24h	3.5h	3h	92%
		11			
Isoquercitrin	Palmitic acid ($C_{16}H_{32}O_2$)	24h	4.5h	3h	93%
		12			

FIG. 4f

Isoquercitrin
Stearic acid
($C_{18}H_{36}O_2$)
24h
4.5h
3h
96%
13
Isoquercitrin
Oleic acid
($C_{18}H_{34}O_2$)
24h
4h
3h
93%
14

FIG. 4g

Isoquercitrin	Linoleic acid ($C_{18}H_{32}O_2$)	24h	4.2h	3h
15				91%

*There is no significant difference in yields of all the three methods.
*Compounds were volatile and some quantity lost during evaporation on rotavap.

FIG. 4h

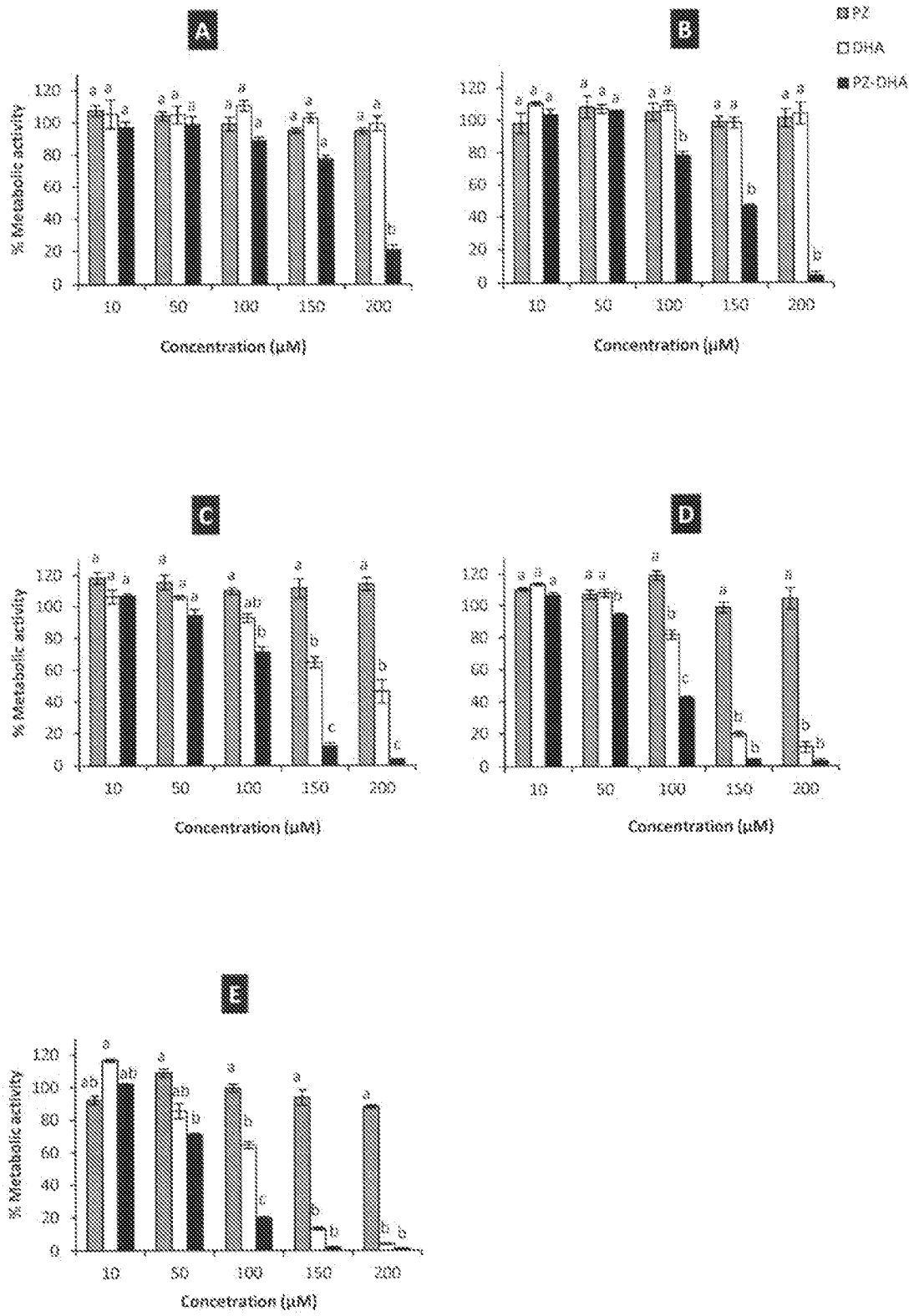

A
B
C
D
E
PZ
DHA
PZ-DHA
% Metabolic activity
Concentration (µM)
Concetration (µM)
0
20
40
60
80
100
120
10
50
100
150
200

Figure 5

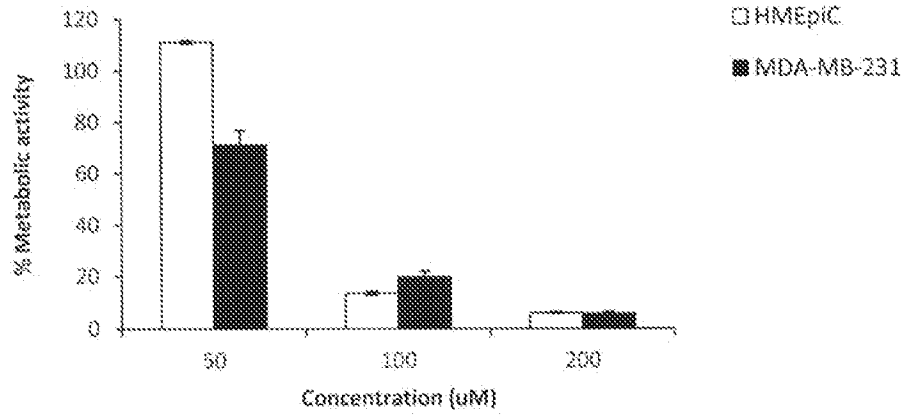

120
100
80
60
40
20
0
% Metabolic activity
50
100
200
Concentration (uM)
HMEpiC
MDA-MB-231

Figure 6

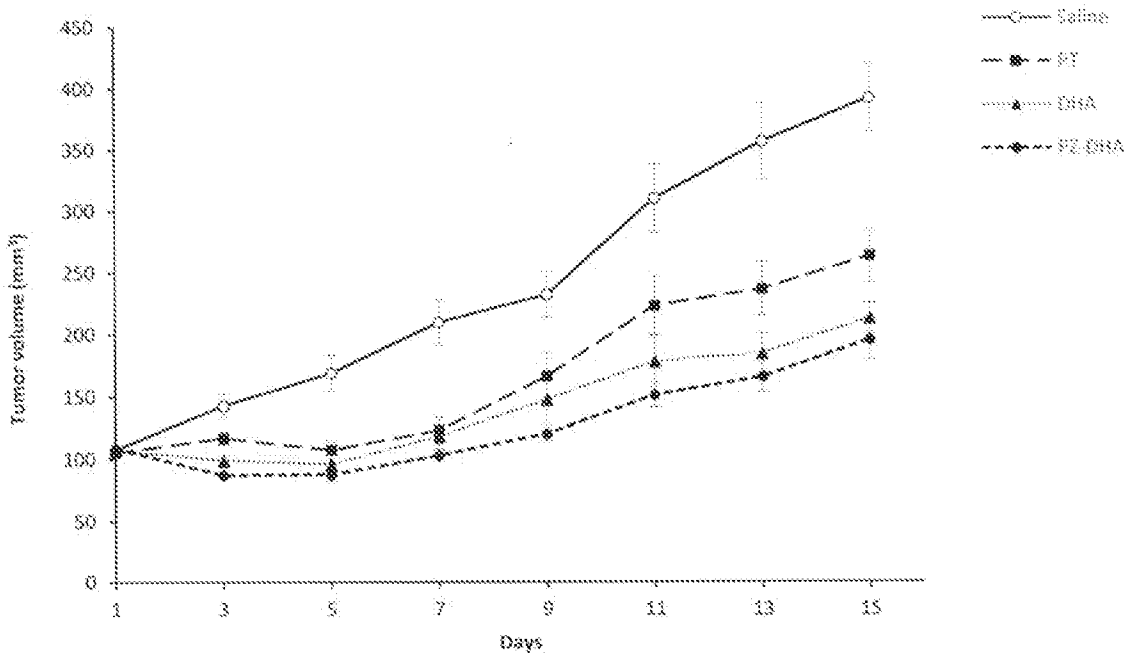


Figure 7A

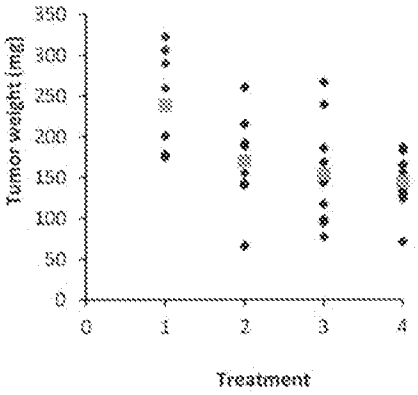


Figure 7B

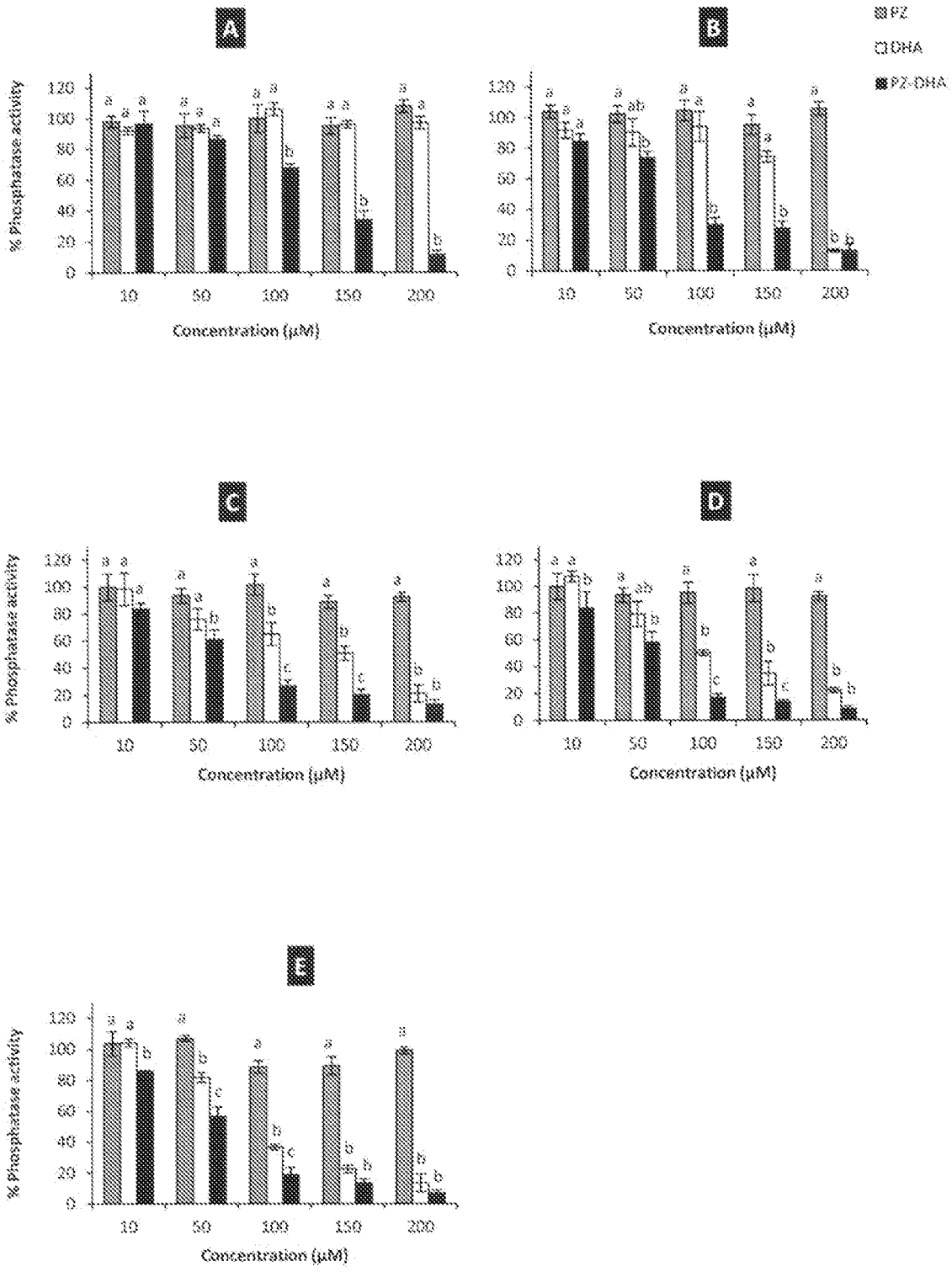

A
B
C
D
E
PZ
DHA
PZ-DHA
% Phosphatase activity
Concentration (µM)
10
50
100
150
200
0
20
40
60
80
100
120

Figure 8

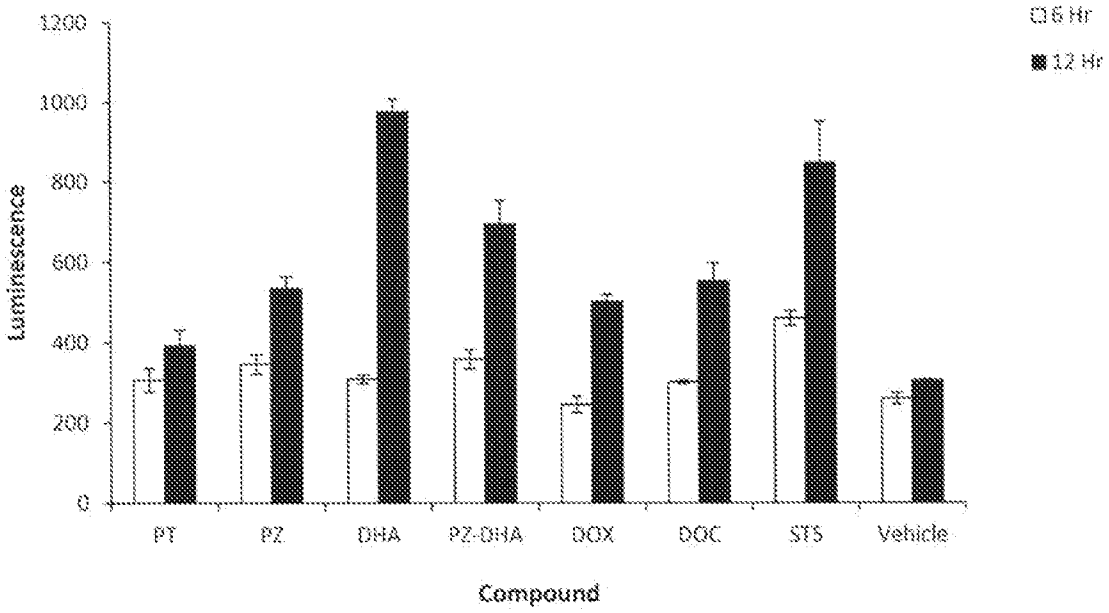

6 Hr
12 Hr
1200
1000
800
600
400
200
0
Luminescence
PT
PZ
DHA
PZ-DHA
DOX
DOC
STS
Vehicle
Compound

Figure 9

		HepG2				MDA-MB-231				THP-1			
		EC50 (μM)				EC50 (μM)				EC50 (μM)			
		3 h	6 h	12 h	18 h	24 h	3 h	6 h	12 h	18 h	24 h	3 h	6 h	12 h	18 h	24 h
Phloridzin Esters	Phloridzin Oleic acid ester	64.6	60.9	54.2	48.5	37.8	63.4	57.4	50.5	37.4	35.2	95.8	61.8	53.8	41.7	40.7
	Phloridzin Stearic acid ester	53.3	48.2	37.6	34.8	31.5	64.8	61.1	57.4	56.7	37.9	162.7	153.7	144.5	122.1	70.7
	Phloridzin Linoleic acid ester	43.3	40.5	35.4	30.8	29.2	41.6	38.8	38.1	36.1	32.3	36.6	18.7	14.7	13.1	6.2
	Phloridzin α-Linolenic acid ester	76.5	66.3	60.5	55.7	53.1	89.9	83.8	80.5	66.1	63.8	88.7	72.4	41.3	37.9	35.7
	Phloridzin DHA ester	76.9	64.4	56.2	54.2	51.9	133.5	83.7	70.2	62.6	55.5	65.8	49.5	42.7	35.8	27.3
	Phloridzin EPA ester	59.4	37.8	36.0	33.1	26.8	37.9	33.6	32.6	31.5	26.5	27.9	19.9	17	15.8	14.8
Parent Compounds	Phloridzin	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
	Oleic acid	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
	Stearic acid	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
	Linoleic acid	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
	A-Linolenic acid	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
	DHA	NA*	NA*	NA*	NA*	119.9	NA*	NA*	NA*	220.4	102.4	NA*	NA*	NA*	NA*	NA*
	EPA	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*	NA*
Drug	Sorafenib	67.5	46.7	17.3	13.4	10.7										
	Doxorubicin						NA*	NA*	9.6	5.9	4.1	89.9	81.3	15.7	9.5	2.1
Aglycone of Phloridzin	Phloretin	147.0	137.7	130.3	118.2	97.20	165.3	142.3	119.9	109.5	104.7	63.9	42.5	31.3	21.0	17.3

*Not Active

FIG. 1O

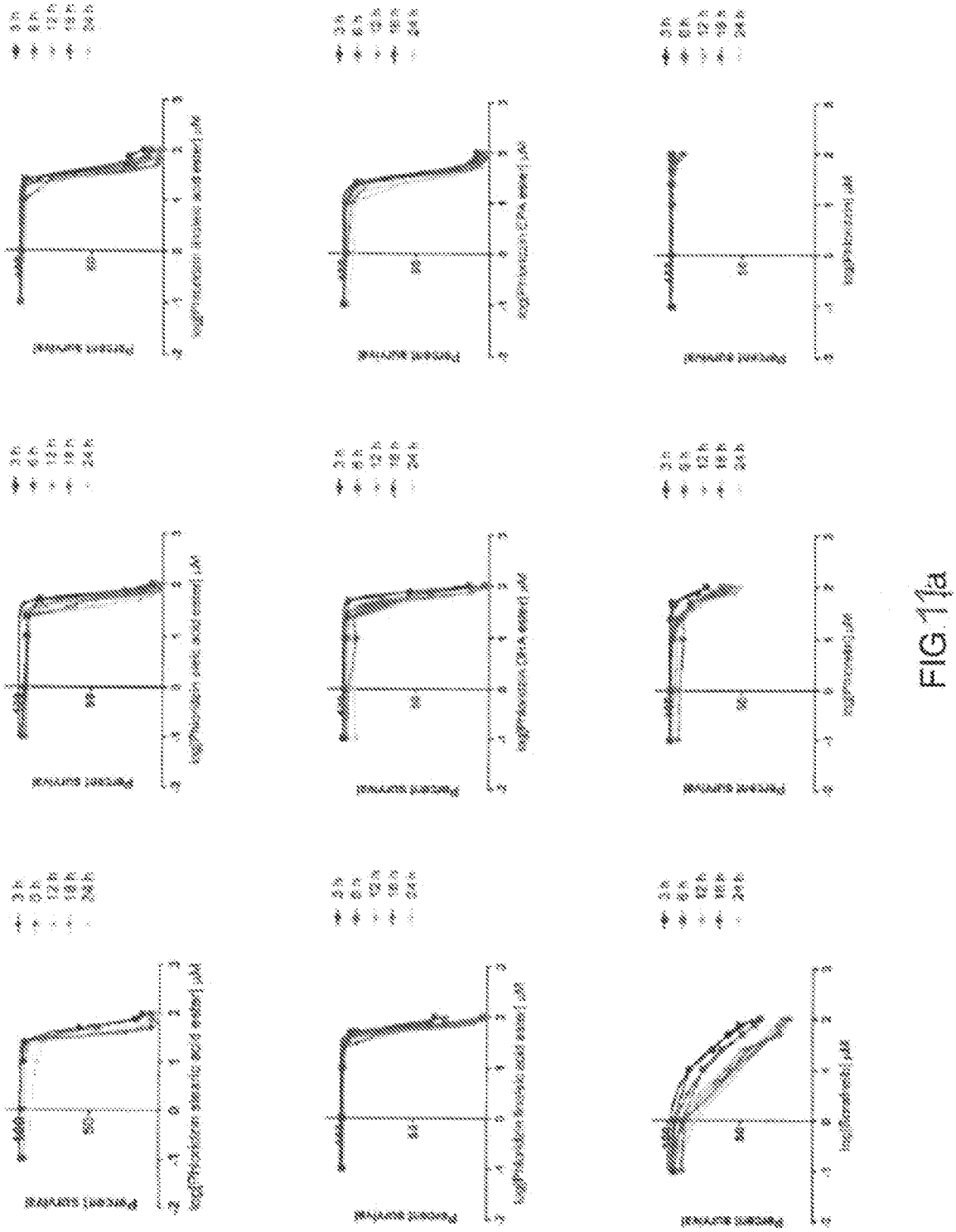


FIG 11a

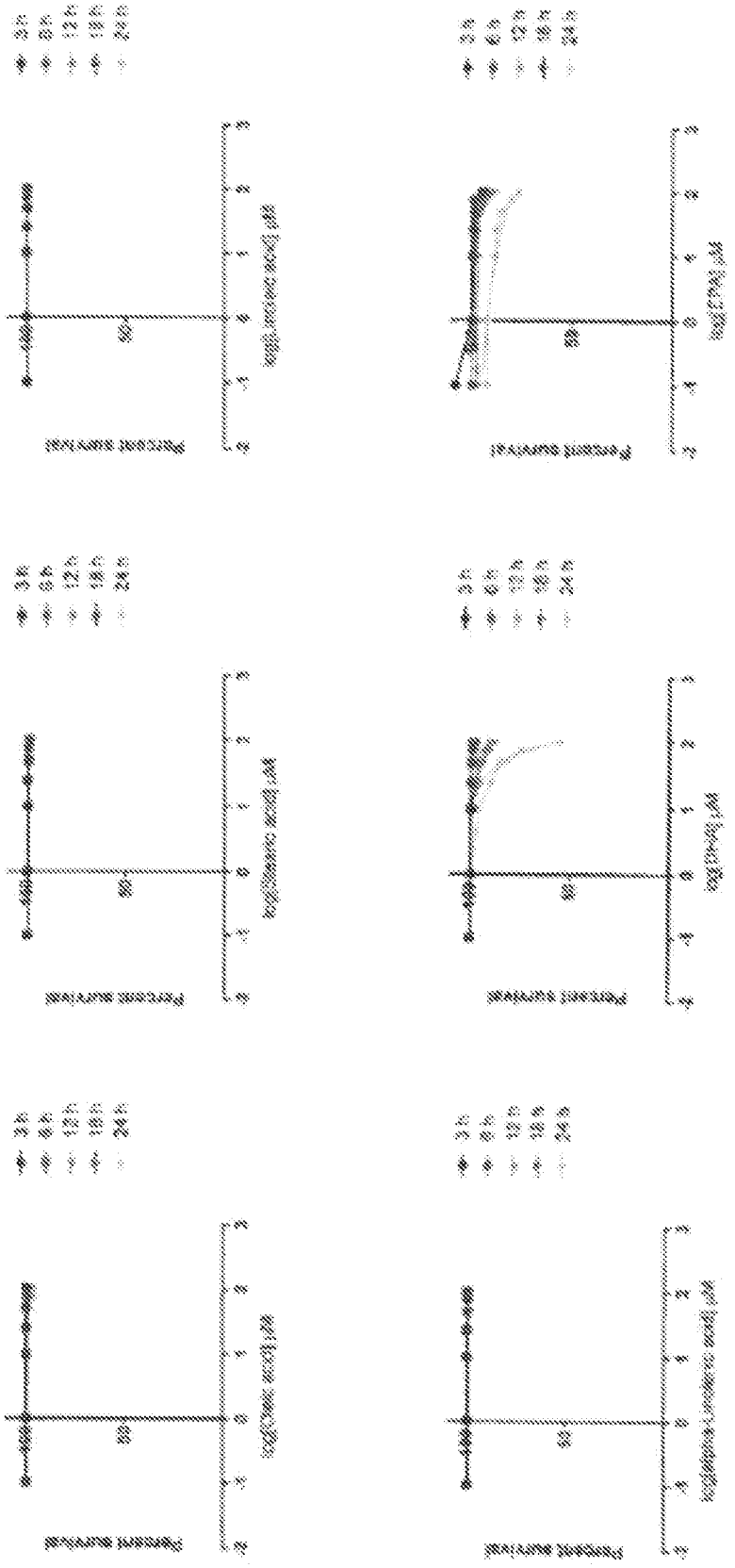


FIG.11b

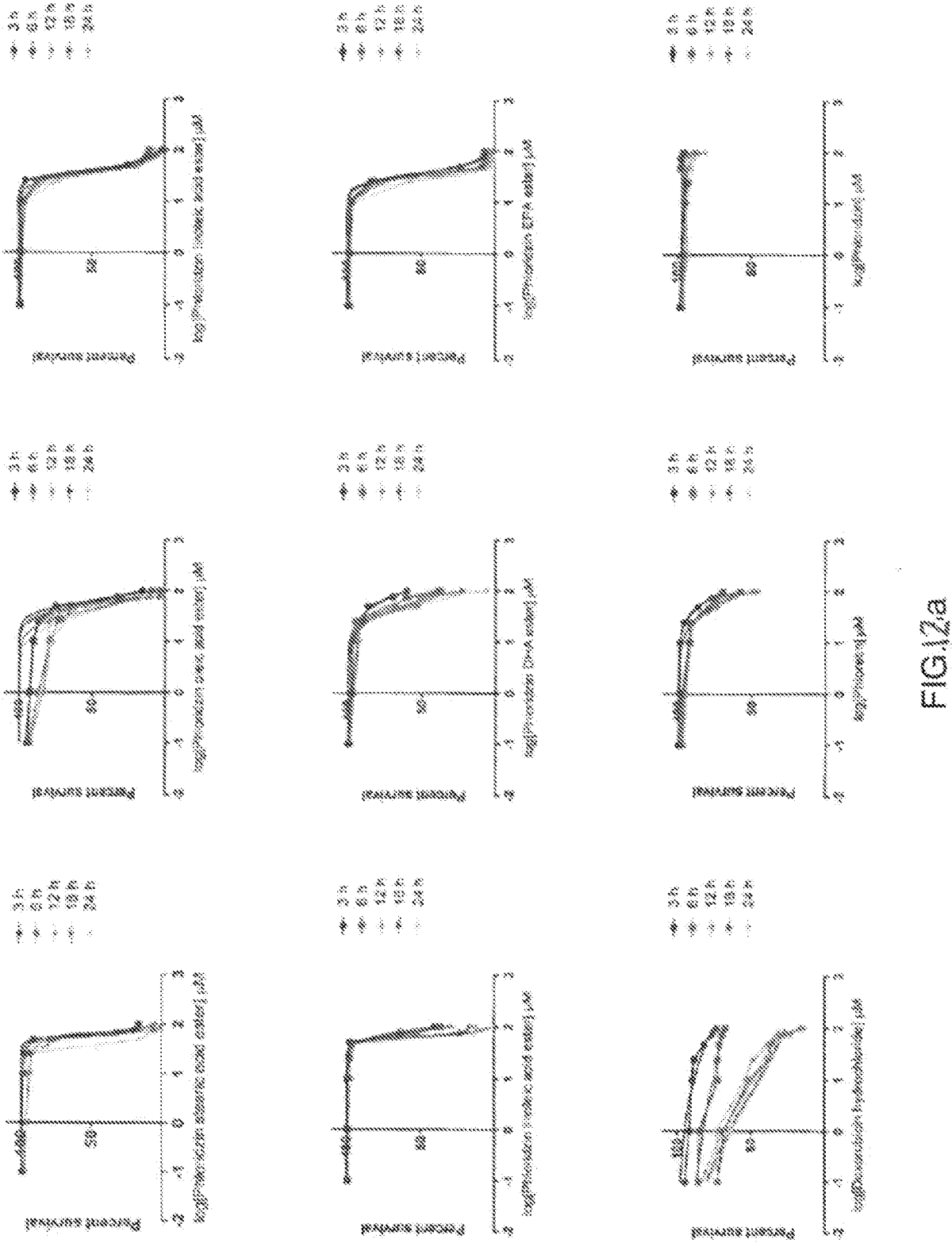


FIG.12a

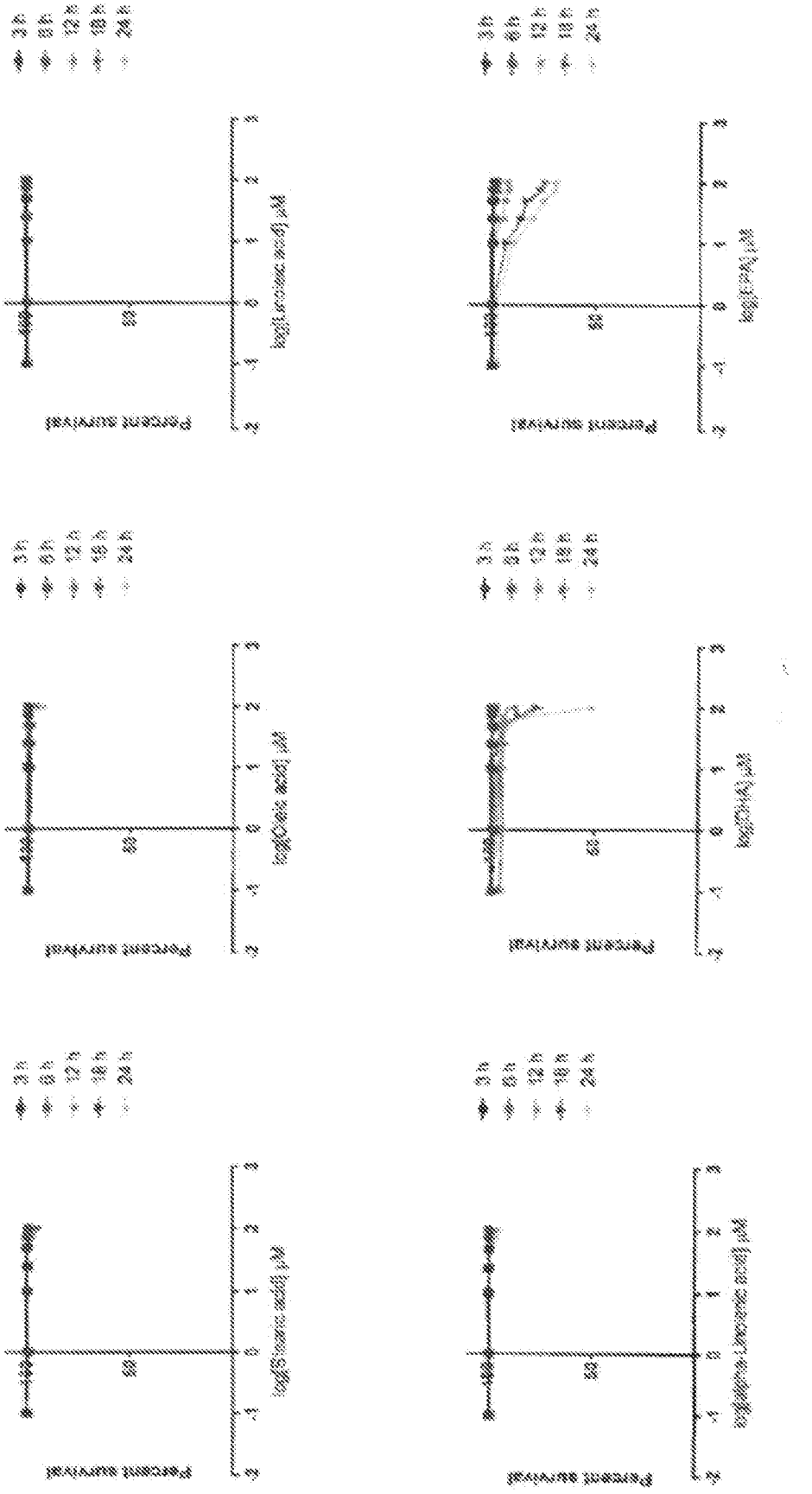


FIG.12b

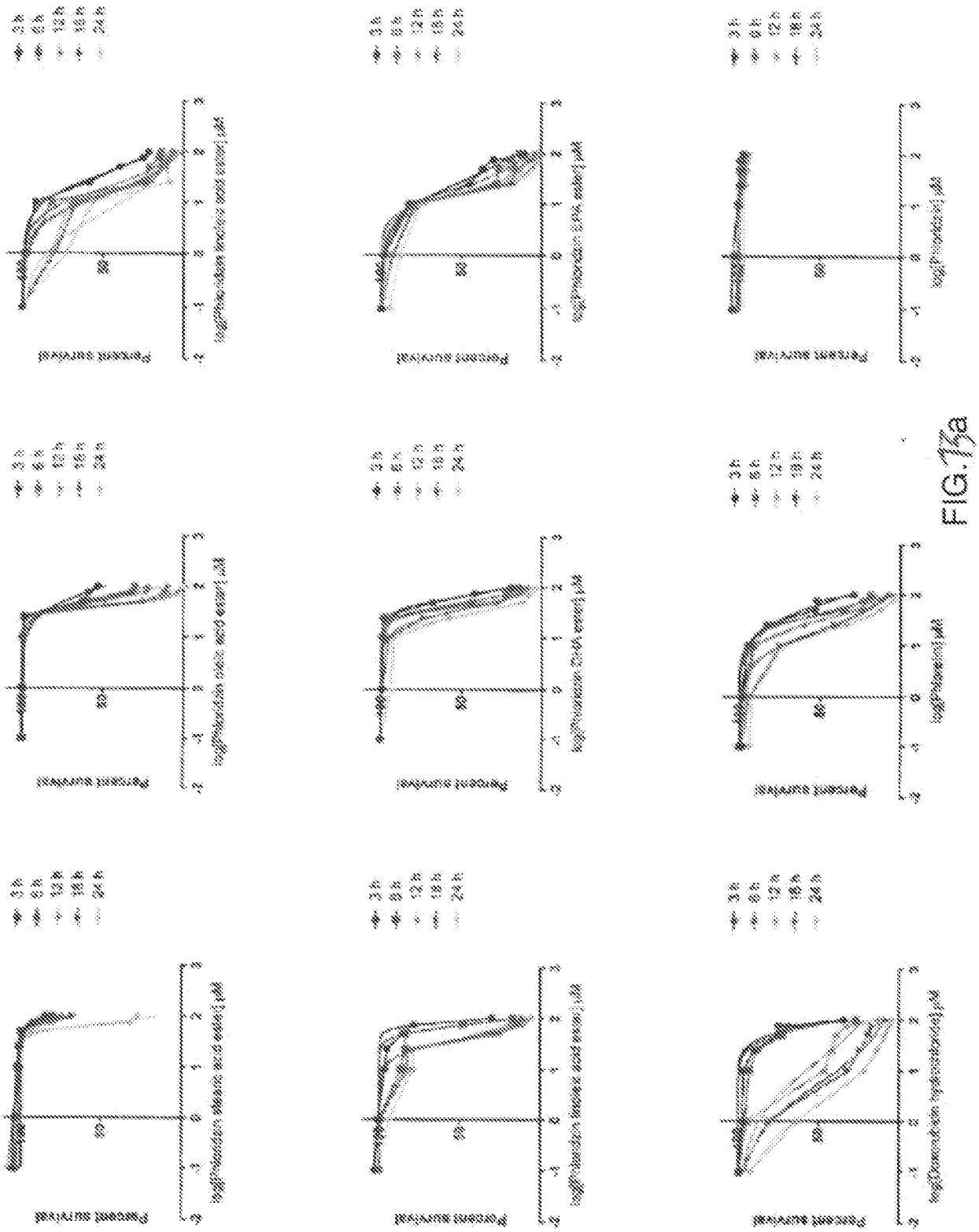


FIG.17a

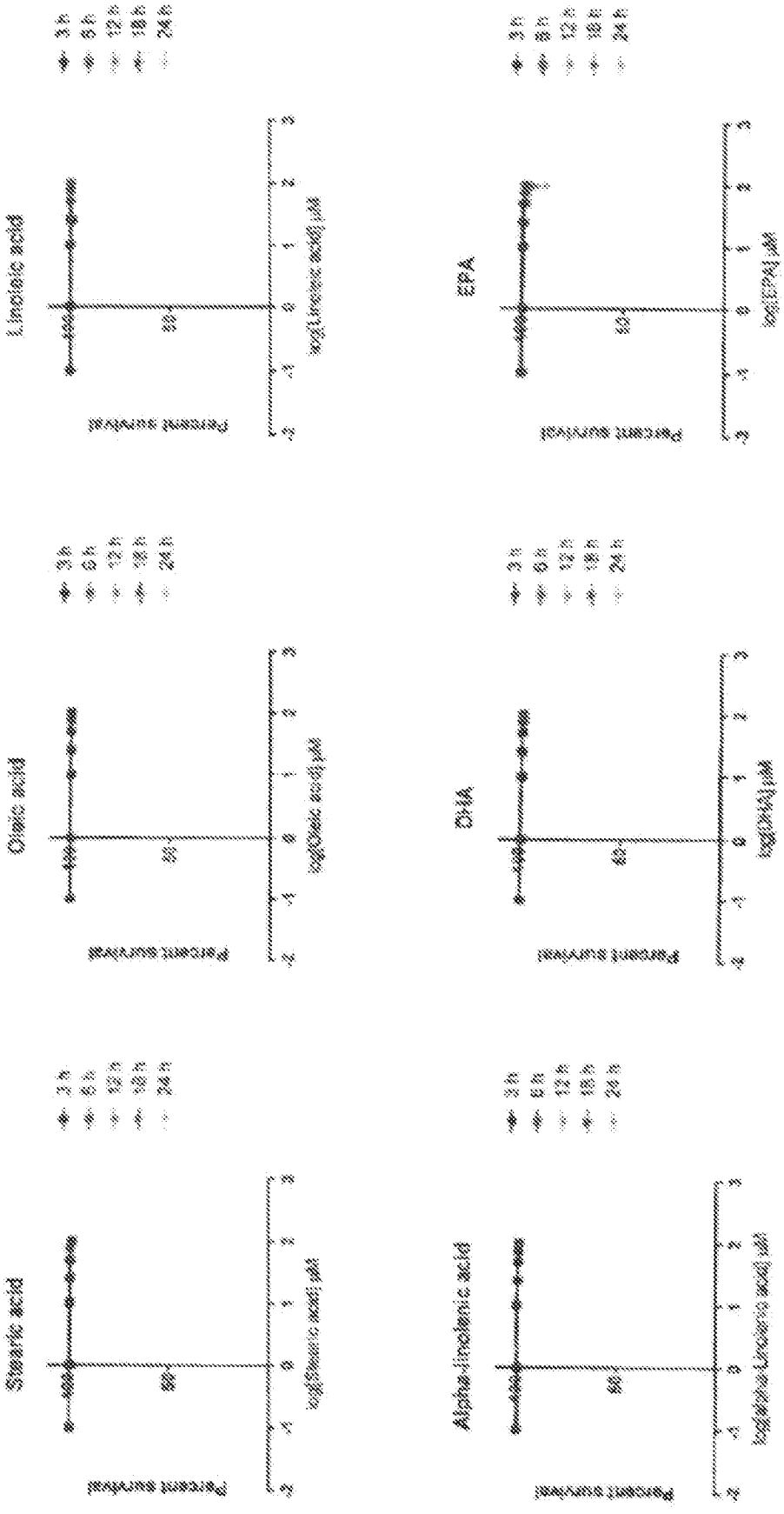

Linoleic acid
Oleic acid
Stearic acid
EPA
DHA
Alpha-linolenic acid
Percent survival
50
3 h
6 h
12 h
18 h
24 h

FIG.12b

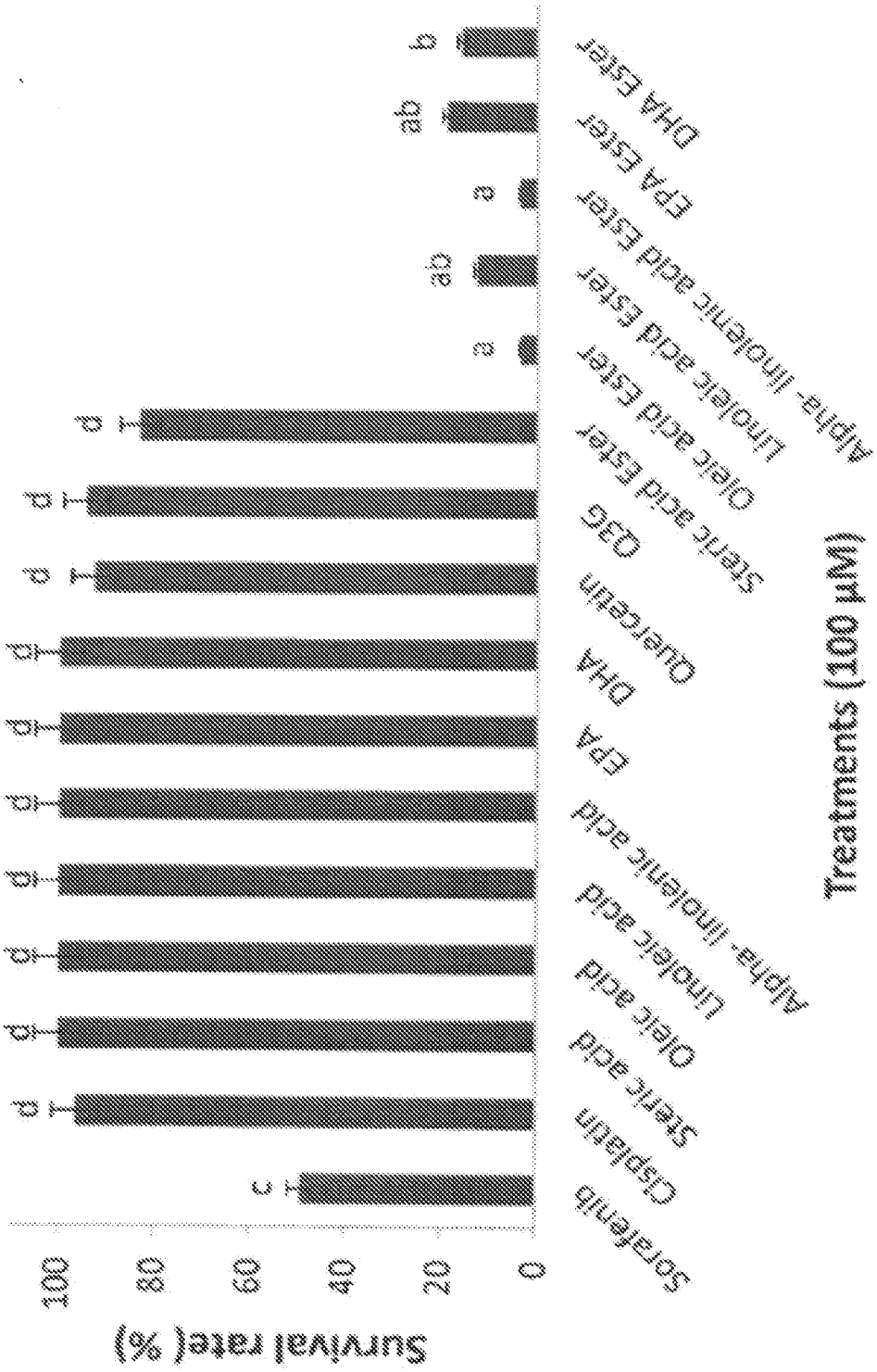

Survival rate(%)
100
80
60
40
20
0
Sorafenib
Cisplatin
Stearic acid
Oleic acid
Linoleic acid
Alpha- linolenic acid
EPA
DHA
Quercetin
Q3G
Stearic acid Ester
Oleic acid Ester
Linoleic acid Ester
Alpha- linolenic acid Ester
EPA Ester
DHA Ester
Treatments (100 µM)

FIG.11a

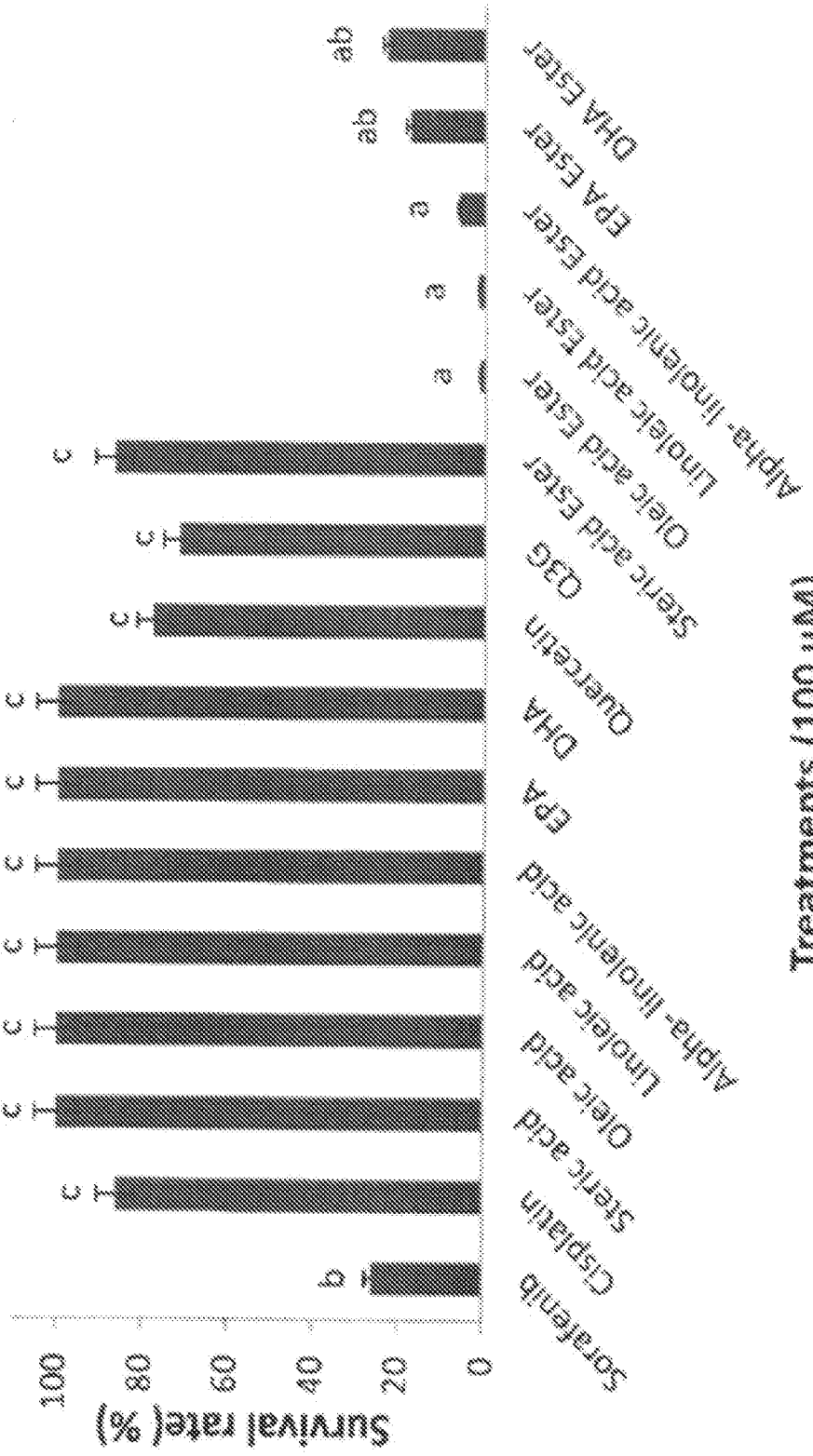

Survival rate(%)
100
80
60
40
20
0
d
c
c
c
c
c
c
c
c
c
c
c
e
e
e
ab
ab
Sorafenib
Cisplatin
Stearic acid
Oleic acid
Linoleic acid
Alpha- linolenic acid
EPA
DHA
Quercetin
Q3G
Stearic acid Ester
Oleic acid Ester
Linoleic acid Ester
Alpha- linolenic acid Ester
EPA Ester
DHA Ester
Treatments (100 µM)

FIG.14b

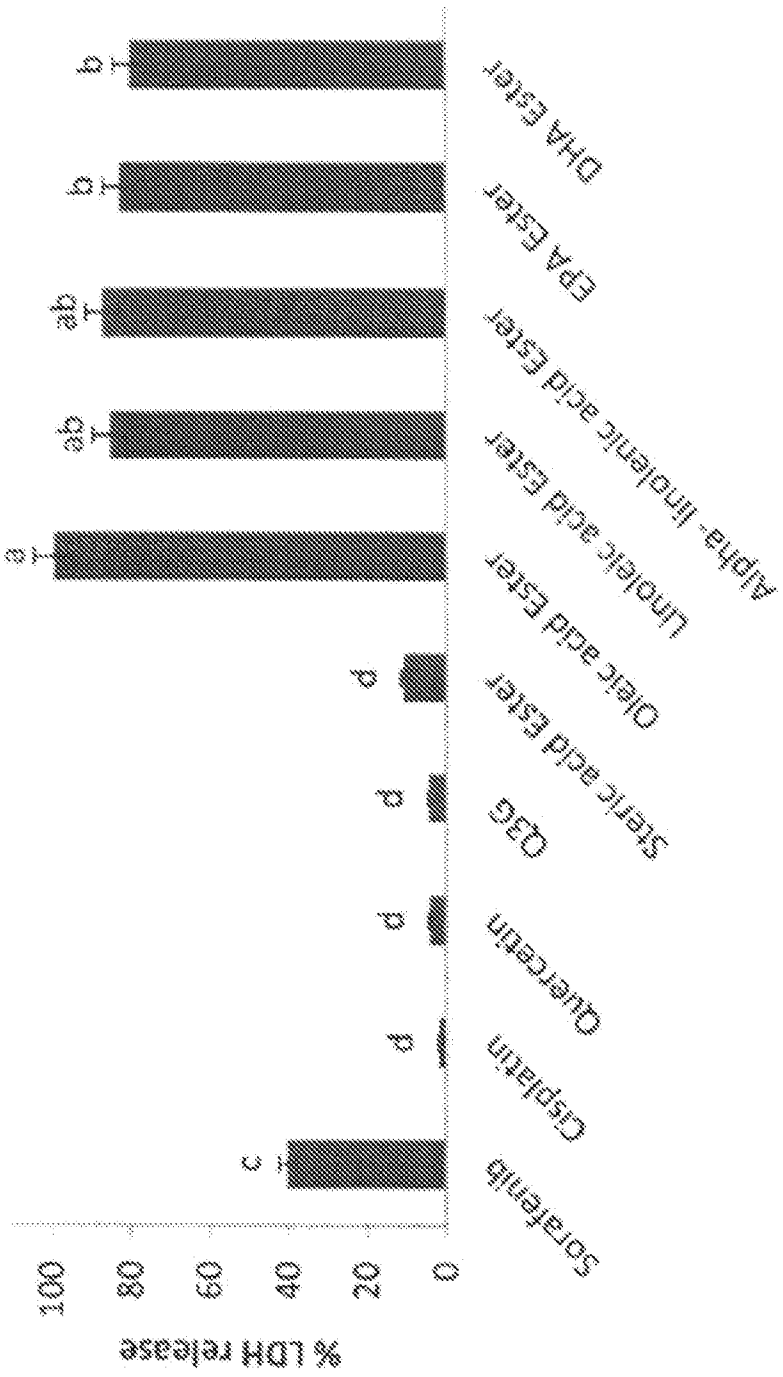

% LDH release
100
80
60
40
20
0
c
d
d
d
d
e
ab
ab
d
d
Sorafenib
Cisplatin
Quercetin
QSG
Stearic acid Ester
Oleic acid Ester
Linoleic acid Ester
Alpha- linolenic acid Ester
EPA Ester
DHA Ester
Treatments (100 µM)

FIG.15a

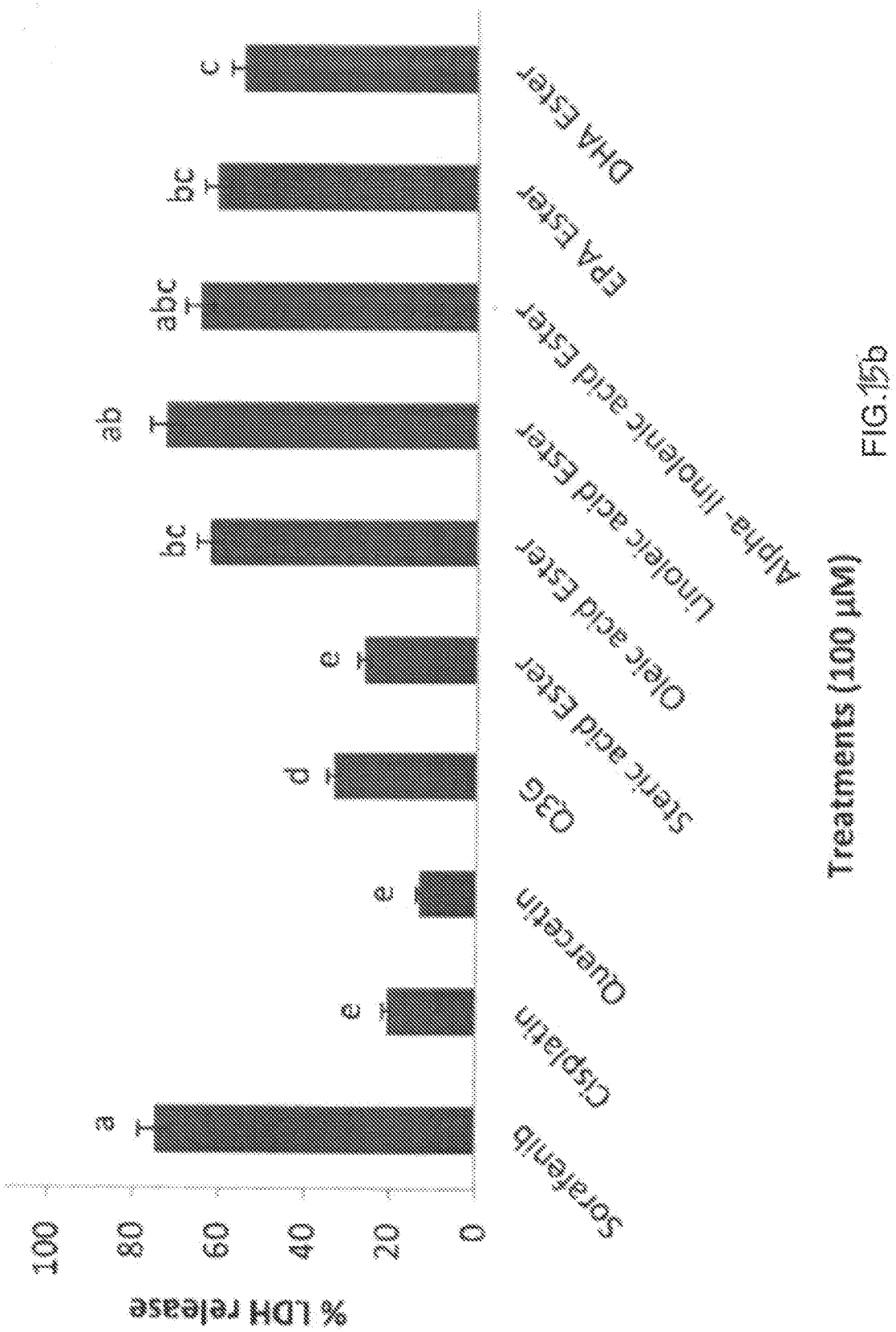

% LDH release
100
80
60
40
20
0
a
e
e
d
e
bc
ab
abc
bc
c
Sorafenib
Cisplatin
Quercetin
Q3G
Stearic acid Ester
Oleic acid Ester
Linoleic acid Ester
Alpha- linolenic acid Ester
EPA Ester
DHA Ester
Treatments (100 µM)

FIG.15b

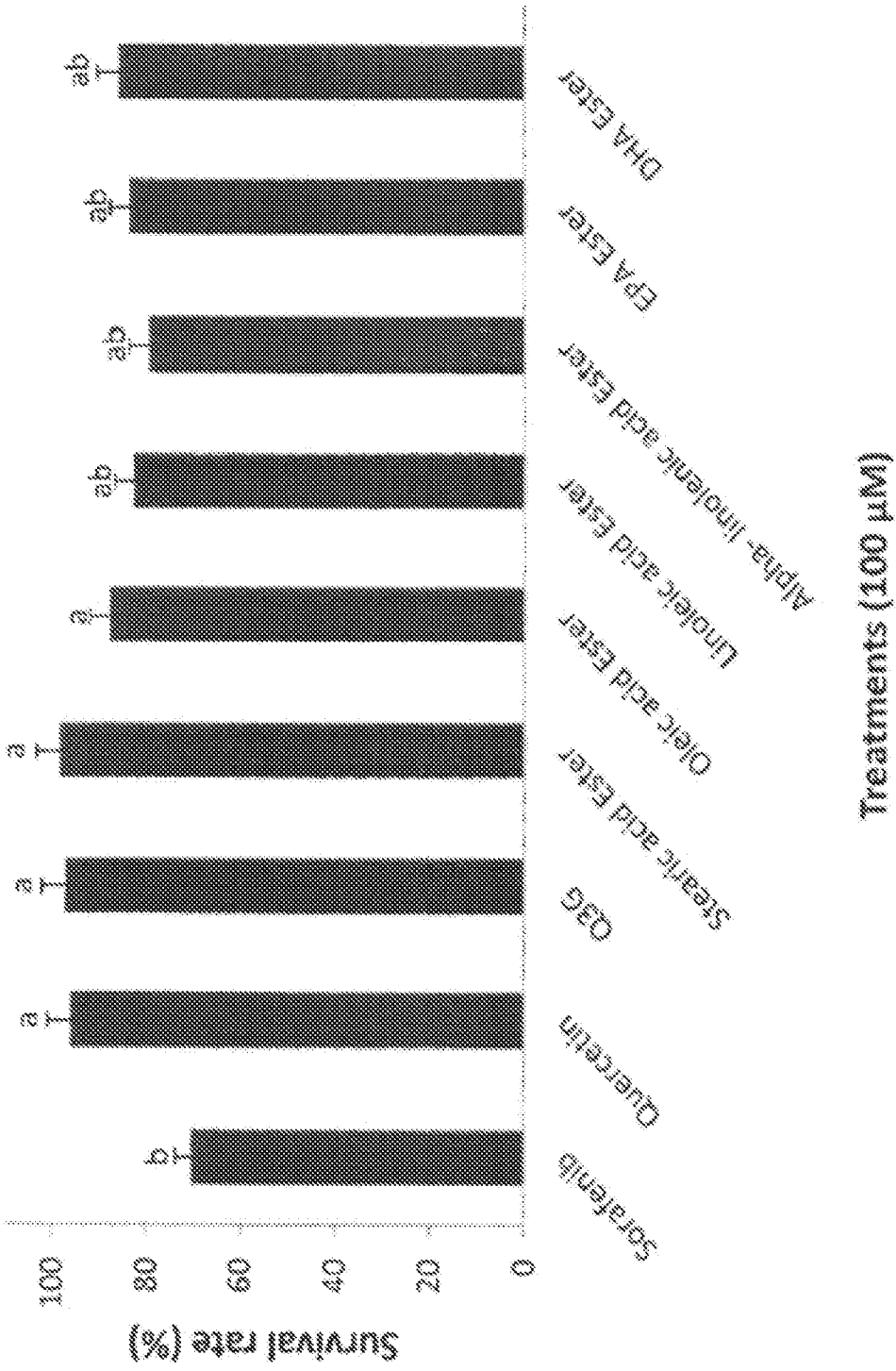

Survival rate (%)
100
80
60
40
20
0
c
a
a
a
a
ab
ab
ab
Sorafenib
Quercetin
Q3G
Stearic acid Ester
Oleic acid Ester
Linoleic acid Ester
Alpha- linolenic acid Ester
EPA Ester
DHA Ester
Treatments (100 µM)

FIG.16

Signaling Group	Gene	Description	Fold Change
Apoptosis	*BCL2*	B-cell CLL/lymphoma 2	+2.2
	BIRC5	Baculoviral IAP repeat containing 5	-11.2
Growth factors and receptors	*EGFR*	Epidermal Growth Factor Receptor	-12.2
	PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	+2.0
Drug Metabolism	*TXN*	Thioredoxin	-2.0
G Protein Signaling	*RHOB*	Ras homolog gene family, member B	+3.3
Heat Shock Proteins	*HSP90AA1*	Heat shock protein 90kDa alpha (cytosolic), class A member 1	-2.1
Receptor Tyrosine Kinase	*AKT2*	V-akt murine thymoma viral oncogene homolog 2	-2.1
	GRB2	Growth factor receptor-bound protein 2	-2.0
Cathepsins	*CTSB*	Cathepsin B	+2.2
Cell Cycle	*CDC25A*	Cell division cycle 25 homolog A (S. pombe)	-2.0
	CDK1	Cyclin-dependent kinase 1	-2.0
	CDK2	Cyclin-dependent kinase 2	-2.0
	CDK8	Cyclin-dependent kinase 8	-12.2
Topoisomerase, Type II	*TOP2A*	Topoisomerase (DNA) II alpha 170kDa	-2.7
Transcription Factors	*HIF1A*	Hypoxia inducible factor 1, alpha subunit	-2.4
	IRF5	Interferon regulatory factor 5	+6.3
	NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	-2.2
Protein Kinases	*AURKB*	Aurora kinase B	-2.0
	PRKCA	Protein kinase C, alpha	-2.0
RAS Signaling	*HRAS*	V-Ha-ras Harvey rat sarcoma viral oncogene homolog	-2.0
Histone Deacetylases	*HDAC2*	Histone deacetylase 2	-2.0
	HDAC6	Histone deacetylase 6	-25.9
Poly ADP-Ribose Polymearses	*PARP1*	Poly (ADP-ribose) polymerase 1	-2.5
	PARP4	Poly (ADP-ribose) polymerase family, member 4	+2.1

FIG. 17