

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
18 October 2007 (18.10.2007)

PCT

(10) International Publication Number
WO 2007/116404 A2

(51) International Patent Classification:
A61K 31/704 (2006.01)

(21) International Application Number:
PCT/IL2007/000454

(22) International Filing Date: 10 April 2007 (10.04.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/790,538 10 April 2006 (10.04.2006) US

(71) Applicant (for all designated States except US): **BAR-ILAN UNIVERSITY** [IL/IL]; Ramat Gan 52900 (IL).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **TANNIN, Tehila** [IL/IL]; 10/9 Tabechnik, Petach Tikva 49720 (IL). **GROSSMAN, Shlomo** [IL/IL]; 15 Shderot Hayered, Ramat Gan 52444 (IL). **BERGMAN, Margalit** [IL/IL]; 12 Zadaf Street, Rishon Le Zion 75437 (IL). **DOVRAT, Sara** [IL/IL]; 60 Hazait Street, Oranit 44813 (IL).

(74) Agent: **WEBB, Cynthia**; Webb & Associates, P.O. Box 2189, 76121 Rehovot (IL).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: CUCURBITACIN GLUCOSIDES AND USE THEREOF IN TREATING CANCER

(57) Abstract: The present invention relates to cucurbitaciii glucosides isolated from Citrullus colocynthis and their synergistic effects on inhibiting growth of human cancer cells including the effects on cellular growth, cell cycle distribution, apoptosis and the expression of proteins involved in cell cycle regulation.

WO 2007/116404 A2

CUCURBITACIN GLUCOSIDES AND USE THEREOF IN TREATING CANCER

FIELD OF THE INVENTION

5 The present invention relates to cucurbitacin glucosides isolated from *Citrullus colocynthis* and their synergistic effects on inhibiting growth of human cancer cells including effects on cellular growth, cell cycle distribution, apoptosis and the expression of proteins involved in cell cycle regulation.

10 BACKGROUND OF THE INVENTION

Breast cancer affects one in every ten women in Western Europe and the US (Chang, 1998; Biomed Pharmacother 52:133-136), and it is the second leading cause of cancer-related deaths (Parker et al., 1997; J Clin 47:5-27). Recently, there is a growing interest in the use of herbs as a potent source for new therapeutic anti cancer drugs.

15 Plants contain a wide variety of chemicals that have potent biological effects, including anticancer activity (Sporn and Suh, 2000; Carcinogenesis 21:525-530). Identification of the active components, their mechanisms of action, and their possible interactions is important in order to assess their potential for clinical use and possible adverse side effects.

20 A number of triterpenoids have shown promise as antineoplastic agents. Members of the cycloartane, lupane, ursane, oleanane, friedelane (especially quinone methides), dammarane, cucurbitacin, and limonoid triterpenoids, have demonstrated anti-proliferative activity on various cancer cell lines (Setzer and Setzer, 2003; Mini Rev Med Chem 3(6):540-56).

25 *Citrullus colocynthis* (L.) Shrad (Cucurbitaceae), locally known as Sherry or Handal, is used in folk medicine by people in rural areas as a purgative, antirheumatic, anthelmintic, and as a remedy for skin infection. The plant contains cucurbitacins A, B, C and D and α -elaterin and probably other biologically active constituents (Adam et al., 2001; Small Rumin Res 40:239-244).

30 The cucurbitacins (highly oxygenated tetracyclic triterpens) are of great interest because of the wide range of biological activity they exhibit in plants and animals. They are predominantly found in the family Cucurbitaceae but are also present in several

other families of the plant kingdom. Despite their toxicity, species of the plants in which they are found have been used for centuries in various pharmacopeia. A number of compounds of this group have been investigated for their cytotoxic, hepatoprotective, anti-inflammatory and cardiovascular effects (Jayaprakasam et al., 2003; Cancer Lett 5 189:11-16).

The general phenomenon that particular types of cucurbitacins are capable of inhibiting growth of certain types of cancer cells is known. For example, Duncan et al. have identified cucurbitacin E as having potent *in vitro* growth inhibitory activity against prostate carcinoma explants. It has been shown that cucurbitacin E causes 10 marked disruption of the actin cytoskeleton, and the anti-proliferative activity was directly correlated with the disruption of the F-actin cytoskeleton. The distribution of vimentin was also altered in cells exposed to cucurbitacin E, as was shown by the association of vimentin with drug-induced membrane blebs (Duncan et al. 1996; Biochem Pharmacol. 52(10):1553-1560). Fang et al. showed the isolation of new 15 cucurbitacin derivative, hexanorcucurbitacin F from *Elaeocarpus dolichostylus* whose structure was determined by spectroscopic and chemical correlation with cucurbitacin F. cucurbitacin F and 23,24-dihydrocucurbitacin F were also isolated in this study (Fang et al., 1984; J Nat Prod. 47(6):988-93).

US Application No. 2004/0138189 discloses use of cucurbitacin I for treating 20 tumors and cancerous tissues through the modulation of JAK/STAT3 intracellular signaling, particularly by inhibiting the STAT3 activation pathways.

However, none of the prior art discloses or suggests bioactivity of cucurbitacin glucosides in general nor any synergistic effect of cucurbitacin glucosides on inhibiting growth of cancer cell lines.

25

SUMMARY OF THE INVENTION

The present invention relates to cucurbitacin B and E glucoside, isolated from *Citrullus colocynthis* and their use for treating cancer, particularly breast cancer.

The present invention discloses for the first time the synergistic effects of 30 cucurbitacin glucosides on inhibiting growth of human cancer cells including effects on cellular growth, cell cycle distribution, apoptosis and the expression of proteins involved in cell cycle regulation.

The present invention provides for the first time isolated cucurbitacin glucosides from *C. colocynthis* and has characterized by means of NMR analysis the molecular structures of both cucurbitacin B and E glucosides. These compounds are now shown to have anti proliferative activity on tumor cells.

5 The present invention is based in part on the finding that *Citrullus colocynthis* and specific constituents extracted therefrom, including cucurbitacins inhibit growth of estrogen dependent (MCF-7) and estrogen-independent (MDA-MB-231) human breast cancer cell lines.

10 Without wishing to be bound by any specific theory or mechanism of action, inhibition of breast cancer cells may be the result of accumulation of cells in the G₂/M phase of the cell cycle accompanied with induction of apoptosis by the cucurbitacin B/E glucosides combination. The inventors of the present invention also identified one of the possible signaling mechanisms through which the cucurbitacin glucosides exert their beneficial effects in the chemoprevention of breast cancer cells. Unexpectedly,
15 cucurbitacin glucosides treatment caused elevation in phosphorylated STAT3, an active form of STAT3, and in p21^{WAF}, which was proven to be a STAT3 positive target in the absence of survival signals. Elevated p21^{WAF} might also contribute to G₂/M arrest by inhibiting the p34^{CDC2}/cyclin B1 complex that is necessary to the regulation of G₂ exit and initiation of mitosis.

20 Thus, according to one aspect, the present invention provides an isolated cucurbitacin glucoside from *Citrullus colocynthis*. According to certain embodiments, the cucurbitacins are characterized by means of NMR. According to one embodiment, the cucurbitacin glucoside is selected from the group consisting of cucurbitacin B glucoside and cucurbitacin E glucoside. According to certain embodiments, the
25 cucurbitacin B and E glucosides have characteristic NMR parameters as presented in Table 1 hereinbelow.

 According to another aspect, the present invention provides a pharmaceutical composition comprising a therapeutically effective amount of at least one isolated cucurbitacin glucoside from *Citrullus colocynthis* or a salt thereof and a
30 pharmaceutically acceptable excipient, diluent or carrier. According to one embodiment, the cucurbitacin glucoside is selected from the group consisting of cucurbitacin B glucoside and cucurbitacin E glucoside. According to another embodiment, the

cucurbitacin B and E glucosides are characterized by certain NMR parameters presented in Table 1 hereinbelow. According to other embodiments, the pharmaceutical composition comprises a plurality of cucurbitacins isolated from *C. colocynthis*. According to certain currently preferred embodiments, the pharmaceutical composition
5 comprises a therapeutically effective amount of a combination of cucurbitacin B glucoside and cucurbitacin E glucoside.

According to yet another aspect, the present invention provides a method for treating a tumor in a subject in need thereof comprising administering a pharmaceutical composition comprising an isolated cucurbitacin glucoside of the invention.

10 According to certain embodiments, the method comprises administering a pharmaceutical composition comprising a plurality of isolated cucurbitacin glucosides that act synergistically to reduce the proliferation of the tumor cells. According to currently preferred embodiments, the cucurbitacin glucosides are cucurbitacin B glucoside and cucurbitacin E glucoside isolated from *C. colocynthis*. According to other
15 embodiments, the cucurbitacin B glucoside and cucurbitacin E glucoside have characteristic NMR parameters as presented in Table 1 hereinbelow.

According to other embodiments, the pharmaceutical composition of the present invention is administered in combination with at least one additional anti-tumor treatment.

20 According to one embodiment, the additional anti tumor treatment is selected from the group consisting of, but not limited to, radiation therapy, chemotherapy, immunotherapy, hormonal therapy and genetic therapy.

According to one embodiment, the tumor is human breast tumor.

Other objects, features and advantages of the present invention will become clear
25 from the following description and drawings.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 shows a chemical structure of cucurbitacin B glucoside (MW=739). Structure of cucurbitacin E glucoside (MW=737) is the same as cucurbitacin B glucoside except to a
30 double bond between carbon 1 and 2.

FIG. 2 illustrates a dose dependent effect of cucurbitacin glucosides combination (B

and E in 1:1 ratio) on proliferation of estrogen-independent MDA-MB-231 cells. The data represent the mean $\pm$ SE of 3 experiments where each treatment was performed in 3 wells.

FIG. 3 shows the effect of cucurbitacin B/E glucosides on cell cycle distribution in MDA-MB-231 and MCF7 cells. A: MDA-MB-231 cells treated with cucurbitacin E glucoside; B: MDA-MB-231 cells treated with cucurbitacin B glucoside; C: MCF-7 cells treated with cucurbitacin E glucoside; D: MCF-7 cells treated with cucurbitacin B glucoside. The data shown are representative of three independent experiments with similar findings.

FIG. 4 shows the effect of cucurbitacin glucosides combination (E+B, 1:1) on cell cycle distribution. A: MCF7 cells treated with cucurbitacin glucosides combination (5.8, 11.6 and 14.5 μ g/ml). The values indicate the percentage of cells in the indicated phases of the cell cycle. The data shown are representative of three independent experiments with similar findings; B: Histograms showing the number of cells per channel (vertical axis) versus DNA content (horizontal axis).

FIG. 5 is a Western blot analysis of extracts obtained from MCF7 and MDA-MB-231 cells treated with the cucurbitacin glucosides combination. A: Cells were treated with 0 (-) and 14.5 μ g/ml (+) cucurbitacin glucosides combination for 24 h. Extracts were prepared and analyzed by Western blotting with an antibody to p34^{CDC2} and cyclin B1; B: MDA-MB-231 cells were treated with 0 (-) and 14.5 μ g/ml (+) cucurbitacin glucosides combination for 1, 4, 24 h. Extracts were prepared and analyzed by Western blotting with an antibody to p34^{CDC2} and phospho- p34^{CDC2}. An antibody to β -actin was used as a loading control. Western blots are representative of three independent experiments.

FIG. 6 shows the effect of cucurbitacin glucosides combination treatment on MDA-MB-231 cell morphology. A: MDA-MB-231 cells were treated with 14.5 μ g/ml of the cucurbitacin glucoside combination (24h) and photographed (light microscopy, 40 $\times$ magnification); B: MDA-MB-231 cells were treated with 14.5 μ g/ml of the cucurbitacin glucosides combination (24h). Cells were fixed and stained with β -tubulin antibody and photographed (confocal microscope, 40 $\times$ magnification).

FIG. 7 is a Western blot analysis of extracts obtained from MCF7 and MDA-MB-231 cells treated with the cucurbitacin glucosides combination. Cells were treated with 0 (-)

and 14.5 µg/ml (+) cucurbitacin glucosides combination for 24 h. Extracts were prepared and analyzed by Western blotting with an antibody to STAT3, phospho-STAT3 (Tyr-705). A: antibody to p21; B: antibody to survivin; C: An antibody to β-actin was used as a loading control. Western blots are representative of three independent experiments.

FIG. 8 shows annexin V binding and propidium iodide uptake induced by the cucurbitacin glucosides combination treatment. A: MDA-MB-231 cells were treated with the cucurbitacin glucosides combination (14.5 µg/ml) for 24 h, stained with annexin V and propidium iodide, and analyzed by flow cytometry. The horizontal (FL1-H) and vertical (FL2-H) axes represent labeling with annexin V and propidium iodide (PI), respectively; B: Graphic presentation of data obtained by Annexin/PI staining after 24h treatment using 5.8 µg/ml and 14.5 µg/ml cucurbitacin glucosides combination. UR represents late apoptotic cells (positive for both Annexin and PI); LL represents live cells. The data shown are representative of three independent experiments with similar findings.

FIG. 9 shows the effect of cucurbitacin glucoside combination on membrane potential. Cells were analyzed on a FACScan cytometer. A dot plots of red fluorescence (FL2) vs. green fluorescence (FL1) showing live cells with intact mitochondrial membrane potential and dead cells with lost mitochondrial potential respectively. The data shown are representative of three independent experiments with similar findings.

DETAILED DESCRIPTION OF THE INVENTION

It is an object of the present invention to determine whether *Citrullus colocynthis* leaves contain constituents that inhibit the growth of human breast cancer cells, and therefore may be useful in the prevention or treatment of breast cancer, or other forms of cancer. Unexpectedly the present invention discloses that these novel ingredients act synergistically to retard the growth of exemplary human breast tumor cell lines.

The present invention provides for the first time a detailed NMR analysis of cucurbitacin glucosides isolated from *C. colocynthis* leaves and their inhibiting activity on breast cancer cells through G₂/M arrest and apoptosis.

Taken together, the results of the present invention show that cucurbitacin glucosides have therapeutic value against both estrogen dependent and estrogen

independent breast cancer.

Definitions

The chemical structure of cucurbitacin B glucoside (MW=739) is presented in Figure 1. The structure of cucurbitacin E glucoside (MW=737) is the same as cucurbitacin B glucoside except to a double bond between carbon 1 and 2.

The term “treating a tumor” or “inhibiting a tumor” as used herein is intended to encompass tumor formation, primary tumors, tumor progression or tumor metastasis.

The term “reduction of growth” in relation to cancer cells, in the context of the present invention refers to a decrease in at least one of the following: number of cells (due to cell death which may be necrotic, apoptotic or any other type of cell death or combinations thereof) as compared to control; decrease in growth rates of cells, i.e. the total number of cells may increase but at a lower level or at a lower rate than the increase in control; decrease in the invasiveness of cells (as determined for example by soft agar assay) as compared to control even if their total number has not changed; progression from a more differentiated cell type to a less differentiated cell type; a deceleration in the neoplastic progress; or alternatively the slowing of the progression of the cancer cells from one stage to the next.

Reduction of growth of cancer cells may be utilized for the treatment of cancer by the administration, to an individual in need of such treatment, of a therapeutically effective amount of the compound of the present invention, as described herein.

As used herein, the terms “subject” or “patient” refers to a human or non-human mammal. Subjects in need of treatment involving inhibition of tumor growth can be identified using standard techniques known to those in the relevant art.

Preferred embodiments of the invention

The cucurbitacin glucosides of the present invention were extracted from the leaves of *Citrullus colocynthis* with water, followed by few more extractions to yield cucurbitacin B glucoside and cucurbitacin E glucoside purified fractions as described hereinbelow. Cucurbitacin I glucoside was also extracted using the same methods. It is to be understood that cucurbitacin B/E glucosides extracted by other methods as are known in the art are also encompassed within the scope of the present invention.

Analogues of the cucurbitacin glucosides of the present invention as well as salt thereof, as along as they exert growth inhibitory activity on tumor cells may be also used according to the teaching of the present invention.

Cucurbitacin B/E glucoside combination (1:1) exhibited growth inhibitory activity of human breast cancer cell lines in a dose- and time-dependent manner as can be seen by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Figure 2). Cucurbitacin B and E combination treatment inhibited cell growth with IC₅₀ value of 5.8 µg/ml after 48h for the estrogen independent MDA-MB-231 cell line. Cell cycle analysis showed that treatment with isolated cucurbitacin glucosides or their combination induced cell cycle arrest in the G₂/M phase of the cell cycle (60% in G₂/M compared to 20% in untreated cells). Unexpectedly, the present invention now shows a significant synergistic effect on cell cycle arrest when a combination of both cucurbitacin was applied to estrogen dependent as well as to estrogen independent cancerous breast cells (Figure 3).

p34^{CDC2} and cyclin B1 are known as the key proteins regulating G₂/M transition. The p34^{CDC2} protein kinase is generally acknowledged to be an important mediator of G₂/M phase transition in all eukaryotic cells (Liang et al. 2003; Biochem Pharmacol 65:1053-1060). The active mitotic kinase (MPF, or mitosis-promoting factor) is a dimer comprised of catalytic subunit, a B-type cyclin and a cyclin dependent kinase termed p34^{CDC2} or CDK1. The cyclins are a class of proteins that are synthesized during the interphase of each cell cycle and are rapidly degraded at the end of mitosis (Xiao et al. 2003; Carcinogenesis 24:891-897). The activity of the p34^{CDC2} kinase not only depends on its association with cyclin B1, but also on its phosphorylation state. Phosphorylation of either Thr14 or Tyr15 inhibits p34^{CDC2} kinase activity, while phosphorylation on Thr161 by CDK7 kinase is required for kinase activity. In addition, the dephosphorylation of Thr14 or Tyr15 by CD^{C25C} phosphatase is a final step for p34^{CDC2} kinase activity (Molinari M. 2000; Cell Prolif 33:261-274) and also serves as a main component in regulating G₂/M arrest in respond to DNA damaging agents. Treating cells with the cucurbitacin B/E glucosides combination of the invention caused a reduction at the protein level of both p34^{CDC2} and Cyclin B1. This reduction was very rapid and could be detected as early as 1 h post treatment. By 24 h only remnant of p34^{CDC2} and cyclin B1 protein were observed (Figure 5A). This phenomenon indicates a highly effective activity, as by 24 h of treatment most of the cells were arrested in G₂/M

stage in which p34^{CDC2} and cyclin B1 are mostly expressed. Moreover, when phosphorylated p34^{CDC2} was followed, a sharp decrease in phosphorylation status was observed, with a similar kinetics compared to the inhibition at the protein level (Figure 5B). Thus, without wishing to be bound to a specific mechanism or theory, treating cancerous cells with cucurbitacin B/E glucosides may cause cell cycle arrest at G₂/M by reducing the amount and hence the activity of p34^{CDC2}/cyclin B1 complex, which is necessary for G₂ to M transition, and not by inhibition of p34^{CDC2} activity by its phosphorylation.

Cucurbitacin B/E glucosides treatment caused an unexpected elevation in the phosphorylated form of STAT3 (Figure 7A). This finding is in contrast to recent studies showing that cucurbitacin I and Q specifically inhibit STAT3 phosphorylation that contributes to the proliferation of many cancerous cells (U.S. Patent Application 2004/0138189; Blaskovich et al. 2003; Cancer Res 63:1270-1279; Sun et al. 2005; Oncogene 24:3236-3245). Cucurbitacin B/E treatment also caused a marked change in cell morphology with a disruption of the elongated shape of the cells toward a round shape appearance, as was demonstrated by both tubulin staining and by light microscopy visualization (Figure 6). These results suggest that cucurbitacin treatment cause impairment in actin filament organization. This profound morphological change might as well influence intracellular signaling by molecules such as PKB, resulting in inhibition in transmission of survival signals. Indeed, as is shown in Figure 7, cucurbitacin glucosides treatment caused a sharp reduction in survivin expression and, on the other hand, induced p21^{waf} expression as expected in a situation where STAT3 is enhanced in the presence of inhibited PKB signaling.

Without wishing to be bound to a specific mechanism or theory, the inhibitory effect of cucurbitacin B/E glucoside on breast cell proliferation can be attributed to both STAT3 activation and at the same time inhibition of PKB signaling through disruption of cytoskeleton filaments causing inhibition in survivin expression.

The preferred outcome of a chemotherapeutic drug is apoptosis. In addition to cell cycle arrest cucurbitacin treatment also induced apoptosis, as was measured by cell surface annexin V binding, which measure the appearance of phosphatidylserine on the external plasma membrane and by binding of a fluorescent dye, known as JC-1 that indicates changes in mitochondrial membrane potential ($\Delta\Psi$) (Bijl et al. 2003; Arthritis

Rheum 48:248-254; Nihal et al. 2005; Int J Cancer 114:513-521). The two methods used clearly showed (Figures 8 and 9) an apoptosis induction resulting in up to 3.5 fold in apoptotic cell fraction following cucurbitacin B/E glucosides treatment.

5 Taken together, these results show that cucurbitacin glucosides effectively inhibit proliferation of both estrogen-dependent and estrogen-independent human breast cancer cells by causing G₂/M phase arrest and apoptosis. Moreover, the use of the cucurbitacin glucosides combination of the present invention is advantageous to the use of non-glucosilated cucurbitacin for the treatment of cancer, as the glucosilated forms were found to have minor toxic effects as compared to cucurbitacins that do not contain a
10 glucoside moiety (Bartalis and Halaweish 2005; J Chromatogr B Analyt Technol Biomed Life Sci 818:159-166).

Pharmaceutical compositions

According to certain embodiments, cucurbitacin E glucoside, cucurbitacin B glucoside, or preferably, a combination thereof are formulated as a pharmaceutical
15 composition to be used for treating a tumor according to the teaching of the present invention.

The pharmaceutical compositions of the invention will be administered to the patient by standard procedures. The amount of compound to be administered and the route of administration will be determined according to the kind of tumor, stage of the disease, age
20 and health conditions of the patient. The pharmaceutical composition of the present invention may be administered by any suitable means, such as topically, orally or parenterally including intranasal, subcutaneous, intramuscular, intravenous, intra-arterial, intraarticular, or intralesional administration. Ordinarily, intravenous (i.v.), intraarticular or oral administration will be preferred.

25 The compound used according to the invention can be formulated by any required method to provide pharmaceutical compositions suitable for administration to a patient.

The cucurbitacin compounds of the present invention include all hydrates and salts that can be prepared by those of skill in the art. Under conditions where the compounds of the present invention are sufficiently basic or acidic to form stable
30 nontoxic acid or base salts, administration of the compounds as salts may be appropriate. Examples of pharmaceutically acceptable salts are organic acid addition salts formed with acids which form a physiological acceptable anion, for example,

tosylate, methanesulfonate, acetate, citrate, malonate, tartarate, succinate, benzoate, ascorbate, alpha-ketoglutarate, and alpha-glycerophosphate. Suitable inorganic salts may also be formed, including hydrochloride, sulfate, nitrate, bicarbonate, and carbonate salts.

5 The novel compositions contain, in addition to the active ingredient, conventional pharmaceutically acceptable carriers, diluents and the like as are known in the art (see, for example, Ansel, *Pharmaceutical Dosage Forms and Drug Delivery Systems*. Malvern, PA: Williams and Wilkins, 1995; Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995). In addition, if desired, the composition
10 can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, flavoring agents, binders, surface active agents, thickeners, lubricants, preservatives, (including antioxidants) and the like.

Pharmaceutical compositions for use in accordance with the present invention may be manufactured by processes well known in the art, e.g., by means of
15 conventional mixing, dissolving, granulating, grinding, pulverizing, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Solid compositions for oral administration, such as tablets, pills, capsules or the like, may be prepared by mixing the active ingredient with conventional, pharmaceutically acceptable ingredients such as corn starch, lactose, sucrose, sorbitol,
20 talc, stearic acid, magnesium stearate, dicalcium phosphate and gums, with pharmaceutically acceptable diluents. The tablets or pills can be coated or otherwise compounded with pharmaceutically acceptable materials known in the art to provide a dosage form affording prolonged action or sustained release. Other solid compositions can be prepared as microcapsules for parenteral administration. Liquid forms may be
25 prepared for oral administration or for injection, the term including subcutaneous, intramuscular, intravenous, and other parenteral routes of administration. The liquid compositions include aqueous solutions, with or without organic cosolvents, aqueous or oil suspensions, emulsions with edible oils, as well as similar pharmaceutical vehicles. In addition, the compositions of the present invention may be formed as encapsulated
30 pellets or other depots, for sustained delivery.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount

effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of a compound effective to prevent, delay, alleviate or ameliorate one or more symptoms associated with a disorder being treated over a period of time. Particularly, a disorder according to the teaching of the present invention is
5 tumor growth.

According to the method of the present invention, cucurbitacin B or E glucoside, or a pharmaceutically acceptable salt or analogue thereof can be administered to a patient as sole active ingredient, or co-administered with another compound. Preferably, a combination of cucurbitacin B/E or a pharmaceutically acceptable salt or analogue
10 thereof is administered. Furthermore, the cucurbitacin B/E combination of the present invention can be administered to a subject as adjunctive therapy with an additional anti-tumor agent. Co-administration can be carried out simultaneously (in the same or separate formulations) or consecutively. According to certain embodiment, the adjunctive therapy is selected from the group consisting of radiation therapy,
15 chemotherapy, immunotherapy, hormonal therapy and genetic therapy.

The cucurbitacin formulations of the present invention can include various other components as additives. Examples of acceptable components or adjuncts which can be employed in relevant circumstances include antioxidants, free radical scavenging agents, peptides, growth factors, antibiotics, bacteriostatic agents, immunosuppressive,
20 anticoagulants, buffering agents, anti-inflammatory agents, antipyretics, time-release binders, anesthetics, steroids, and corticosteroids. Such components can provide additional therapeutic benefit, act to affect the therapeutic action of the cucurbitacin compound, or act towards preventing any potential side effects which may be posed as a result of administration of the cucurbitacin compound. The cucurbitacin compounds of
25 the subject invention can be conjugated to a therapeutic agent, as well.

Additional agents that can be co-administered to a subject in the same or as a separate formulation include those that modify a given biological response, such as immunomodulators. For example, proteins such as tumor necrosis factor (TNF), interferon (such as alpha-interferon and beta-interferon), nerve growth factor (NGF),
30 platelet derived growth factor (PDGF), and tissue plasminogen activator can be administered. Biological response modifiers, such as lymphokines, interleukins (such as interleukin-1 (IL-1), interleukin-2 (IL-2), and interleukin-6 (IL-6)), granulocyte

macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), or other growth factors can be administered.

Toxicity and therapeutic efficacy of the cucurbitacin compounds described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., by determining the IC₅₀ (the concentration which provides 50% inhibition) and the LD₅₀ (lethal dose causing death in 50% of the tested animals) for a subject compound. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition (e.g. Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1, Laurence L. Brunton, Ed.).

It will be understood that the dosage may be an escalating dosage so that low dosage may be administered first, and subsequently higher dosages may be administered until an appropriate response is achieved. Also, the dosage of the composition can be administered to the subject in multiple administrations in the course of the treatment period in which a portion of the dosage is administered at each administration. Depending on the severity and responsiveness of the condition to be treated, dosing can also be a single administration of a slow release composition, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

It will be apparent to those of ordinary skill in the art that the therapeutically effective amount of the molecule according to the present invention will depend, *inter alia* upon the administration schedule, the unit dose of molecule administered, whether the molecule is administered in combination with other therapeutic agents, the immune status and health of the patient, the therapeutic activity of the molecule administered and the judgment of the treating physician.

The foregoing formulations and administration methods are intended to be illustrative and not limiting. It will be appreciated that, using the teaching provided herein, other suitable formulations and modes of administration can be readily devised.

The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Material and Methods

Extraction and isolation procedures

Fresh leaves of *Citrullus colocynthis* (L.) Shrad (collected from an open field at Bar-Ilan University), were mixed 1:4 (w/v) with distilled water and homogenized in a blender for about 5 min. The homogenate was filtered and centrifuged at 20,000×g for 10 min. The supernatant was frozen in liquid nitrogen and was dried in lyophilizer (0.07 mbar, -48°C). The powder was further extracted in 70% chloroform/methanol overnight, filtered and evaporated under reduced pressure. The resulting extract was examined by thin layer chromatography (silica gel 60 F₂₅₄ plates, Merck Eurolab SA, Strasbourg, France) using the solvent system: chloroform/methanol (9:1). The fraction with R_f = 0.56 was a mixture of two cucurbitacins, cucurbitacin B and E glucoside (1:1). The two cucurbitacin glucosides were separated by another TLC (0.25 mm layer thickness) using the solvent system benzene-ethanol (8:2). The compounds were visualized under UV.

Identification of Molecular Structure by NMR

NMR spectra of the cucurbitacin glucosides were obtained on a Bruker DMX-600 spectrometer, at 600.1 (¹H) and 150.9 (¹³C) MHz, respectively, at room temperature. The solvent used was a mixture of 10% CD₃OD and 90% CDCl₃, containing 0.1% TMS as internal reference. Table 1 presents NMR assignments for cucurbitacin B and E glucosides. The aglycone of the latter (cucurbitacin E, Carl-Roth, GmbH) was also analyzed in the same solvent combination for comparison purposes. NMR analysis was facilitated by the use of 2D experiments such as COSY (¹H×¹H correlation), HMQC (one-bond ¹³C×¹H correlation), and HMBC (long-range ¹³C×¹H correlation).

Cell culture

MCF7 (estrogen receptor positive) and MDA-MB-231 (estrogen receptor negative) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI-1640 medium (Biological Industries, Inc., Kibbutz Beit Haemek, Israel) supplemented with 10% (v/v) fetal calf serum (FCS), 1% penicillin-streptomycin-nystatin. The cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air.

Cell proliferation and viability assay

The effect of the cucurbitacin glucoside treatment on survival/proliferation of MCF7 and MDA-MB-231 cells was measured by a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, MO) assay based on the ability of live cells to cleave in active mitochondria the tetrazolium ring to a molecule that absorbs at 570 nm (Mosmann 1983; J Immunol Methods 65:55-63). 7×10^3 cells were grown on a 96-well microliter plate and incubated with cucurbitacin B/E glucoside or their combination (2.9, 5.8 and 14.5 $\mu\text{g/ml}$) in RPMI-10% FCS medium. After 24, 48, and 72h, the medium was changed and 130 $\mu\text{l/well}$ of fresh RPMI-1640 media were added. Next, 20 μl MTT reagent (5 mg/1 ml PBS) was added to each well, and the cells were further incubated at 37°C for 2h. To determine lysis of the cells, 100 μl N,N-dimethyl formamid solution (50% final concentration of N,N-dimethyl formamid and 20% of sodium dodecyl sulphate, pH 4.7) was added to each well for an additional 7h, followed by reading on a scanning multi-well spectrophotometer.

15 Flow Cytometry

MCF-7 and MDA-MB-231 cells (5×10^5) were seeded in 100 mm culture dishes, and allowed to attach overnight. The medium was replaced with fresh complete medium containing desired concentration of cucurbitacin B/E glucoside (B: 15 and 23 $\mu\text{g/ml}$; E: 10 and 18 $\mu\text{g/ml}$) or their combination (5.8, 11.6, 14.5 $\mu\text{g/ml}$). Cells were incubated for 24 h at 37°C. The cells were washed with PBS and then spun at 300g. The pellet was resuspended in 250 μl PBS and 250 μl propidium iodide (50 $\mu\text{g/ml}$ final concentration) for 15 min, and analyzed using a flow cytometer.

AnnexinV/PI Flow Cytometric Staining Technique

Apoptosis was determined based on morphological change. Apoptotic cells were quantified by annexin V-FITC and propidium iodide (PI) double staining by using a staining kit purchased from MBL Co., Ltd. (Watertown, MA).

JC-1 mitochondrial membrane potential detection assay

JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzamidazolocarbocyanin iodide, sigma, MO) was used for *in-situ* detection of mitochondrial membrane transition events in live cells, which provides an early indication of the initiation of cellular apoptosis. JC-1 used to indicate the collapse in the electrochemical gradient across the

mitochondrial membrane ($\Delta\Psi$). In non-apoptotic cells, JC-1 exists as a monomer in the cytosol (green) and also accumulates as aggregates in the mitochondria (red). In apoptotic and necrotic cells, JC-1 exists in monomeric form and stains the cytosol green. For this assay, cells were treated with the cucurbitacin glucoside combination (0- control, 5.8, 14.5 $\mu\text{g/ml}$) and maintained at 37°C in a humidified 5% CO₂ atmosphere for 24h. Cells were washed with PBS and spun at 300g. The pellet was resuspended in 500 μl PBS, and 2 μl of 1 mg/ml JC-1 reagent was added for 20 minutes in 37°C in the dark. Cells were washed with PBS and analyzed using a flow cytometer. The analysis was performed using Cell Quest software (BD Biosciences, San Jose, CA) for apoptosis.

Western blot analysis

MCF7 and MDA-MB-231 cells (1×10^6) were seeded in 100 mm culture dishes. The cells were treated with the cucurbitacin glucosides combination (14.5 $\mu\text{g/ml}$) in RPMI-1640 media for 24h. The media was then aspirated, and the cells were washed with cold PBS. The cells were scraped and washed twice by centrifugation at 500 $\times$ g for 5 min at 4°C. The pellet was resuspended in lysis buffer supplemented with proteases and phosphatase inhibitors and incubated for 1h at 4°C. The lysate was collected by centrifugation at 14,000 $\times$ g for 40 min at 4°C, and the supernatant (total cell lysate) was stored at -20°C. For Western blot analysis, 30 μg protein were resolved over 12% polyacrylamide gels and transferred to a nitrocellulose membrane. The blot was blocked in blocking buffer (1% nonfat dry milk/1% Tween 20 in PBS) for 1 h at room temperature, incubated with appropriate monoclonal primary antibodies (human reactive anti- p34^{CDC2}, anti-Survivin, anti- STAT3 from Santa Cruz Biotechnology, anti-Cyclin B1 from Biosource and anti-p21^{WAF} from BD Biosciences) or polyclonal primary antibodies (human reactive anti-phospho- p34^{CDC2} (Thr14 and Tyr15) from Santa Cruz and anti-phospho- STAT3 from cell signaling) in blocking buffer overnight at 4°C. The blot was then incubated with anti-mouse secondary antibody horseradish peroxidase conjugate and detected by chemiluminescence and autoradiography using X-RAY film. β -actin detected on the same membrane and used as a loading control.

Microscopy

Treated cells (14.5 $\mu\text{g/ml}$) and untreated cells (for 24h), were examined under a light microscope CK40 (Olympus).

Immunocytochemistry

Cells were grown on glass coverslips and fixed after treatment (14.5 $\mu\text{g/ml}$ of the cucurbitacin glucosides combination for 24h) in 4% paraformaldehyde solution for 30 min. Cells were washed twice with PBS, and blocked by 90% blocking solution (1% BSA, 0.5% Triton in PBS) and 10% Fetal calf serum, for 1h. Incubation with β -tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was performed in blocking solution over night at 4°C. Cells were washed four times with PBST (0.2% Tween) and incubated with secondary antibody (in blocking solution) for 1h. The glass slides were analyzed using a confocal microscope.

10 Statistical analysis

All experiments were performed at least 3 times. Where appropriate, the data are expressed as the mean $\pm$ standard error of the mean (SEM).

Example 1: Spectroscopic identification of the isolated fraction

Fractions were isolated from the water extract of *Citrullus colocynthis* leaves using TLC as described in the Materials and Methods section hereinabove, and their structure was determined by NMR spectroscopy (Table 1; Fig. 1). As can be seen from the NMR data the fractions isolated are glycosylated cucurbitacin B and E.

Recently Seger et al. (Seger et al. 2005; Magn Reson Chem 43:489-491) pointed out that some works in the literature report NMR data for cucurbitacins, but without the benefit of modern, two-dimensional NMR assignment techniques. As a consequence, the inventors of the present invention found that the published data contain inconsistencies and contradictions. NMR characteristic assignments for cucurbitacin B and E glucosides are presented in Table 1. For comparison purposes, NMR for the aglycone of cucurbitacin E glucoside (commercial) was performed in the same solvent combination. The NMR was facilitated by the use of 2D experiments such as COSY ($^1\text{H} \times ^1\text{H}$ correlation), HMQC (one-bond $^{13}\text{C} \times ^1\text{H}$ correlation), and HMBC (long-range $^{13}\text{C} \times ^1\text{H}$ correlation).

Table 1: NMR data for Cucurbitacin B glucoside (Table 1A) and Cucurbitacin E glucoside (Table 1B)**Table 1A**

Peak No.	¹³ C chemical shift (ppm)	¹ H chemical shift (ppm)	Multiplicity (Numbers represent J-coupling in Hz)	Peak No.	¹³ C Chemical shift (ppm)	¹ H Chemical shift (ppm)	Multiplicity (Numbers represent J-coupling in Hz)
1	35.24	2.43	bd 13	18	20.02	0.95	bs, 3H
		1.41	m (superimposed)	19	19.94	1.06	s, 3H
2	80.22	4.60	dd 13, 5.5	20	78.63	—	
3	211.62	—		21	24.15	1.43	s, 3H
4	51.43	—		22	203.26	—	
5	139.79	—		23	120.63	6.56	d, 16
6	120.43	5.79	bd, 6	24	151.58	7.03	d, 16
7	23.91	2.03	m (superimposed)	25	79.81	—	
		1.96	m (superimposed)	26	26.10	1.56	s, 3H
8	42.48	1.98	m (superimposed)	27	26.33	1.56	s, 3H
9	48.61	—		28	29.02	1.29	s, 3H
10	34.18	2.80	bd, 13	29	21.24	1.30	s, 3H
11	214.47	—		30	18.75	1.34	s, 3H
12	48.79	3.28	bd, 14.5	Ac	170.84	—	
		2.65	d, 14.5	Ac	21.97	2.03	s, 3H
13	50.53	—		1'	104.20	4.28	d, 7.5
14	48.12	—		2'	73.85	3.36	m (superimposed)
15	45.27	1.86	bdd, 13, 9	3'	76.39	3.45	t, 9
		1.43	m (superimposed)	4'	70.16	3.36	m (superimposed)
16	70.88	4.38	ddd, 9, 7.5, 1.5	5'	76.62	3.31	ddd, 10, 6, 2.5
17	58.34	2.51	d, 7	6'	62.35	3.92	dd, 12.5, 2.5
						3.77	dd, 12.5, 6

- 5 b – broad; d – doublet; m – multiplet; s – singlet; t - triplet; bs - broad singlet; bd - broad doublet; bdd - broad double doublet; dd - double doublet; ddd - double double doublet

Table 1B

Peak No.	¹³ C chemical shift (ppm)	¹ H chemical shift (ppm)	Multiplicity (Numbers represent J-coupling in Hz)	Peak No.	¹³ C Chemical shift (ppm)	¹ H Chemical shift (ppm)	Multiplicity (Numbers represent J-coupling in Hz)
1	122.98	6.16	bd, 2.5	18	20.03	0.96	bs, 3H
2	145.95	—		19	20.03	1.00	s, 3H
3	198.85	—		20	78.65	—	
4	49.29	—		21	24.10	1.42	s, 3H
5	135.56	—		22	203.24	—	
6	121.48	5.81	dt, 5, 2.5	23	120.67	6.58	d, 16
7	23.71	2.39	ddt, 20, 9.5, 3	24	151.57	7.03	d, 16
		2.06	ddd 20, 5, 2	25	79.81	—	
8	41.58	2.04	m (superimposed)	26	26.17	1.56	s, 3H
9	49.05	—		27	26.32	1.56	s, 3H
10	35.27	3.52	m (superimposed)	28	28.05	1.24	s, 3H
11	215.41	—		29	20.24	1.30	s, 3H
12	48.97	3.23	bd, 14.5	30	18.15	1.38	s, 3H
		2.69	d, 14.5	Ac	170.83	—	
13	50.55	—		Ac	21.97	2.03	s, 3H
14	48.09	—		1'	100.57	4.61	d, 8
15	45.46	1.88	bdd, 13.5, 9	2'	72.78	3.45	dd, 9.5, 8
		1.48	bd, 13.5	3'	75.72	3.51	m (superimposed)
16	70.89	4.42	ddd. 9, 7, 1.5	4'	69.77	3.51	m (superimposed)
17	58.30	2.50	d, 7	5'	77.01	3.46	ddd, 10, 4.5, 2.5
				6'	61.95	4.07	dd, 12.5, 2.5
						3.98	dd, 12.5, 4.5

b – broad; d – doublet; m – multiplet; s – singlet; t - triplet; bs - broad singlet; bd - broad doublet; bdd - broad double doublet; dd - double doublet; ddd - double double doublet

Example 2: Effect of cucurbitacin B and E combination on MCF-7 and MDA-MB-231 cell proliferation

To evaluate the effect of cucurbitacin glucoside on proliferation of human breast cancer cell lines MCF-7 and MDA-MB-231, both cell lines were initially treated with escalating doses of the cucurbitacin glucoside combination (1:1). Cell proliferation was estimated by the MTT assay (as described in the Materials and Methods section hereinabove). The effect at various concentrations was studied after 24, 48, and 72h of cell growth. Figure 2 demonstrates that cucurbitacin glucosides combination inhibited cellular proliferation in a dose-dependent manner. The proliferation of MDA-MB-231 cells was reduced by 50% upon a 48 h exposure to 5.8 µg/ml cucurbitacin glucoside combination, while using cucurbitacin B or E glucoside alone in this concentration was less effective. Antiproliferative activity of the cucurbitacin glucoside combination was further confirmed by counting live cells (using trypan blue exclusion dye). Proliferation of MDA-MB-231 cells was inhibited upon treatment in dose- and time-dependent manner.

Example 3: Effect of cucurbitacin B or E glucoside and their combination on cell cycle

To gain insights into the mechanism by which cell reduction is achieved the effect of cucurbitacin B or E glucoside or their combination on the cell cycle distribution was investigated by fluorescence-activated cell sorting (FACS) analysis. The results are summarized in Figures 3 and 4. A 24 h exposure of MDA-MB-231 and MCF-7 cells to 15/23 µg/ml cucurbitacin B glucoside or 18 µg/ml cucurbitacin E glucoside caused ~3-fold enrichment of cells in G₂/M phase (Figure 3). The combination of cucurbitacin B and E glucosides (1:1) was more active than each component alone showing a synergistic effect, as was found by using the MTT assay. A 24 h exposure of MCF7 cells to 11.6 µg/ml (which correlate to 8 µM) of the cucurbitacin glucoside combination resulted in the accumulation of 60% cells in G₂/M phase which is ~3-fold enrichment of cells in G₂/M phase compared to control untreated cells. The G₂/M enrichment was accompanied by a 93% decrease in S phase cells (Fig. 4A). A histogram representing this G₂/M arrest is shown in Figure 4B. Since the combination was more effective than each component alone the combination of B and E cucurbitacin glucosides (1:1) was

used for the following experiments. These results clearly show the synergistic activity of cucurbitacin B and E glucosides combination.

Example 4: Effect of the cucurbitacin glucosides combination on cell protein

expression

The molecular events involved in the cucurbitacin glucosides activity on cell cycle progression, were studied by examining the effect of the cucurbitacin glucosides combination on the expression of proteins that are pivotal for G₂/M transition, including p34^{CDC2} and cyclin B1. The G₂/M transition requires activity of cyclin-dependent kinase, p34^{CDC2}. p34^{CDC2} is positively regulated by association with cyclin B1, and negatively regulated by phosphorylation of amino acids Thr14 and Tyr15. Figure 5A represent a typical Western blot from treated (14.5 µg/ml of the cucurbitacin glucosides combination) and untreated cells. A decrease in the level of cyclin B1 (~3 fold reduction compared with control in MCF-7 cells and ~6 fold reduction in MDA-MB-231) and in the level of p34^{CDC2} protein was observed at 24 h post treatment. In many cases, particularly those elicited by DNA-damaging agents, reduction in p34^{CDC2} expression is preceded by p34^{CDC2} phosphorylation at the inhibitory positions Thr14 and Tyr15. Thus, the Thr14 and Tyr15 phosphorylation status of p34^{CDC2} at early time points was followed, in order to examine whether cucurbitacin glucosides combination treatment induces inhibitory phosphorylation of p34^{CDC2}. As is shown in Figure 5B a reduction in phospho p34^{CDC2} was observed as early as 1 h post treatment. These results show that cucurbitacin glucosides combination causes rapid elimination of p34^{CDC2} protein which is responsible for blocking the G₂ to M transition. The mechanism by which this profound phenomenon occurs is unknown. These results indicate that changes in expression of G₂/M regulating proteins may contribute to the cucurbitacin glucosides mediated cell cycle arrest in MCF-7 and MDA-MB-231 cells.

Example 5: Effect of the cucurbitacin glucosides combination on cell morphology

Cucurbitacin E was previously shown to interfere with cell cytoskeleton (Duncan and Duncan 1996; 1997; *supra*), and to cause marked disruption of the actin cytoskeleton. Staining analysis using light microscopy was employed to measure the

effect of the cucurbitacin glucosides combination of the invention on cell morphology, particularly on cytoskeleton elements. Viewing cells after treatment (14.5 $\mu\text{g/ml}$) compared to control, using light microscopy, showed clearly a profound change in the overall morphology of the cell from an elongated typical MDA-MB-231 cells to a round shape cells (Figure 6A). Histochemical staining with β -tubulin confirmed this result (Figure 6B). This effect of the cucurbitacin combination is probably not a result of an apoptotic process which is also characterized by round cell morphology, since it appeared shortly after treatment when there is no evidence for cells undergoing apoptosis, but rather to the alteration of the cytoskeleton network, causing both changes in cell morphology and consequently G₂/M arrest and apoptosis.

Example 6: Effect of Cucurbitacin on STAT3 activation and p21^{waf} expression

It has been demonstrated that cucurbitacin I and Q can interfere with STAT3 signaling by inhibiting specifically STAT3 phosphorylation (Blaskovich et al. 2003; *supra*). The effect of cucurbitacin glucosides combination on STAT3 phosphorylation was therefore examined. Unexpectedly, it was found that cucurbitacin glucosides combination enhance rather than decrease STAT3 phosphorylation (Figure 7A). Constitutive activation of STAT3 has been shown in many different tumors. This activation usually results in antiapoptotic effect and promotes cell proliferation (Yu and Jove 2004; Nat Rev Cancer 4:97–105). The effect of cucurbitacin on STAT3 phosphorylation is therefore incompatible with the previous results presented herein showing decrease in cell proliferation. This phenomenon may be explained in view of two different papers published lately, concerning both STAT3 signaling and the consequences of cytoskeleton disruption. First, STAT3 was found to exhibit inhibitory activity on cell proliferation by enhancing p21^{waf} expression in a specific situation in which both STAT3 activation and inhibition of PKB signaling occur simultaneously (Barré et al. 2003; J Biol Chem 278:2990-2996). Another published study showed that disruption of actin cytoskeleton network reduces PKB signaling culminating in reduction in the expression level of survivin (an IAP family member) which leads to both G₂/M arrest and apoptosis (Liang et al., *supra*). Since in the present invention both STAT3 activation and cell transition to round cell morphology were observed, which is a hallmark of cytoskeleton disruption, cucurbitacin treatment may brings about the

specific condition in which STAT3 activity is exploited in favor of preventing cell proliferation rather than enhancing it. Indeed, the present invention now demonstrates that cucurbitacin treatment elevates p21^{waf} expression and inhibits survivin expression, exactly as expected (Figure. 7B-7C). Thus, without wishing to be bound to a specific mechanism or theory, it is postulated that cucurbitacin exhibit pleiotropic effects on cells causing both disruption of actin which reduces PKB signaling and enhancement of STAT3 activity culminating in both elevated expression of p21^{waf} and reduction in survivin expression level.

Example 7: Cucurbitacin glucosides combination induces apoptosis

The apoptosis inducing effect of cucurbitacin glucoside combination was evaluated by annexin V/PI binding. One of the earliest events of apoptosis is loss of plasma membrane polarity, which is accompanied by translocation of phosphatidylserine (PS) from the inner to outer membrane leaflets, thereby exposing PS to the external environment (Vermes et al. 1995; J Immunol Methods 184:39-51). The phospholipid-binding protein Annexin V has a high affinity for PS and can bind to cells with externally exposed PS. Positive staining with fluorescently labeled annexin V correlates with loss of membrane polarity but precedes the complete loss of membrane integrity that accompanies later stages of cell death resulting from either apoptosis or necrosis. In contrast, PI can only enter cells after loss of membrane integrity. Thus, dual staining with annexin V and PI allows clear discrimination between unaffected cells (annexinV negative, PI negative), early apoptotic cells (annexin V positive, PI negative), and late apoptotic cells (annexin V positive, PI positive). As is shown in Figure 8, the cucurbitacin glucoside combination treatment (14.5 µg/ml) increased the percentage of late apoptotic (UR) cells in MDA-MB-231 cells from 18.43% in control untreated cells to 61.35% in treated cells (Figure 8A). Similar data were obtained for MCF-7 cells (data not shown). When cucurbitacin combination was tested in two escalating concentrations (5.8 µg/ml and 14.5 µg/ml) induction of apoptosis showed dose dependency which indicates that the apoptotic effect resulted specifically from cucurbitacin treatment.

The data were further confirmed using additional *in-situ* detection of apoptosis by measuring the effects of treatment on mitochondrial membrane potential ($\Delta\Psi$) using the

fluorescent cationic dye JC-1. This technique effectively detects apoptosis even at early stages. In healthy cells, JC-1 exists as a monomer in the cytosol (FL1 positive; green) and also accumulates as aggregates in the mitochondria (FL2 positive; red). In apoptotic and necrotic cells, JC-1 exists exclusively in monomer form and produces a green
5 cytosolic signal. As shown in Figure 6 cucurbitacin glucosides combination treatment in MDA-MB-231 cells elevated the fraction of apoptotic cells from 11.17% in control cells to 48.58% in cells treated with 14.5µg/ml. A dose response was also observed in this assay (Figure 9).

The foregoing description of the specific embodiments will so fully reveal the
10 general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood
15 that the phraseology or terminology employed herein is for the purpose of description and not of limitation. The means, materials, and steps for carrying out various disclosed functions may take a variety of alternative forms without departing from the invention.

CLAIMS

1. An isolated cucurbitacin glucoside from *Citrullus colocynthis*.
2. The isolated cucurbitacin glucoside from *Citrullus colocynthis* of claim 1,
selected from the group consisting of cucurbitacin B glucoside and
cucurbitacin E glucoside.
3. The isolated cucurbitacin glucosides of claim 2, having characteristic NMR
parameters as presented in Table 1.
4. A pharmaceutical composition comprising at least one isolated cucurbitacin
glucoside from *Citrullus colocynthis* or a pharmaceutically acceptable salt
thereof.
5. The pharmaceutical composition of claim 4 wherein the isolated
cucurbitacin glucoside is selected from the group consisting of cucurbitacin
B glucoside and cucurbitacin E glucoside.
6. The pharmaceutical composition of claim 5, wherein the isolated
cucurbitacin glucoside has characteristic NMR parameters as presented in
Table 1.
7. The pharmaceutical composition of claim 4 comprising a plurality of
isolated cucurbitacin glucosides.
8. The pharmaceutical composition of claim 7 comprising a combination of
cucurbitacin B glucoside and cucurbitacin E glucoside.
9. A method of treating a tumor in a subject in need thereof comprising
administering to the subject a pharmaceutical composition comprising a
therapeutically effective amount of at least one isolated cucurbitacin
glucoside.
10. The method of claim 9, wherein the cucurbitacin glucoside is isolated from
Citrullus colocynthis.
11. The method of claim 9, wherein the cucurbitacin glucoside is selected from
the group consisting of cucurbitacin B glucoside and cucurbitacin E
glucoside.
12. The method of claim 11, wherein the cucurbitacin glucoside has

characteristic NMR parameters as presented in Table 1.

13. The method of claim 9, wherein the pharmaceutical composition comprises a plurality of isolated cucurbitacin glucosides.

5 14. The method of claim 13, wherein the pharmaceutical composition comprises a combination of cucurbitacin B glucoside and cucurbitacin E glucoside.

15. The method of claim 9, wherein the pharmaceutical composition is administered in combination with at least one additional anti-tumor treatment.

10 16. The method of claim 15, wherein the additional anti tumor treatment is selected from the group consisting of radiation therapy, chemotherapy, immunotherapy, hormonal therapy and genetic therapy.

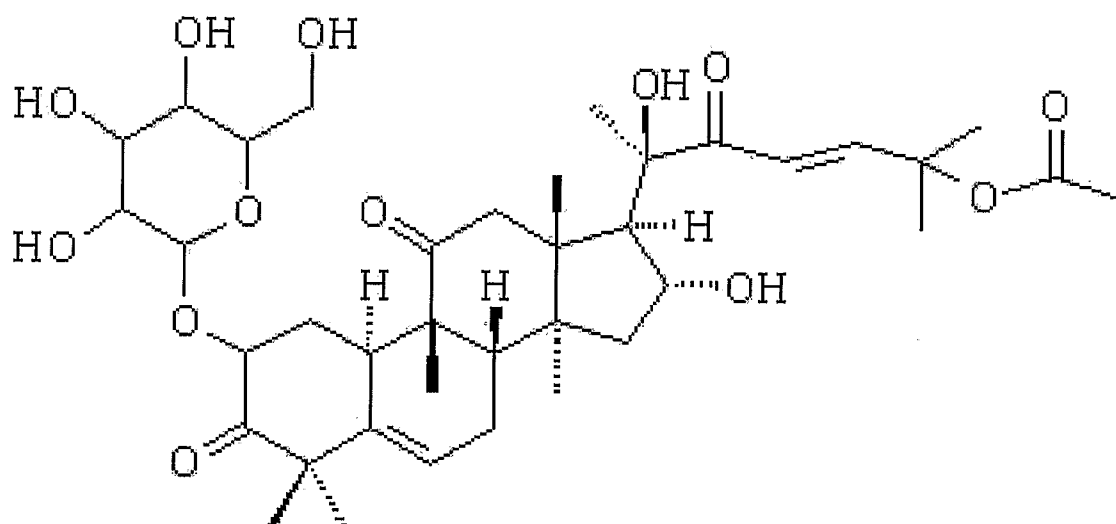
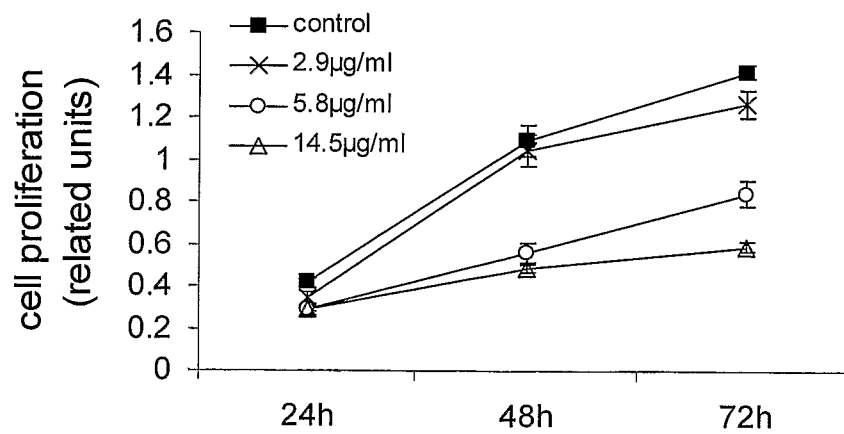
17. The method of claim 9 wherein the tumor is human breast tumor.

15 18. A method of treating a tumor in a subject in need thereof comprising administering to the subject a pharmaceutical composition comprising a plurality of isolated cucurbitacin glucosides that act synergistically to reduce the proliferation of the tumor cells.

19. The method of claim 18 wherein the pharmaceutical composition comprises cucurbitacin B glucoside and cucurbitacin E glucoside.

20 20. The method of claim 18 wherein the tumor is human breast cancer.

1/8

**FIGURE 1****FIGURE 2**

2/8

FIGURE 3A

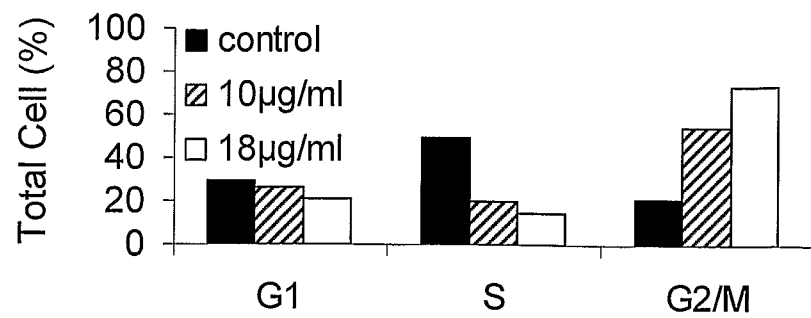


FIGURE 3B

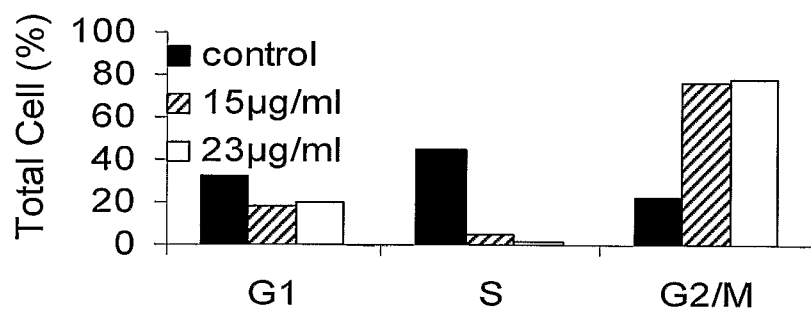


FIGURE 3C

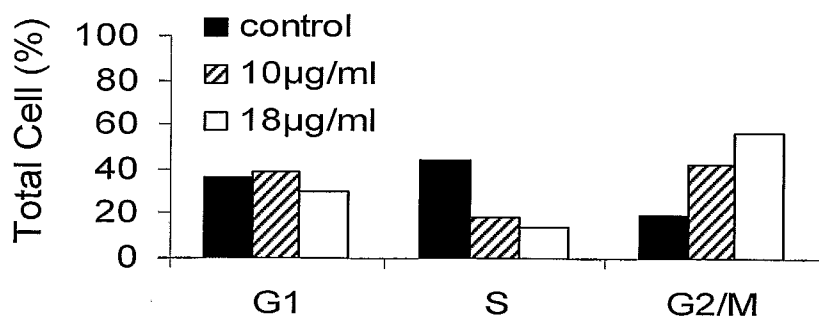
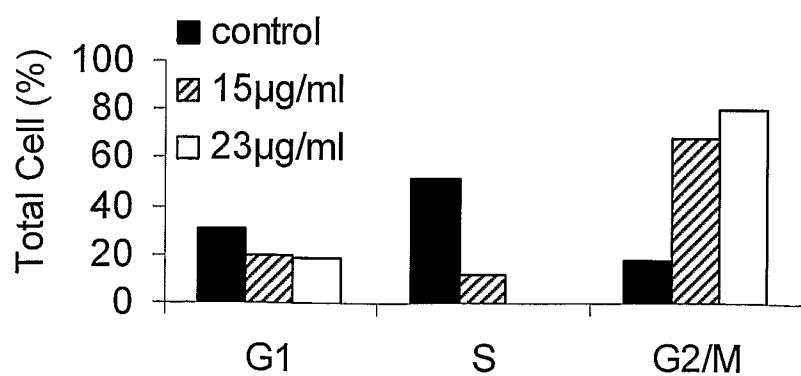


FIGURE 3D



3/8

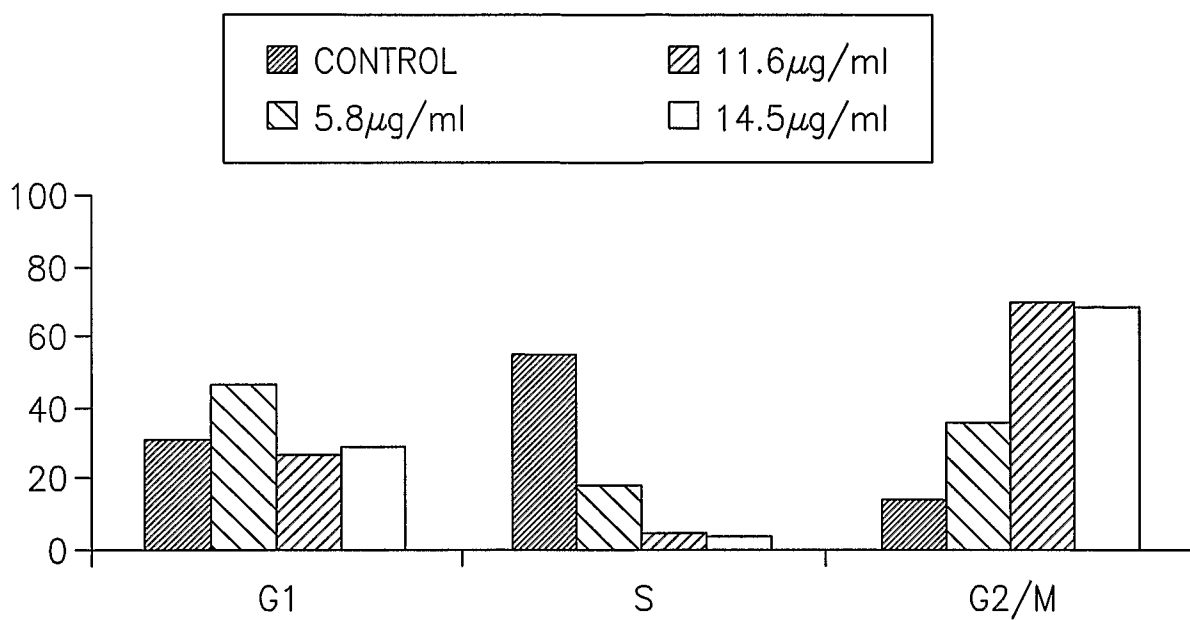


FIG.4A

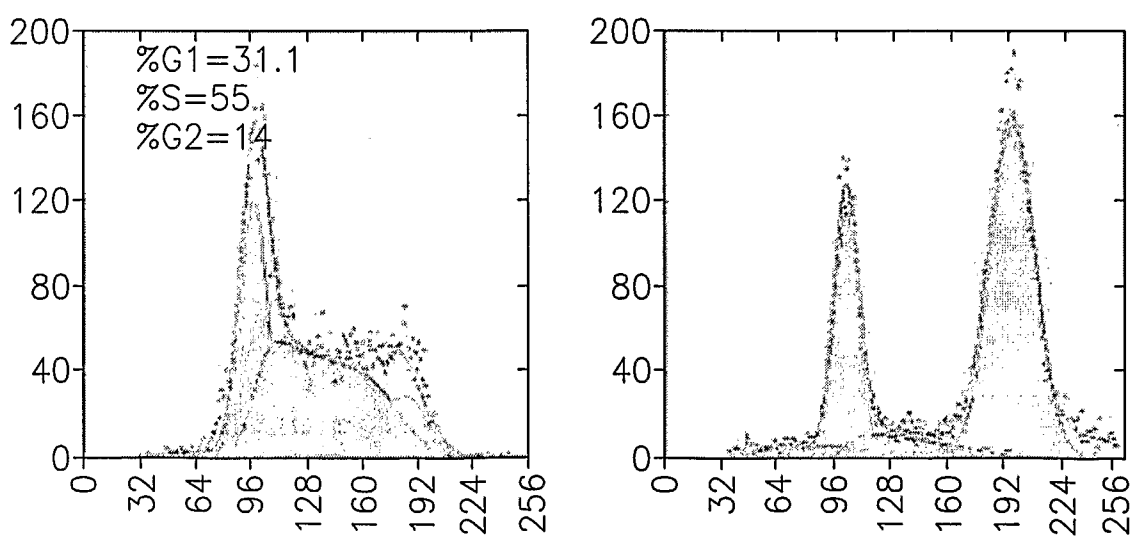


FIG.4B

4/8

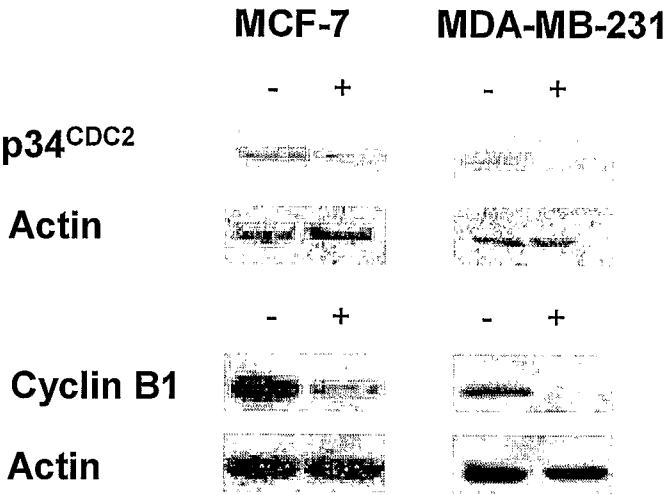


FIGURE 5A

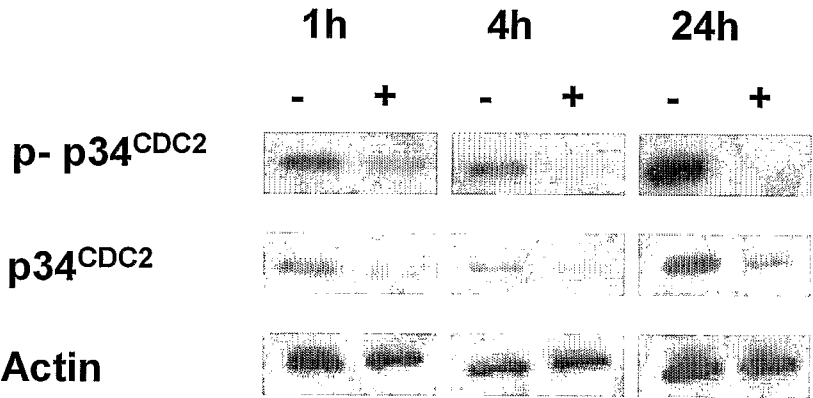


FIGURE 5B

5/8

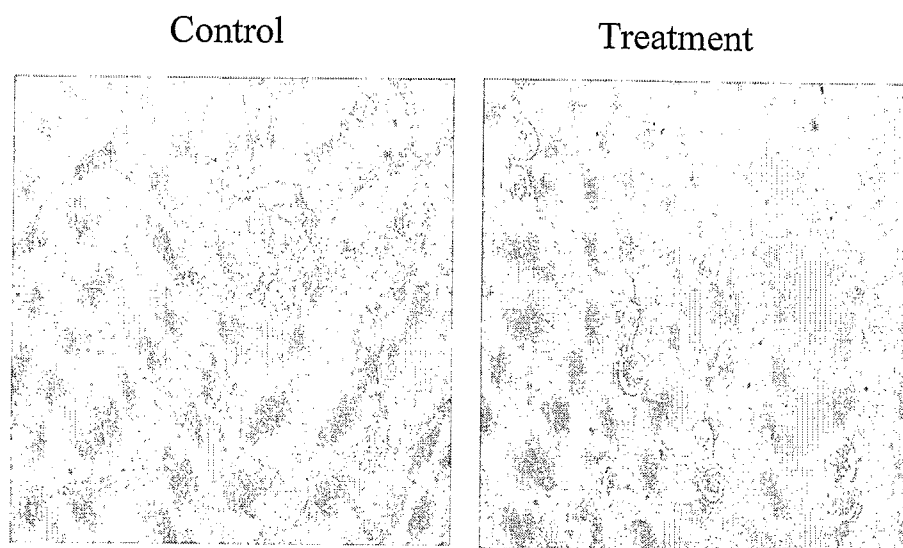


FIGURE 6A

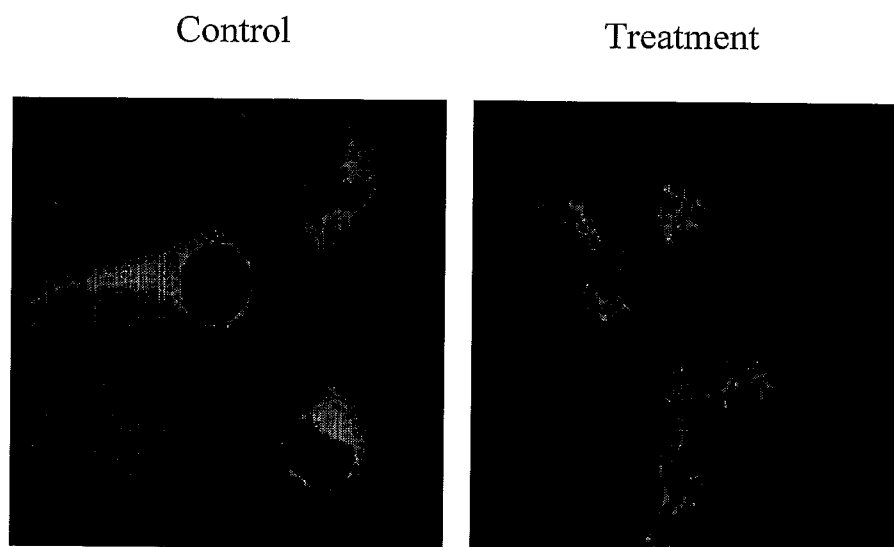


FIGURE 6B

6/8

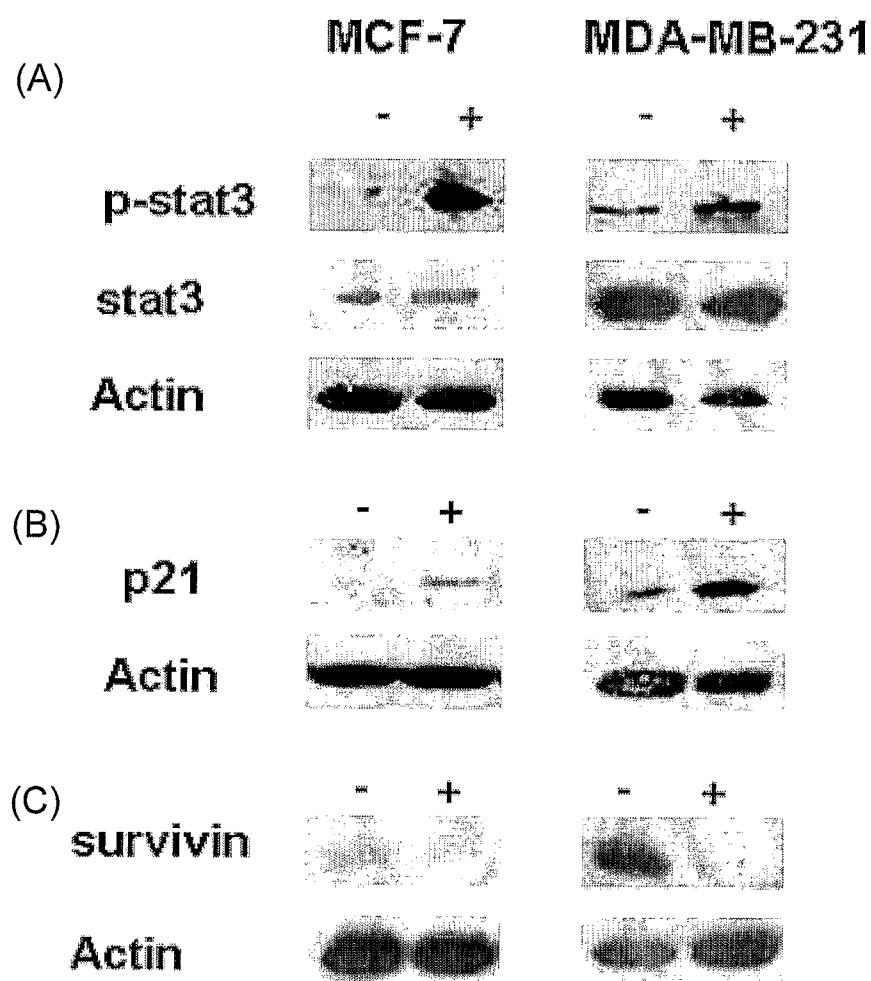


FIGURE 7

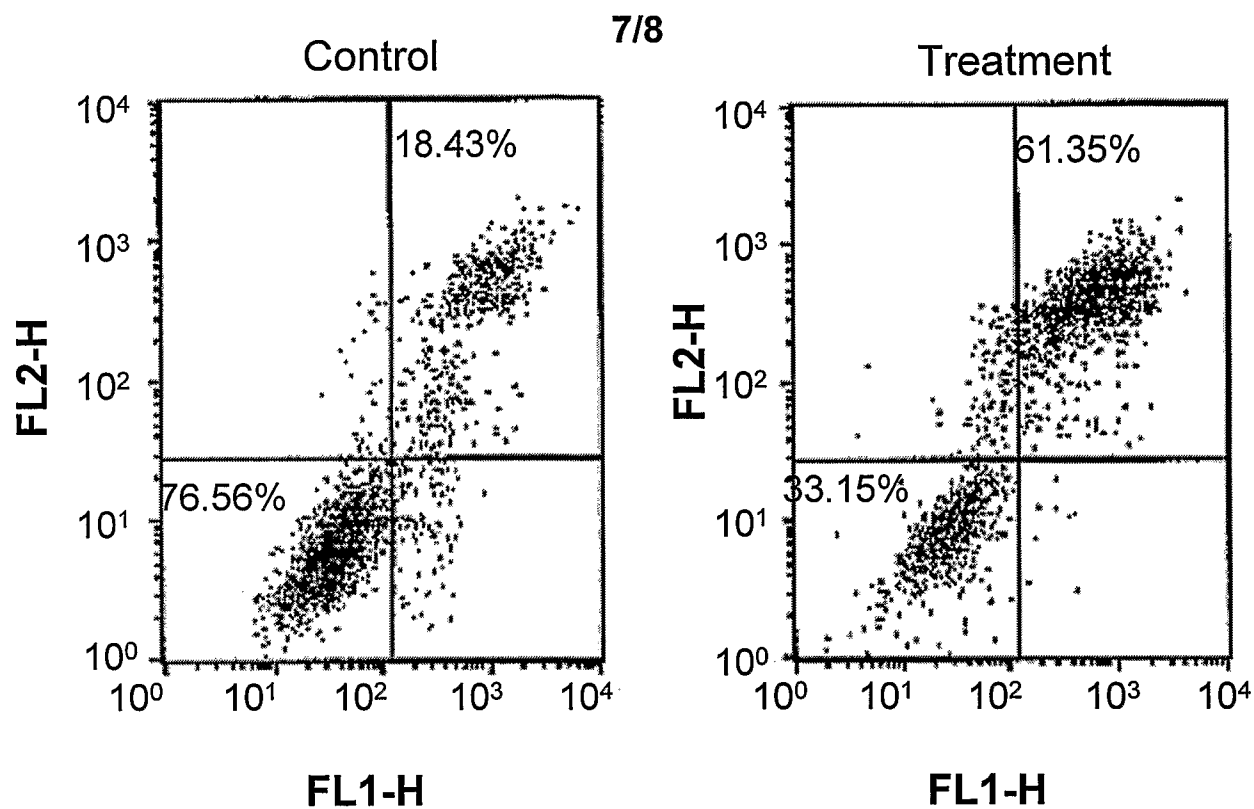


FIGURE 8A

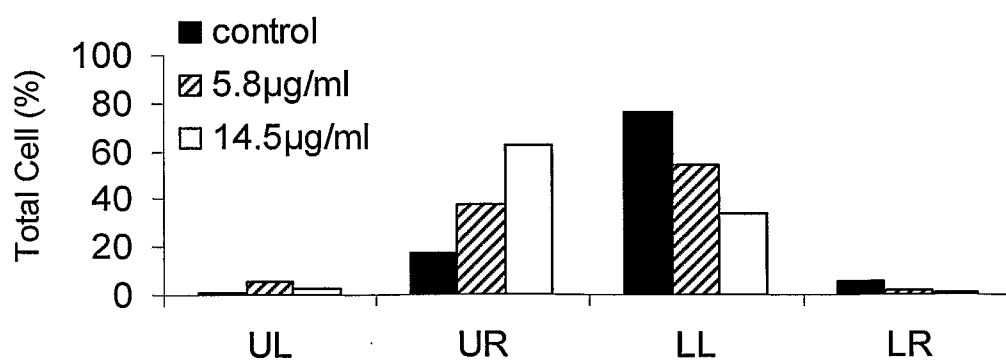


FIGURE 8B

8/8

Control

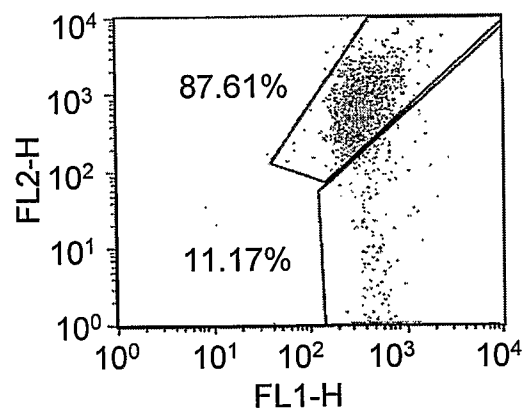
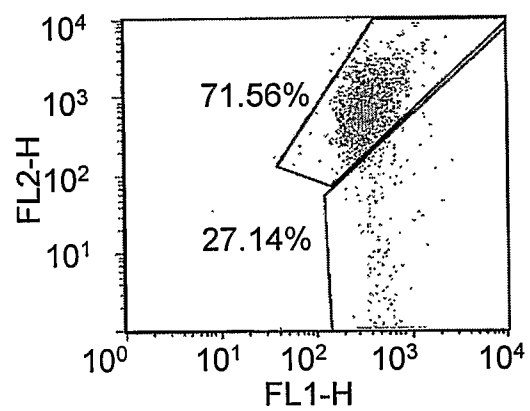
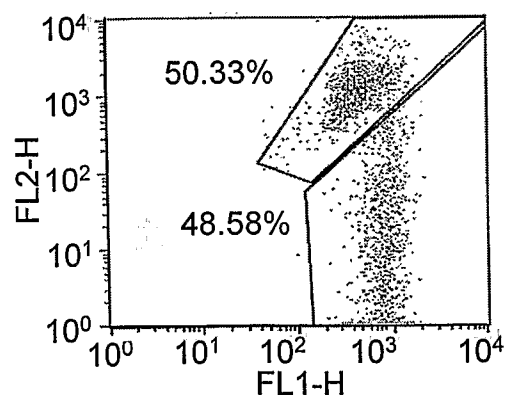
5.8 $\mu\text{g/ml}$ 14.5 $\mu\text{g/ml}$ 

FIGURE 9