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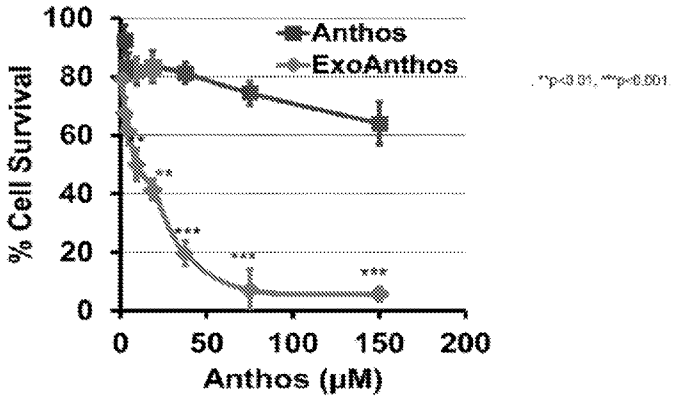

FIGURE 3

(57) Abstract: The present development is a method for delivering anthocyanidins or curcumin using exosomes (ExoAnthos or ExoCUR). The delivery of the anthocyanidins taught herein are directed to the management of ovarian cancer or cervical cancer. The ExoAnthos elicits a potent therapeutic activity against both drug-sensitive and drug-resistant human ovarian cancer cells, as well as synergistically enhance therapeutic activity when used in combination with cisplatin. Furthermore, combination of ExoAnthos and exosomal formulation of Paclitaxel (ExoPAC) treatment sensitized cisplatin resistant ovarian cancer cells by decreasing PgP expression levels. The ExoCUR elicits enhanced CUR levels in brain, liver and lung tissues, and exhibits enhanced biological efficacy measured as antiproliferative, anti-inflammatory, and a ntitumor activities without adverse side effects.

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Exosomal delivery of anthocyanidins and of curcumin

Cross-Reference to Prior Applications

[001] The present application claims priority to U.S. Patent Application **62/567,911** filed 2017-OCT-04, currently pending, and to U.S. Patent Application **62/568,026** filed 2017-OCT-04, currently pending, both of which are incorporated by reference in their entireties.

Field of the Invention

[002] The present invention generally relates to microvesicle compositions and methods for providing enhanced bioavailability and efficacy, particularly for treating cancers and more particularly for treating ovarian cancer and cervical cancer. In particular, certain embodiments of the presently disclosed subject matter relate to microvesicle compositions including microvesicles encapsulating anthocyanidins and microvesicle encapsulating curcumin and methods for using these compositions in the treatment of specific cancers.

Background of the Invention

[003] Ovarian cancer is the most lethal gynecological malignancy in women in the United States. Five to 10 percent of ovarian cancers diagnosed are hereditary. Front-line treatment for this lethal disease is cytoreductive surgery followed by intraperitoneal (i.p.) and/or intravenous (i.v.) platinum chemotherapy. Within 5 years after initial treatment, the disease recurs in 60–70% of patients and often exhibits cross-resistance to many structurally related or unrelated drugs. In addition, ~25% of ovarian cancers are ''innately'' resistant to platinum and respond poorly to initial chemotherapy. For the treatment of ovarian carcinomas, therefore, cisplatin resistance presents a very serious problem.

[004] Attempts have been made to overcome this chemoresistance, such as combining platinum-based chemotherapy with new molecularly-targeted drugs and other chemotherapeutic drugs. To date, paclitaxel (PAC) has been shown to be one of the most effective agents in such patients

with relapsed platinum-refractory disease. Many of these drug combinations have severe toxic side effects and fail to increase the survival rates of patients with drug resistant tumors. Therefore, there is an urgent need to develop effective treatment strategies for the management of platinum-resistant ovarian cancers. Therapeutic dosing with non-toxic plant bioactives in combination with chemotherapeutic drugs to treat the ovarian cancer and overcome drug resistance is a viable strategy.

[005] Despite optimal diagnosis and early therapeutic interventions, the prognosis for ovarian cancer patients remains dismal because the efficacy of chemotherapy is limited by the development of resistance and off-site toxicity. Berry bioactives indicate preventive and therapeutic activities against various cancer types. Epidemiologic studies have shown constant inverse associations between the intake of dark-colored fruits and vegetables and incidence of several chronic diseases. Dark-colored fruits and vegetables have generally been accepted as cancer preventive. Berries have shown potential to prevent chemically-induced colon and esophageal cancers in animal models. Further, the present inventors have shown chemopreventive and therapeutic activity of diet supplemented with freeze-dried berry powder against estrogen (E2)-mediated mammary cancer. More recently, the inventors demonstrated significant therapeutic activity of dietary berry against lung cancer xenograft.

[006] Translatability of dietary berries for therapeutic effects to large population, however, is not practical due to the need for rather large daily doses of berries. In order to achieve effective doses for clinical translation, researchers have used the putative berry bioactives, namely, anthocyanins/anthocyanidins (Anthos) instead of whole fruit. Berry bioactives possess potent anti-oxidant, anti-proliferative, apoptotic and anti-inflammatory properties. The berry phytochemicals have significant therapeutic activity against breast cancer *in vivo* and Anthos have significant therapeutic activity against lung cancer in both cell culture and animal studies. Anthocyanins can overcome trastuzumab-resistance to inhibit breast cancer growth.

[007] However, berry bioactives exhibit poor oral bioavailability and stability, thus limiting their effectiveness to full potential for clinical applications. Identifying effective oral delivery systems that

can achieve high oral bioavailability and are safe when used for prolonged periods are needed to overcome the limitations of Anthos for clinical translatability.

[008] Extensive preclinical studies over the past four decades suggest therapeutic activity by curcumin (CUR) against various diseases including cancer. CUR is a chemopreventive agent which exists as a native mixture of curcumin (75%), demethoxycurcumin (18-20%), and bisdemethoxycurcumin (5-7%). Studies have indicated CUR can attenuate hormonal breast cancer in rats when administered by subcutaneous polymeric implants. However, hydrophobic nature of CUR results in low water solubility and rapid intestinal/hepatic metabolism limits its oral bioavailability, impeding clinical development of CUR as potential therapeutic agent.

[009] Several approaches and drug delivery systems have been used to improve solubility and enhance oral bioavailability of CUR. Liposome, polymeric nanoparticles, micelles and other formulations have provided leads as drug delivery vehicles, however, they are associated with inherent limitations, such as, short circulation time and stability issues when used as unmodified liposomes *in vivo*. Micelles show instability in aqueous or biological environment due to small hydrophobic spaces and dissociates upon dilution. Furthermore, a number of manufactured nanoparticles have recently been shown to cause adverse effects.

[0010] Exosomes are extracellular microvesicles or endogenous nanoparticles secreted by a wide variety of cells having a particle size of 30 nm – 100 nm and carry a cargo of proteins, lipids, RNA, and DNA. Their properties of shuttling in-and-out of the cells suggest that these particles can be exploited as a nano drug carrier. Milk exosomes, in particular, are potential carriers for drug-delivery because of their nano size, a scalable source, biocompatibility, ability of exosomes to stabilize drugs, lack of toxicity, tumor targetability, among other factors. Due to their known stability in acidic environment, milk exosomes may provide a desirable oral drug delivery carrier, with wide preventive and therapeutic applications, including for carrying anthocyanidins and curcumin.

Summary of the Present Invention

[0011] The present development is a method for delivering anthocyanidins and for delivering curcumin using exosomes, particularly for the treatment of cancers and more particularly for the treatment of ovarian cancer and cervical cancer.

[0012] The antiproliferative activity of berry anthocyanidins (Anthos) against drug-sensitive (A2780) and drug-resistant (A2780/CP70, OVCA432 and OVCA433) ovarian cancer cells is reported herein. These drug-resistant ovarian cancer cell lines overexpress p-glycoproteins (PgP) and show >100-fold resistance to chemotherapeutic drug cisplatin compared A2780. The inventors observed dose-dependent growth inhibition of ovarian cancer cells with the Anthos. Further, treatment of drug-resistant ovarian cancer (OVCA432) cells with cisplatin in combination with the Anthos (75 μM) resulted in significantly higher cell kill. The cisplatin dose required to achieve this effect was 10-15 fold lower than the IC_{50} of cisplatin alone.

[0013] However, many plant bioactives including Anthos face the challenge of poor oral bioavailability and stability. Herein, the inventors further disclose a method to overcome these limitations by delivering Anthos via milk-derived exosomes. The exosomal Anthos (ExoAnthos) significantly enhanced antiproliferative activity against the growth of ovarian cancer cells and inhibited tumor growth compared to Anthos alone and vehicle control. Further, exosomal formulations of PAC (ExoPAC) for oral delivery demonstrates fewer side effects than systemic administration of PAC, but with similar therapeutic efficacy as free PAC delivered intraperitoneally.

[0014] Further, it has been observed that an exosomal-CUR (ExoCUR) formulation is stable upon long-term storage, and that oral administration achieves significantly higher CUR tissue levels as compared to free CUR. The inventors have found that ExoCUR exhibits enhanced biological efficacy measured as antiproliferative, anti-inflammatory, and antitumor activities, and that the exosomal formulation was well tolerated without any adverse effects.

Brief Description of the Figures

[0015] Figure 1 is a graph showing the antiproliferative activity of the chemotherapeutic drug, Cisplatin against drug-sensitive (A2780) and drug-resistant (A2780/CP70, OVCA432 and OVCA433) human ovarian cancer cells by MTT assay;

[0016] Figure 2 is a graph showing the antiproliferative activity of the native mixture of Anthos from bilberry (B) against drug-sensitive (A2780) and drug-resistant (A2780/CP70, OVCA432 and OVCA433) human ovarian cancer cells by MTT assay;

[0017] Figure 3 is a graph showing the antiproliferative activity of Anthos-loaded milk exosomes vs free Anthos against human drug-resistant ovarian cancer OVCA433 cells by MTT assay, wherein exosomal protein concentration was maintained constant (50 μg/ml), and each data point represents an average of 3 to 4 replicates ± SD, and wherein the Students' t-test was conducted to calculate the statistical significance;

[0018] Figure 4 is a graph showing the antiproliferative activity of Anthos (6 and 30 mg/kg b. wt.) and exosomal formulations of Anthos (6 mg/kg b. wt., 60 mg/kg Exo protein) given by oral gavage and compared with vehicle control against ovarian cancer A2780 tumor xenograft;

[0019] Figure 5 is a graph showing the cell viability of drug-resistant human ovarian cancer (OVCA433) cells treated with Cisplatin alone or in combination with 75 μM Anthos;

[0020] Figure 6 is a bar graph showing the effect of Anthos and PAC on the PgP expression wherein cells were treated with the indicated concentrations of PAC, Anthos and a combination thereof for 24 h before the cells were collected and proteins were separated on SDS-PAGE and probed for the p-glyoprotein;

[0021] Figure 7 is a graph showing the effect of the exosomal formulations on ovarian cancer (A2780) tumor xenograft for tumors that grew to about 80 mm^3 and then the animals were treated with vehicle, PAC alone, ExoPAC (4 mg/kg), ExoAnthos (6 mg/kg) and combinations of the ExoPAC and ExoAnthos by oral gavage on alternate days;

[0022] Figure 8 is a graph showing the particle-size determination of milk-derived exosomes and ExoCUR by Zetasizer and atomic force microscopy (AFM);

[0023] Figure 9 is a graph showing the cell viability of H1299 cells treated with CUR, freshly prepared ExoCUR, and ExoCUR that was stored at -80C for six months;

[0024] Figure 10 is a set of bar graphs showing the tissue distribution of CUR in rats treated with ExoCUR or free CUR, wherein the tissues examined were from the liver, lung and brain;

[0025] Figure 11 is a set of bar graphs showing the anti-proliferative activity of ExoCUR compared to curcumin and isolated milk exosomes with no drug loading for three different cell lines; and

[0026] Figure 12 is a graph demonstrating the antitumor activity of CUR and ExoCUR against human cervical cancer (CaSki) xenografts in nude mice.

Detailed Description of the Present Development

[0027] The following description is intended to provide the reader with a better understanding of the invention. The description is not intended to be limiting with respect to any element not otherwise limited within the claims.

[0028] **ExoAnthos**

[0029] The presently-disclosed subject matter is based, at least in part, on the discovery that an exosomal formulation of anthocyanidins (Anthos) elicits potent therapeutic activity against both drug-sensitive and drug-resistant human ovarian cancer cells, and also synergistically enhance therapeutic activity when used in combination with cisplatin. Furthermore, it was unexpectedly found that a combination of Exosomal anthocyanidins (ExoAnthos) and Exosomal Paclitaxel (ExoPAC) treatment sensitized cisplatin resistant ovarian cancer cells by decreasing PgP expression levels.

[0030] In an exemplary study, a native mixture of Anthos is isolated from 36% anthocyanin-enriched bilberry extract (Indena, Seattle, WA) using a recently developed solvent-solvent extraction procedure (Patent # 8,987,481 B1). The Anthos thus isolated has over 85% purity, and can be further purified by C18 Bond-Pak column to ~95% purity as determined by UPLC. The final Anthos mixture

contains delphinidin (Dp), cyanidin (Cy), malvidin (Mv), peonidin (Pe) and petunidin (Pt) in the proportion of 33:28:16:16:7. The molecular weight of the Anthos mixture represents the average molecular weight of these five individual anthocyanidins calculated by multiplying its content with respective molecular weights. Authentic Anthos standards were purchased from Chromadex (Irvine, CA). Primary P-glycoprotein antibody and secondary antibodies were purchased from cell signaling technology (Danvers, MA). All other chemicals received were of analytical grade.

[0031] Exosomes are isolated from the mature bovine milk by differential centrifugation. The isolated exosomes are characterized by analysis of size and surface protein markers (including CD63 and CD81). The size distribution of the isolated exosomes is measured by NanoSight and Zetasizer, and confirmed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). The proteins markers are characterized by western blot. The exosomes are suspended in PBS and stored at -80°C until use.

[0032] Drug loading is achieved by dissolving the drug to be loaded in ethanol or 1:1 mixture of ethanol and acetonitrile and then mixing with the milk exosomes. Free drug is removed by a low-speed centrifugation (8000 x g, 5 min) and the drug-loaded exosomes are collected by either Amicon ultra filtration using 500 kDa filter (for ExoAnthos), or by ultracentrifugation (135,000 x g, 90 min). Drug load is determined by analysis of the drug and protein in the formulation.

[0033] Human ovarian cancer cells (A2780, A2780/CP70, OVCA432, and OVCA433) were obtained and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 0.2 units/ml insulin (Sigma-Aldrich, St. Louis, MO). Cells were cultured in an atmosphere of 95% humidity and 5% CO_2 at 37°C. As is known in the art, these drug-resistant ovarian cancer cell lines overexpress p-glycoproteins (PgP) and show >100-fold resistance to chemotherapeutic drug cisplatin compared A2780.

[0034] Antiproliferative activity of the Anthos, PAC, cisplatin and combination of the drugs and their exosomal formulations is assessed against ovarian cancer cells by MTT assay. All the cell lines are plated (5000 cells per well) and antiproliferative activity is determined after 72 h.

[0035] As shown in Figures 1 and 2 and in Table 1, the antiproliferative activity of Anthos against drug sensitive and cisplatin-resistant ovarian cancer cells was studied and the results compared to the efficacy of cisplatin. As shown in Figure 1, the cisplatin-resistant (A2780/CP70, OVCA432 and OVCA433) cell overexpress PgP and show 100-fold resistance to cisplatin compared to the drug-sensitive (A2780) ovarian cancer cell lines. Anthos resulted in dose-dependent inhibition of ovarian cancer cells, as shown in Figure 2. The IC_{50} concentration, as calculated by CalcuSyn Software, for cisplatin compared to Anthos is shown for the four cell lines in Table 1. As indicated, Anthos demonstrated an IC_{50} concentration of nearly 25 μM against the sensitive A2780 cells; however, it was almost 10-fold higher for the drug resistant OVCA432 cells. This is the first demonstration showing the ability of Anthos to inhibit growth of drug-resistant ovarian cancer cells.

Table 1

Cell Line	IC_{50} Concentration (μM)	
	Cisplatin	**Anthos**
A2780	$<0.8^a$	22.7^a
A2780/CP70	6.5^b	56.5^b
OVCA432	$>100^c$	195^c
OVCA433	28.5^d	155^c

IC_{50} values followed by different letters are significantly different at $p \leq 0.05$

[0036] The relative antiproliferative effects of ExoAnthos and Anthos were determined against cisplatin-resistant human ovarian cancer OVCA433 cells by MTT assay. As shown in Figure 3, ExoAnthos showed a significant reduction in the drug concentration to achieve growth inhibition versus the free Anthos. A 20-fold difference in the IC_{50} value was observed between ExoAnthos and Anthos. With another cisplatin-resistant ovarian cell line (OVCA432), the Anthos exhibited an IC_{50} of 112 μM which was decreased to 7 μM with the ExoAnthos. It is known that the exosomes *per se* resulted in moderate (about 18%) growth inhibition of the OVCA433 cancer cells, but the combination with the Anthos is significantly better than either the exosomes or the Anthos alone. Without being bound by theory, it is postulated that the enhanced activity of the ExoAnthos could be due to the

stability of the Anthos after encapsulation into/onto exosomes and/or the greater inhibition could also be due to the higher uptake of the Anthos in the exosomal formulation.

[0037] To determine the therapeutic potential of Anthos against ovarian cancer, female athymic nude mice (5-6-week old) were obtained from Harlan Laboratories (Indianapolis, IN), acclimated for a week and then inoculated with human ovarian cancer A2780 cells (1.5×10^6 cells) in Matrigel. Once tumor grew to around 80 mm^3, animals were divided into several groups (n = 8 – 10 in each group) and were treated by oral gavage with vehicle, two doses of Anthos (6 and 30 mg/kg b. wt.) and exosomal formulation of Anthos containing 6 mg/kg Anthos and 60 mg/kg Exo three times a week. Animal weight, diet intake and tumor growth was monitored weekly. Due to the highly aggressive nature of the A2780 cells, all studies were terminated after three weeks by euthanizing animals by CO_2 asphyxiation.

[0038] As shown in Figure 4, the Anthos treatment demonstrated an inhibition of about 63% with compared with vehicle control (575 ± 217 vs. 1535 ± 340). The sub-optimal concentration of Anthos (6 mg/kg) was included in the evaluation to demonstrate the synergistic effect of the exosomes with the Anthos (comparison being the ExoAnthos composition). Without being bound by theory, it is postulated that the ExoAnthos demonstrate greater efficacy with respect to ovarian cancer because the Anthos is protected in the exosomal formulation and remain viable after tolerating GI conditions to elicit biological response. Higher efficacy of ExoAnthos *in vivo* suggest that Exo formulations possibly provide greater stability, higher uptake, and longer circulation time and slow release of the Anthos from the exosomes to boost the anticancer effects. It is believed that these studies are the first to show that the Anthos are capable of inhibiting ovarian cancer.

[0039] In a clinical scenario, drug-resistant ovarian tumors are often treated by combining platinum-based chemotherapy with other chemotherapeutic drugs to overcome chemoresistance. Therefore, we examined if the Anthos can chemosensitize the drug-resistant OVCA432 cells to cisplatin. Data in Figure 5 demonstrate significantly higher kill (10-15 fold lower IC_{50}) of cisplatin-resistant OVCA432 cells by cisplatin when combined with 75 µM Anthos. These results suggest that

the Anthos have potential to chemosensitize the drug-resistant ovarian cancer cells and reduce the effective cisplatin dose required to achieve therapeutic response.

[0040] Paclitaxel (PAC) is known in the art for treating drug-resistant recurrent ovarian cancers as they often retain sensitivity to PAC. To determine if PAC and Anthos affect PgP expression levels in order to overcome drug resistance in ovarian cancer cells, drug-resistant OVCA432 cells were treated with the Anthos and PAC, alone and in combination, and whole cell lysates were analyzed by Western blot. For the Western blot analysis, the ovarian cancer OVCA432 cells are treated with the Anthos and PAC, individually and in combination at different concentrations. After 24 h cells are collected and whole cell lysates are prepared in RIPA buffer. Protein concentration is determined using the BCA method. Proteins are separated on SDS-PAGE gel, and after transfer membranes are blocked with 4% non-fat dairy milk in TBS-T and incubated with the primary antibodies overnight at 4°C. After washing, the membrane is probed with secondary antibody and proteins bands are visualized with chemiluminescence detection kits (Amersham, ECL kits, Sunnyvale, CA). It was observed that the Anthos and PAC alone do not have significant effect on the expression of PgP. However, PgP expression was significantly reduced (~30%) in cells treated with the combination regimen, and this effect was dose-dependent, as shown in Figure 6. The inhibition of PgP represents a promising therapeutic strategy for ovarian cancer patients. These data provide preliminary evidence suggesting potential benefit of combining PAC and Anthos to treat drug-resistant ovarian tumors.

[0041] To determine if Anthos could enhance the sensitivity of human ovarian cancer xenografts to the chemotherapeutic drug, PAC *in vivo,* the same protocol was followed using the female athymic nude mice as for the therapeutic potential studies except that the mice were (1) treated with a combination of ExoAnthos and ExoPAC containing 6 mg/kg Anthos and 60 mg/kg Exo three times a week (on alternate days), or (2) treated with an exosomal formulation of PAC (4 mg PAC/kg b. wt. and 60 mg Exo/kg b. wt.) given weekly, or (3) treated with a combination of ExoAnthos (6 mg/kg Anthos and 60 mg/kg Exo) and ExoPac (4 mg PAC/kg b. wt. and 60 mg Exo/kg b. wt.) three times a week. As

shown in Figure 7, significant tumor growth inhibition is observed with the ExoAnthos (65%) and combination (78%) treatment, while ExoPAC shows only modest but insignificant tumor inhibition.

[0042] Thus, the inventors have found that berry Anthos are highly effective against ovarian cancer and that the milk exosomes serve as an excellent nano carrier to enhance the drug's oral bioavailability for management of ovarian cancer.

[0043] **ExoCUR**

[0044] CUR is one of the most investigated plant bioactives for benefits against various diseases; however medicinal benefits of this compound are limited because of its low oral bioavailability. In spite of the extensive research, poor aqueous solubility of CUR remains a major barrier in its bioavailability and clinical efficacy. Several attempts have been made to addressed pharmacokinetics, safety, and efficacy of CUR in clinical trials. Efforts taken to increase its solubility and bioavailability include encapsulation of CUR in liposomes, polymeric nanoparticles, biodegradable microspheres, phospholipid mixtures and hydrogels. However, none of these delivery systems has advanced to translational setting due to inherent toxicity, high costs and/or non-scalability. The present invention demonstrates that milk-derived exosomes can overcome the bioavailability issues of CUR.

[0045] Exosomal formulation of CUR qualifies as ideal nanoformulation due to physical attributes such as average size of about 100 nm with a PDI of 0.19 ± 0.02. Sufficiently high drug load (~20-25%) can be achieved by simple mixing of CUR in presence of solvents concentration 10% which preserves the quality attributes of the exosomes. Without being bound by theory, it is postulated that loading of CUR on to exosomes presumably occurs due to the interactions between highly lipophilic CUR and hydrophobic domains on the surface exosomal proteins, however, we cannot rule out if part of the loading is "into" the exosome lumen and lipid membrane. ExoCUR formulations stored at -80°C are stable for at least 6 months with unaltered drug load and particle size. Moreover, the storage of ExoCUR has no effects on its antiproliferative potency.

[0046] It has been found that CUR can be delivered effectively using milk-derived exosomes. CUR when mixed with exosomes in the presence of 10% ethanol: acetonitrile (1:1), provided a drug load of

18- 24%, and the formulation was stable for 6 months as determined by particle size analysis, drug load and anti-proliferative activity. The uptake of exosomes by cancer cells involved caveolae/clathrin-mediated endocytosis. Oral administration of exosomal CUR (ExoCUR) in Sprague-Dawley rats demonstrated 3-5 times higher levels in various organs versus free agent. ExoCUR showed enhanced antiproliferative activity against multiple cancers including, breast, lung and cervical. ExoCUR showed significantly higher anti-inflammatory activity measured as NF-κB activation in lung and breast cancer cells. To determine *in vivo* antitumor activity, nude mice bearing the cervical CaSki tumor xenograft were treated with ExoCUR by oral gavage, CUR diet, exosomes alone and PBS as controls. While CUR via dietary route failed to elicit any effect, exosomes had a modest (25-30%) tumor growth inhibition. However, ExoCUR showed significant inhibition (61%; $p < 0.01$) of the tumor xenograft. No gross or systemic toxicity was observed in the rats administered with the exosomes or ExoCUR. These results suggest that exosomes can be developed as potential nano-carriers for delivering CUR which otherwise has encountered significant tissue bioavailability issues in the past.

[0047] For the preparation of the ExoCUR, exosomes were isolated from raw bovine milk using differential centrifugation performed at 13,000 × g, 100,000 × g and 135,000 × g for 30, 60 and 90 min, respectively, to isolate the exosomes. The exosome pellet was suspended in PBS and stored at -80 °C. The size distribution was measured by Zetasizer and atomic force microscopy (AFM). The buoyant density of exosomes and the ExoCUR was determined using 10–60% (w/v) sucrose density-gradient medium and centrifuged at 135,000 × g in a swing bucket rotor (SW 41Ti, Beckman Coulter Inc.) at 4 °C for 8 h. CUR was loaded to the exosomes by mixing a solution of CUR in ethanol: acetonitrile (1:1) with the exosomes at room temperature (22 °C). The concentration of organic solvent was kept less than 10%. A low speed centrifugation at 10,000 × g for 10 min was done to remove the unbound CUR, and the CUR-loaded exosomes were collected by ultracentrifugation. The pellet was suspended in PBS and stored at -80 °C until use. For drug load analysis, ExoCUR (50 µl) was mixed with 950 µl of acetonitrile and centrifuged at 10,000 x g for 10 min to separate precipitated proteins. CUR was analyzed in the supernatant by UPLC as described below. Protein pellet was dissolved in water and

exosomal proteins were analyzed by BCA method. Exo and ExoCUR formulations were prepared and stored at -80°C prior to testing in cell culture and for animal experiments. To ensure that formulations were stable, storage stability at -80°C was tested. ExoCUR was stored in PBS at -80 °C and analyzed for CUR load after 3 and 6 months using UPLC. Stability of the exosomal formulation was determined by analyzing the drug load, size of the exosomes and antiproliferative activity at different time intervals.

[0048] The efficacy of the ExoCUR was assessed using human lung cancer H1299 and A549 cells, cervical cancer (HeLa) and breast cancer (MDA-MB-231 and T47D) cells. The cells were maintained in DMEM glutamax medium (A549 and H1299 cells), RPMI (HeLa, CaSki and T47D cells) and L15 (MDA-MB-231 cells) containing 10% fetal bovine serum at 37 °C in 5% CO2 and humidified air. To determine the cellular uptake, lung cancer H1299 cells were plated in 8-well chamber slide and treated with different endocytosis inhibitors for 2 h prior to incubation with PKH-67-labelled exosomes (50 μg exosomal proteins/mL) for 4 h. Green fluorescent cell linker (PKH-67) was used to label the milk exosomes as per manufacturer's instructions. The inhibitors of caveolae (colchicine and genistein), clathrin (sucrose and phenothiazine), microtubule (actin) (cytochalasin D), golgi bodies (berfeldin A), and microtubule/macropinocytosis (nocodazole) were used (20). Actin and nuclei were stained with Alexa Fluor 555 Phalloidin and DAPI, respectively. To determine the metabolic inhibition, cells were treated with the metabolic inhibitor, sodium azide or incubated at 4 °C in the presence of PKH-67-labelled exosomes. After incubation with PKH-67-labelled exosomes for 8 h, H1299 cells were fixed and incubated with endocytosis markers caveolin-1, clathrin, early endosome markers EEA1, late endosome Rab 7 and recycling endosome Rab11 (Cell Signaling, Danvers, MA) primary antibody for 1h followed by anti-rabbit alexa fluor 546 (Thermo Fisher Scientific, Waltham, MA). CellLight® Lysosomes-RFP reagent was used to label lysosomes (red). Nuclei were visualized by staining with DAPI. Images were taken at 20x - 40x magnification on a confocal microscope. For tissue drug analysis and toxicity testing, female (5-6 week-old) Sprague-Dawley rats (n=4) were procured from Envigo (Indianapolis, IN) and acclimatized for one week. Following acclimation, rats were treated daily with either free CUR (2.5 mg/kg b. wt.) or ExoCUR (1.25 and 2.5 mg/kg b. wt.) by oral gavage for 14 days. Animals were

euthanized by CO2 asphyxiation, blood and different tissues were collected and stored at -80 °C until analysis. Liver, lung and brain tissues (~500 mg) were homogenized and extracted with 2 vol of ethyl acetate in the presence of 0.5 M sodium acetate. The extracted CUR was analyzed by UPLC. Antiproliferative activity was determined against lung, cervical and breast cancer cells. Cells were plated at an initial density of 3 x 10^3 (lung cancer) and 5 x 10^3 (cervical and breast cancer) cells per well and treated with CUR or ExoCUR (0.25 – 50 μM) and incubated for 72 h. Exosome concentration was maintained constant at 50 μg/mL. Inhibition of cell proliferation was analyzed by MTT assay as described. DNA binding of NF-κB was measured in the vehicle, CUR and ExoCUR treated lung and breast cancer cell by EMSA. In vivo tumor xenograft studies were conducting using athymic nude mice (Female; 5–6-week old) were purchased from Envigo (Indianapolis, IN). Animals were maintained under a 12-h light/12-h dark cycle in accordance with the Institutional Animal Care and Use Committee guidelines. Antitumor activity was determined against cervical cancer Caski cells. A suspension of 5 × 10^6 Caski cells mixed with Matrigel (100 μL; 1:1) was injected subcutaneously. When the tumor xenografts grew to 50 - 60 mm^3, the mice were randomized and treated on alternate days by oral gavage with either vehicle (PBS), Exo (80 mg/kg b. wt.) or ExoCUR (CUR - 20 mg/kg b. wt. and Exo - 80 mg/kg b. wt.). An additional group of animals was provided diet supplemented with CUR (400 ppm) that is equivalent to 80 mg/kg b. wt. based on average 4 g diet consumption. Tumor dimensions (length, width and height) of the tumors were measured with a digital calipers. The animals were euthanized after 7-8 weeks of the treatment by CO_2 asphyxiation.

[0049] Exosomes were isolated by differential centrifugation from bovine milk and characterized for structure morphology, buoyant density, particle size, and presence of exosomal markers. The average size of isolated exosomes as determined by zetasizer was 84 ± 7 nm and had polydispersity index (PDI) of 0.19 ± 0.02 as shown in Figure 8. The size and morphology of exosomes was confirmed by AFM and it was found that the size was somewhat smaller (60- 80 nm) than reported from the Zetasizer, which could have been due to shrinkage during drying. CUR was loaded onto the exosomes in the presence of ethanol and acetonitrile (1:1; 10%) and resulted in a drug load of 18-24%. Unbound

CUR was removed by slow-speed centrifugation and ExoCUR was harvested by ultracentrifugation. Size analysis of ExoCUR revealed that there was slight but insignificant increase in the size (93 ± 6 nm) after CUR loading with a PDI of 0.21 ± 0.04, as shown in Figure 8. The stability of CUR in exosomal formulation upon long-term storage was determined by solvent extraction and UPLC analysis. As shown in Figure 9, there was essentially no change in the CUR levels after 6 months of storage at -80°C. Exosomal formulation of CUR showed almost unaltered particle size (104 ± 11 nm) after 6 months of storage. The antiproliferative efficacy of the ExoCUR against A549 lung cancer cells after storing for six months remained similar to the efficacy determined with the fresh formulation.

[0050] In order to explore the cellular uptake mechanism of milk exosomes, the H1299 cells were pretreated with various endocytosis inhibitors or incubated at 4 °C prior treatment with PKH67-labeled exosomes. Incubation of cells at 4 °C prevented uptake of exosomes compared to incubation under physiological condition (37 °C) and pretreatment of cells with 0.1% sodium azide caused nearly 50% decrease in uptake, suggesting that cellular uptake of exosomes to be an energy-dependent process. The caveolae inhibitor, colchicine and clathrin inhibitor, phenothiazine resulted in 53% and 48% decrease in uptake of exosomes, respectively; microtubule inhibitor, nocodazole and actin inhibitor, cytochalasin D caused 32% and 21 % inhibition of exosome internalization by cells, respectively. Involvement of calveolin-1 and clathrin proteins was confirmed by observation of colocalization with PHK-67-labeled exosomes in lung cancer cells.

[0051] To understand the subcellular trafficking of exosomes, we examined the colocalization of PKH67-labeled exosomes with early endosome EEA1, late endosome Rab7 and recycling-endosomal (Rab11) markers. After 1 h to 8 h incubation, PKH67-labeled exosomes were found to co-localize mainly with EEA1. However, after longer incubation of 24 h, exosomes showed decreased co-localization with early endosomal markers and increase association with late endosomal marker Rab7 and Rab11 indicating that the exosomes move from early endosome into late endosome with time. Further, labeling of cell lysosomes with red fluorescent protein (RFP) indicated co-localization of exosomes with lysosomes.

[0052] The tissue distribution of CUR in the lung, liver and brain was also examined. Female Sprague-Dawley rats (n = 5) were treated daily with either free CUR (2.5 mg/kg b. wt.) or ExoCUR (1.25 and 2.5 mg/kg b. wt.) by oral gavage for 14 days and indicated tissues were analyzed by ultra-performance liquid chromatography (UPLC). Data represent average ± SD of four animals. Student's t-test was done for statistical analysis and ExoCUR was compared with CUR alone. As shown in Figure 10, in all tissues examined, significantly higher ($p<0.05$) levels of CUR were found with ExoCUR versus free CUR at equivalent doses (2.5 mg/kg b. wt.). Liver and brain tissues showed higher levels of CUR even at half the dose of ExoCUR as compared to free CUR (Figure 10). The maximum enhancement (6-fold) was found in the brain, suggesting that exosomes could facilitate higher bio-accumulation of CUR in the brain. The three curcuminoids in CUR were detected in similar ratios in the tissues, irrespective of whether the animals were treated with free CUR or ExoCUR.

[0053] CUR is a well-established anti-inflammatory agent. ExoCUR inhibited both constitutive and TNFα-induced NF-κB levels in lung and breast cancer cells, while free CUR was essentially ineffective. Similar effects were observed against LPS-induced NF-κB activation. A modest anti-inflammatory effect of the exosomes alone was also evident against the constitutive and TNFα-induced NF-κB levels. These protective effects are presumably exerted by the endogenous cargo of immune factors, miRNAs and proteins in the exosomes.

[0054] Anti-proliferative activity of ExoCUR was determined against several human cancer cell line. As shown in Figure 11, compared with free CUR, an increased anti-proliferative efficacy of ExoCUR with all cancer types examined was observed. The growth inhibition increased from 23% to 66% against H1299 lung cancer cells and from 34% to 61% against A549 lung cancer cells with free CUR and ExoCUR at 1.56 μM concentration each. ExoCUR showed even greater inhibition of cell growth against cervical and breast cancer cells - 70-80% compared to 10-35% with CUR. Milk exosomes *per se* exhibited significant growth inhibitory effects ranging from 15-45%, different cancer cell types.

[0055] As shown in Figure 12, the efficacy of ExoCUR *in vivo* was tested using human cervical cancer (CaSki) xenografts. Animals were treated with vehicle, exosomes or ExoCUR at (20 mg/kg b.

wt.) by oral gavage three times a week. An additional group received free CUR in diet at 400 ppm (equivalent to 80 mg/kg b. wt.). The CaSki cells were injected subcutaneously in athymic nude mice (n = 8-10). When tumors grew to around 50 mm^3, animals were treated with vehicle (PBS), Exo alone (80 mg/kg b. wt.) or ExoCUR (CUR - 20 mg/kg b. wt. and Exo - 80 mg/kg b. wt.) on alternate days by oral gavage. An additional group was provided diet supplemented with CUR at 400 ppm. Data represent average of 8-10 animals ± SE. Statistical analysis was done using student's t-test. Dietary CUR showed no effect on the growth of tumor xenograft. However, a significantly higher inhibition of tumor growth was observed with ExoCUR (61%; $p < 0.01$) compared to Exo alone (21%).

[0056] To test systemic toxicity, wild-type SD rats were treated with vehicle, exosomes alone, CUR and ExoCUR. Specifically, wild-type rats were treated with PBS, CUR (2.5 mg/kg b. wt.) or two doses ExoCUR (1.25 and 2.5 mg CUR/kg b. wt., each containing 25 mg Exo proteins/kg b. wt.) daily by oral gavage. After 14 days, animals were euthanized by CO_2 asphyxiation. Blood was collected at the time of euthanasia, and hematological and various biochemical parameters were analyzed. Data represent average ± SD of four animals. No differences were observed when statistical analysis was done using student's t-test. Neither CUR nor ExoCUR showed any signs of any gross toxicity based on the body weight gain and diet intake. Based on blood analysis, CUR and ExoCUR did not alter hematological and liver/kidney function parameters. There was a slight but insignificant increase in cholesterol levels with ExoCUR.

[0057] Exosomal formulation of CUR was prepared with the goal of improving its tissue bioavailability. This nanoformulation overcomes limitations of low oral bioavailability of CUR, and reduces its effective dose. Exosomal formulation resulted in increased tissue accumulation of CUR presumably due to higher uptake, prolonged circulation, and protection from rapid hepatic degradation. ExoCUR demonstrated enhanced anti-proliferative, anti-inflammatory and anti-tumor activities compared to free CUR. Furthermore, the milk-derived exosomes and ExoCUR showed lack of any cross-species reaction. Thus, exosomes may offer an effective nanodelivery method for CUR and other plant bioactives many of which otherwise face oral bioavailability issues.

[0058] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the presently disclosed subject matter pertains. Representative methods, devices, and materials are described herein, but are not intended to be limiting unless so noted.

[0059] Although any methods, devices, and materials similar or equivalent to those described herein can be used in the practice or testing of the presently-disclosed subject matter, representative methods, devices, and materials are described herein. For example, the size distribution of the isolated exosomes is measured by NanoSight and Zetasizer and the size is confirmed by scanning electron microscopy (SEM) and atomic force microscopy (AFM). These are methods currently recognized by those skilled in the art as acceptable methods for the intended application. It is anticipated that other methods to accomplish the same goal may currently be available or may be developed in the future. There is no intention to limit the current invention based on the specific methods cited herein.

[0060] All results presented herein are presented as the average and standard error or standard deviation of three experiments done in triplicate. Statistical analysis was performed using the GraphPad Prizm statistical software. Student's t-test was performed for the statistical analysis and a p-value of <0.05 was considered significant. Data were further analyzed for statistical significance using analysis of variance (one way ANOVA).

[0061] As used herein, the abbreviations for any protective groups, amino acids and other compounds, are, unless indicated otherwise, in accord with their common usage, recognized abbreviations, or the IUPAC-IUB Commission on Biochemical Nomenclature.

[0062] As used herein, "microvesicles" are defined as particles that are in the form of small assemblies of lipid particles, are about 30 to 1000 nm in size, and are not only secreted by many types of *in vitro* cell cultures and *in vivo* cells, but are commonly found *in vivo* in body fluids, such as blood, urine and malignant ascites. Indeed, microvesicles include, but are not limited to, particles such as exosomes, epididimosomes, argosomes, exosome-like vesicles, microparticles, promininosomes, prostasomes, dexosomes, texosomes, dex, tex, archeosomes, and oncosomes. Microvesicles can be

formed by a variety of processes, including the release of apoptotic bodies, the budding of microvesicles directly from the cytoplasmic membrane of a cell, and exocytosis from multivesicular bodies. For example, exosomes are commonly formed by their secretion from the endosomal membrane compartments of cells as a consequence of the fusion of multivesicular bodies with the plasma membrane. The multivesicular bodies (MVBs) are formed by inward budding from the endosomal membrane and subsequent pinching off of small vesicles into the luminal space. The internal vesicles present in the MVBs are then released into the extracellular fluid as so-called exosomes. As is known in the art, as part of the formation and release of microvesicles, unwanted molecules are eliminated from cells. However, cytosolic and plasma membrane proteins are also incorporated during these processes into the microvesicles, resulting in microvesicles having particle size properties, lipid bilayer functional properties, and other unique functional properties that allow the microvesicles to potentially function as effective nanoparticle carriers of therapeutic agents. In this regard, the term "microvesicle" is used interchangeably herein with the terms "nanoparticle," "liposome," "exosome," "exosome-like particle," "nano-vector" and grammatical variations of each of the foregoing.

[0063] The term "milk" is used herein to describe the opaque liquid that contains proteins, fats, lactose, and various vitamins and minerals and that is produced by the mammary glands of mature female mammals including, but not limited to, after the mammals have given birth to provide nourishment for their young. In this regard, in some embodiments, the term "milk" is further inclusive of colostrum. The term "colostrum" is used herein to describe the liquid that is secreted by the mammary glands of mammals at the time of parturition and that is rich in antibodies and minerals. In some embodiments, the compositions of the presently-disclosed subject matter are comprised of colostrum-derived microvesicles.

[0064] The phrase "milk-derived" or "colostrum-derived," when used in the context of a microvesicle derived from milk or colostrum, refers to a microvesicle that, by the hand of man, exists apart from its native environment and is therefore not a product of nature. In this regard, the phrases

"milk-derived" microvesicles or "colostrum-derived" microvesicles is used interchangeably herein with the phrases "milk microvesicles" or "colostrum microvesicles," respectively to refer to microvesicles that have been isolated from milk or colostrum. Additionally, in some embodiments, the phrase "milk-derived" can be used interchangeably with the phrase "isolated from milk" to describe a microvesicle of the presently-disclosed subject matter that is useful for encapsulating therapeutic agents.

[0065] In some embodiments, the isolation of microvesicles is achieved by centrifuging raw (i.e., unpasteurized milk or colostrum) at high speeds to isolate the microvesicles. In one preferred embodiment, the microvesicles of the presently-disclosed subject matter are isolated in a manner that allows for the isolation of clinical-grade microvesicles. In this regard, in some embodiments, a method of isolating a microvesicles is further provided that includes the steps of: obtaining an amount of milk (e.g., raw milk or colostrum). In some embodiments, the series of sequential centrifugations comprises a first centrifugation at 20,000 x g at 4°C for 30 min, a second centrifugation at 100,000 x g at 4°C for 60 min, and a third centrifugation at 120,000 x g at 4°C for 90 min. In some embodiments, the isolated microvesicles can then be stored at a concentration of about 5 mg/ml to about 10 mg/ml as such a concentration has been found to prevent coagulation and allow the isolated microvesicles to effectively be used for the encapsulation of one or more therapeutic agents. In some embodiments, the isolated microvesicles are passed through a 0.22 μm filter to remove any coagulated particles as well as microorganisms, such as bacteria.

[0066] The phrase "encapsulated by a microvesicle," or grammatical variations thereof is used herein to refer to microvesicles whose lipid bilayer surrounds a therapeutic agent. For example, a reference to "microvesicle curcumin" refers to a microvesicle whose lipid bilayer encapsulates or surrounds an effective amount of curcumin. In some embodiments, the encapsulation of various therapeutic agents within microvesicles can be achieved by mixing the one or more of the phytochemical agents or chemotherapeutic agents with isolated microvesicles in a suitable solvent, such as ethanol. After a period of incubation sufficient to allow the therapeutic agent to become

encapsulated during the incubation period, the microvesicle/therapeutic agent mixture is then subjected to a low-speed centrifugation (e.g., 10,000 x g) to remove any unbound therapeutic agent and one or more high-speed centrifugation centrifugations to isolate the microvesicles encapsulating the therapeutic agents.

[0067] As used herein, the term "therapeutic agent" is used to refer to an agent that is capable of "treating" a disease, as defined herein below. As noted above, in some embodiments, the therapeutic agent can comprise an anthocyanidin. In some embodiments, the phytochemical agent is a bilberry anthocyanidin mixture, which includes, in certain embodiments, a mixture of five anthocyanidins isolated from anthocyanin-enriched bilberry extract following acid hydrolysis and purification by solvent-solvent extraction. In some embodiments, the mixture of five anthocyanidins is a mixture of delphinidin, cyanidin, malvidin, peonidin, and petunidin.

[0068] As also noted herein above, in some embodiments of the presently-disclosed subject matter, the therapeutic agent that is encapsulated within the exosome is a chemotherapeutic agent. Examples of chemotherapeutic agents that can be used in accordance with the presently disclosed subject matter include, but are not limited to, platinum coordination compounds such as cisplatin, carboplatin or oxalyplatin; taxane compounds, such as paclitaxel or docetaxel; topoisomerase I inhibitors such as camptothecin compounds for example irinotecan or topotecan; topoisomerase II inhibitors such as anti-tumor podophyllotoxin derivatives for example etoposide or teniposide; anti-tumor vinca alkaloids for example vinblastine, vincristine or vinorelbine; anti-tumor nucleoside derivatives for example 5-fluorouracil, gemcitabine or capecitabine; alkylating agents, such as nitrogen mustard or nitrosourea for example cyclophosphamide, chlorambucil, carmustine or lomustine; anti-tumor anthracycline derivatives for example daunorubicin, doxorubicin, idarubicin or mitoxantrone; HER2 antibodies for example trastuzumab; estrogen receptor antagonists or selective estrogen receptor modulators for example tamoxifen, toremifene, droloxifene, faslodex or raloxifene; aromatase inhibitors, such as exemestane, anastrozole, letrazole and vorozole; differentiating agents such as retinoids, vitamin D and retinoic acid metabolism blocking agents (RAMBA) for example

accutane; DNA methyl transferase inhibitors for example azacytidine; kinase inhibitors for example flavoperidol, imatinib mesylate or gefitinib; farnesyltransferase inhibitors; HDAC inhibitors; other inhibitors of the ubiquitin-proteasome pathway for example VELCADE® (Millennium Pharmaceuticals, Cambridge, MA); or YONDELIS® (Johnson & Johnson, New Brunswick, NJ). In some embodiments, the chemotherapeutic agent that is encapsulated by an exosome in accordance with the presently-disclosed subject matter is paclitaxel, which can be encapsulated in an exosome separate from the exosome encapsulating the anthocyanidin.

[0069] In some embodiments of the presently-disclosed subject matter, pharmaceutical compositions including the milk-derived exosomes are further provided. In some embodiments, a pharmaceutical composition is provided that comprises a milk-derived microvesicle composition disclosed herein and a pharmaceutical vehicle, carrier, or excipient. In some embodiments, the pharmaceutical composition is pharmaceutically-acceptable in humans. Also, as described further below, in some embodiments, the pharmaceutical composition can be formulated as a therapeutic composition for delivery to a subject.

[0070] A pharmaceutical composition as described herein preferably comprises a composition that includes pharmaceutical carrier such as aqueous and non-aqueous sterile injection solutions that can contain antioxidants, buffers, bacteriostats, bactericidal antibiotics and solutes that render the formulation isotonic with the bodily fluids of the intended recipient; and aqueous and non-aqueous sterile suspensions, which can include suspending agents and thickening agents. The pharmaceutical compositions used can take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and can contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Additionally, the formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a frozen or freeze-dried or room temperature (lyophilized) condition requiring only the addition of sterile liquid carrier immediately prior to use.

[0071] In some embodiments, solid formulations of the compositions for oral administration can contain suitable carriers or excipients, such as corn starch, gelatin, lactose, acacia, sucrose,

microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, calcium carbonate, sodium chloride, or alginic acid. Disintegrators that can be used include, but are not limited to, microcrystalline cellulose, corn starch, sodium starch glycolate, and alginic acid. Tablet binders that can be used include acacia, methylcellulose, sodium carboxymethylcellulose, polyvinylpyrrolidone, hydroxypropyl methylcellulose, sucrose, starch, and ethylcellulose. Lubricants that can be used include magnesium stearates, stearic acid, silicone fluid, talc, waxes, oils, and colloidal silica. Further, the solid formulations can be uncoated or they can be coated by known techniques to delay disintegration and absorption in the gastrointestinal tract and thereby provide a sustained/extended action over a longer period of time. For example, glyceryl monostearate or glyceryl distearate can be employed to provide a sustained-/extended-release formulation. Numerous techniques for formulating sustained release preparations are known to those of ordinary skill in the art and can be used in accordance with the present invention.

[0072] Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional techniques with pharmaceutically-acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations can also contain buffer salts, flavoring, coloring and sweetening agents as appropriate. Preparations for oral administration can be suitably formulated to give controlled release of the active compound. For buccal administration, the compositions can take the form of capsules, tablets or lozenges formulated in conventional manner.

[0073] Various liquid and powder formulations can also be prepared by conventional methods for inhalation into the lungs of the subject to be treated or for intranasal administration into the nose and sinus cavities of a subject to be treated. For example, the compositions can be conveniently

delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the desired compound and a suitable powder base such as lactose or starch.

[0074] The compositions can also be formulated as a preparation for implantation or injection. Thus, for example, the compositions can be formulated with suitable polymeric or hydrophobic materials (e.g., as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives (e.g., as a sparingly soluble salt).

[0075] The compositions can further be formulated as topical semi-sold ointment or cream formulations can contain a concentration of the presently-described microvesicle compositions in a carrier such as a pharmaceutical cream base. Various formulations for topical use include drops, tinctures, lotions, creams, solutions, and ointments containing the active ingredient and various supports and vehicles. The optimal percentage of the therapeutic agent in each pharmaceutical formulation varies according to the formulation itself and the therapeutic effect desired in the specific pathologies and correlated therapeutic. In some embodiments, such ointment or cream formulations can be used for trans-dermal delivery of the pharmaceutical compositions described herein or for delivery to organs such as vagina or cervix in women.

[0076] Injectable formulations of the compositions can contain various carriers such as vegetable oils, dimethylacetamide, dimethylformamide, ethyl lactate, ethyl carbonate, isopropyl myristate, ethanol, polyols (glycerol, propylene glycol, liquid polyethylene glycol), and the like. For intravenous injections, water soluble versions of the compositions can be administered by the drip method, whereby a formulation including a pharmaceutical composition of the presently-disclosed subject matter and a physiologically-acceptable excipient is infused. Physiologically-acceptable excipients can include, for example, 5% dextrose, 0.9% saline, Ringer's solution or other suitable excipients. Intramuscular preparations, e.g., a sterile formulation of a suitable soluble salt form of the

compounds, can be dissolved and administered in a pharmaceutical excipient such as Water-for-Injection, 0.9% saline, or 5% glucose solution. A suitable insoluble form of the composition can be prepared and administered as a suspension in an aqueous base or a pharmaceutically-acceptable oil base, such as an ester of a long chain fatty acid, (e.g., ethyl oleate).

[0077] In addition to the formulations described above, the microvesicle compositions of the presently-disclosed subject matter can also be formulated as rectal compositions, such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides. Further, the microvesicle compositions can also be formulated as a depot preparation by combining the compositions with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

[0078] Further provided, in some embodiments of the presently-disclosed subject matter, are methods for treating a cancer that include administration of a microvesicle composition described herein. In some embodiments, the cancer is ovarian cancer.

[0079] For administration of a therapeutic composition as disclosed herein (e.g., a milk-derived microvesicle encapsulating a therapeutic agent), conventional methods of extrapolating human dosage based on doses administered to a murine animal model can be carried out using the conversion factor for converting the mouse dosage to human dosage: Dose Human per kg = Dose Mouse per kg × 1/12. Doses can also be given in milligrams per square meter of body surface area because this method rather than body weight achieves a good correlation to certain metabolic and excretionary functions. Moreover, body surface area can be used as a common denominator for drug dosage in adults and children as well as in different animal species. Briefly, to express a mg/kg dose in any given species as the equivalent mg/sq m dose, multiply the dose by the appropriate kg factor. In an adult human, 100 mg/kg is equivalent to 100 mg/kg x 37 kg/sq m=3700 mg/m^2.

[0080] Suitable methods for administering a therapeutic composition in accordance with the methods of the presently-disclosed subject matter include, but are not limited to, systemic

administration, parenteral administration (including intravascular, intramuscular, and/or intraarterial administration), oral delivery, buccal delivery, rectal delivery, subcutaneous administration, intraperitoneal administration, inhalation, dermally (e.g., topical application), intratracheal installation, surgical implantation, transdermal delivery, local injection, intranasal delivery, and hyper-velocity injection/bombardment. Where applicable, continuous infusion can enhance drug accumulation at a target site. In some embodiments of the therapeutic methods described herein, the therapeutic compositions are administered orally, intravenously, intranasally, or intraperitoneally to thereby treat a disease or disorder.

[0081] Regardless of the route of administration, the compositions of the presently-disclosed subject matter typically not only include an effective amount of a therapeutic agent, but are typically administered in an amount effective to achieve the desired response. As such, the term "effective amount" is used herein to refer to an amount of the therapeutic composition (e.g., a microvesicle encapsulating a therapeutic agent, and a pharmaceutically vehicle, carrier, or excipient) sufficient to produce a measurable biological response (e.g., a decrease in inflammation). Actual dosage levels of active ingredients in a therapeutic composition of the present invention can be varied so as to administer an amount of the active compound(s) that is effective to achieve the desired therapeutic response for a particular subject and/or application. Of course, the effective amount in any particular case will depend upon a variety of factors including the activity of the therapeutic composition, formulation, the route of administration, combination with other drugs or treatments, severity of the condition being treated, and the physical condition and prior medical history of the subject being treated. Preferably, a minimal dose is administered, and the dose is escalated in the absence of dose-limiting toxicity to a minimally effective amount. Determination and adjustment of a therapeutically effective dose, as well as evaluation of when and how to make such adjustments, are known to those of ordinary skill in the art.

[0082] As used herein, the terms "treatment" or "treating" relate to any treatment of a condition of interest (e.g., an inflammatory disorder or a cancer), including but not limited to prophylactic

treatment and therapeutic treatment. As such, the terms "treatment" or "treating" include, but are not limited to: preventing a condition of interest or the development of a condition of interest; inhibiting the progression of a condition of interest; arresting or preventing the further development of a condition of interest; reducing the severity of a condition of interest; ameliorating or relieving symptoms associated with a condition of interest; and causing a regression of a condition of interest or one or more of the symptoms associated with a condition of interest.

[0083] As used herein, the term "subject" includes both human and animal subjects. Thus, veterinary therapeutic uses are provided in accordance with the presently disclosed subject matter. As such, the presently-disclosed subject matter provides for the treatment of mammals such as humans, as well as those mammals of importance due to being endangered, such as Siberian tigers; of economic importance, such as animals raised on farms for consumption by humans; and/or animals of social importance to humans, such as animals kept as pets or in zoos. Examples of such animals include but are not limited to: carnivores such as cats and dogs; swine, including pigs, hogs, and wild boars; ruminants and/or ungulates such as cattle, oxen, sheep, giraffes, deer, goats, bison, and camels; and horses. Also provided is the treatment of birds, including the treatment of those kinds of birds that are endangered and/or kept in zoos, as well as fowl, and more particularly domesticated fowl, i.e., poultry, such as turkeys, chickens, ducks, geese, guinea fowl, and the like, as they are also of economic importance to humans. Thus, also provided is the treatment of livestock, including, but not limited to, domesticated swine, ruminants, ungulates, horses (including race horses), poultry, and the like.

[0084] The practice of the presently-disclosed subject matter can employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Some exemplary reference materials are listed in U.S. Patent Application 62/567,911 and U.S. Patent Application 62/568,026, and are incorporated herein by reference.

[0085] The terms "a", "an", and "the" refer to "one or more" when used in the subject specification, including the claims. The term "ambient temperature" as used herein refers to an environmental temperature of from about 0°F to about 120°F, inclusive.

[0086] Unless otherwise indicated, all numbers expressing quantities of components, conditions, and otherwise used in the specification and claims are to be understood as being modified in all instances by the term "about". Accordingly, unless indicated to the contrary, the numerical parameters set forth in the instant specification and attached claims are approximations that can vary depending upon the desired properties sought to be obtained by the presently disclosed subject matter.

[0087] As used herein, the term "about", when referring to a value or to an amount of mass, weight, time, volume, concentration, or percentage can encompass variations of, in some embodiments ± 20%, in some embodiments ± 10%, in some embodiments ± 5%, in some embodiments ± 1%, in some embodiments ± 0.5%, and in some embodiments to ± 0.1%, from the specified amount, as such variations are appropriate in the disclosed application. As used herein, ranges can be expressed as from "about" one particular value, and/or to "about" another particular value. It is also understood that there are a number of values disclosed herein, and that each value is also herein disclosed as "about" that particular value in addition to the value itself. For example, if the value "10" is disclosed, then "about 10" is also disclosed. It is also understood that each unit between two particular units are also disclosed. For example, if 10 and 15 are disclosed, then 11, 12, 13, and 14 are also disclosed.

[0088] All compositional percentages used herein are presented on a "by weight" basis, unless designated otherwise.

[0089] Specific quantities or material sources relevant to the compositions described herein are provided for the purpose of demonstrating the invention, but these quantities and sources are not intended to limit the scope of the invention. It is understood that, in light of a reading of the foregoing description, one with ordinary skill in the art may make alterations and/or modifications to the present

invention, and specifically to the embodiments shown and described herein, without departing from the scope of the invention.

[0090] All patents, patent applications, published applications and publications, GenBank sequences, databases, websites and other published materials referred to throughout the entire disclosure herein, unless noted otherwise, are incorporated by reference in their entirety.

[0091] As used herein, "optional" or "optionally" means that the subsequently described event or circumstance does or does not occur and that the description includes instances where said event or circumstance occurs and instances where it does not. For example, an optionally variant portion means that the portion is variant or non-variant.

[0092] Where reference is made to a URL or other such identifier or address, it understood that such identifiers can change and particular information on the internet can come and go, but equivalent information can be found by searching the internet. Reference thereto evidences the availability and public dissemination of such information.

Claims

What is claimed is:

1. A composition, comprising an effective amount of an anthocyanidin or an effective amount of curcumin encapsulated by a milk-derived microvesicle.
2. The composition of claim 1, wherein the milk-derived microvesicle is a colostrum-derived microvesicle.
3. The composition of claim 1, wherein the anthocyanidin comprises bilberry anthocyanidins.
4. The composition of claim 3, further comprising cisplatin.
5. The composition of claim 1, further comprising an effective amount of a chemotherapeutic agent encapsulated by a milk-derived microvesicle.
6. The composition of claim 5, wherein the chemotherapeutic agent is paclitaxel.
7. A pharmaceutical composition, comprising a composition according to claim 1 and a pharmaceutically-acceptable vehicle, carrier, or excipient.
8. A method of treating an ovarian cancer in a subject, comprising administering to a subject in need thereof an effective amount of a composition including an effective amount of an anthocyanidin encapsulated by a milk-derived microvesicle.
9. The method of claim 8, further comprising administering to the subject a composition including an effective amount of an chemotherapeutic agent encapsulated by a milk-derived microvesicle.
10. The method of claim 9, wherein administering the composition comprises orally, intravenously, intranasally, or intraperitoneally administering the composition.
11. A method of treating a cervical cancer in a subject, comprising administering to a subject in need thereof an effective amount of a composition including an effective amount of an curcumin encapsulated by a milk-derived microvesicle.

12. The method of claim 11, further comprising administering to the subject a composition including an effective amount of an chemotherapeutic agent encapsulated by a milk-derived microvesicle.

13. The method of claim 12, wherein administering the composition comprises orally, intravenously, intranasally, or intraperitoneally administering the composition.

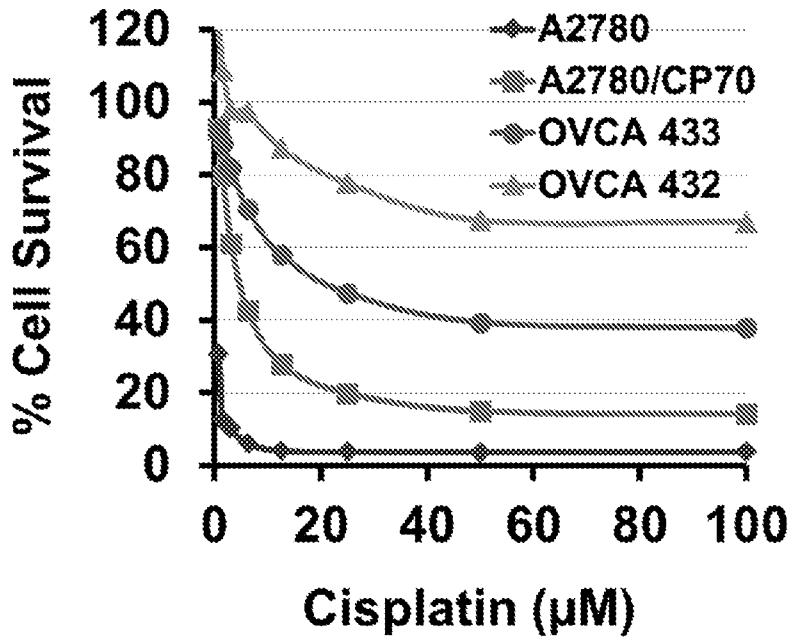


FIGURE 1

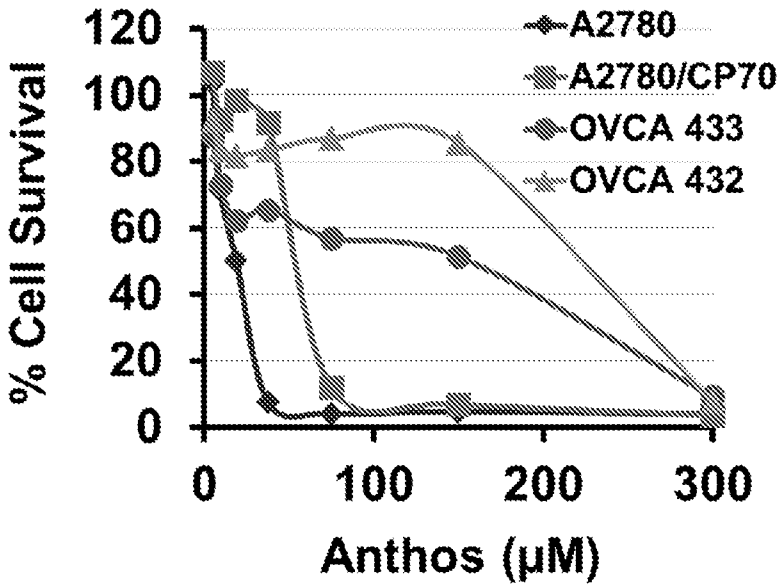
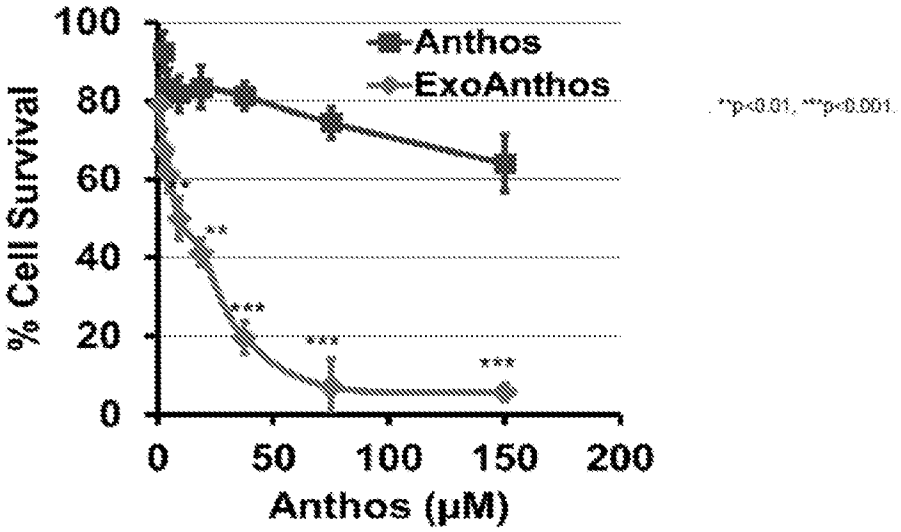


FIGURE 2



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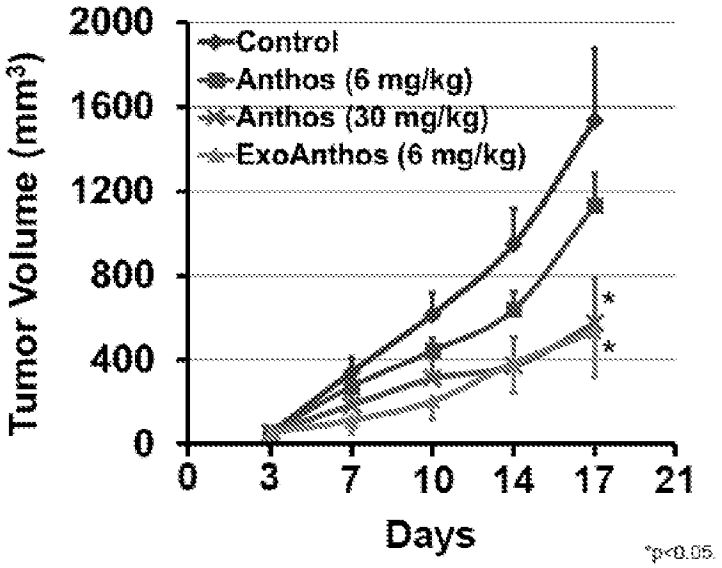
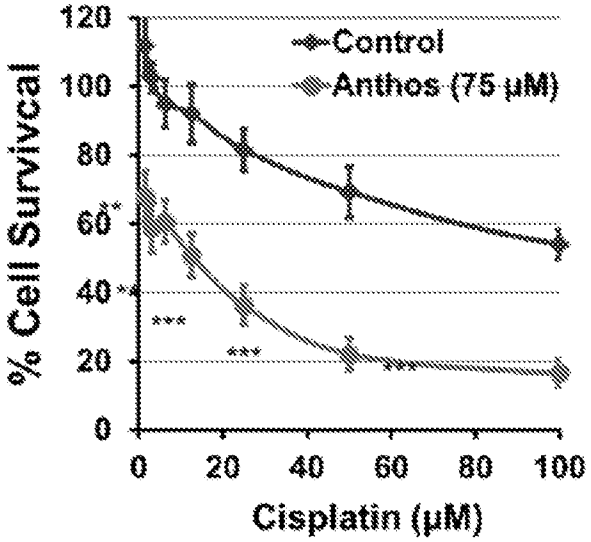


FIGURE 4



*p<0.05, **p<0.01, ***p<0.001.

FIGURE 5

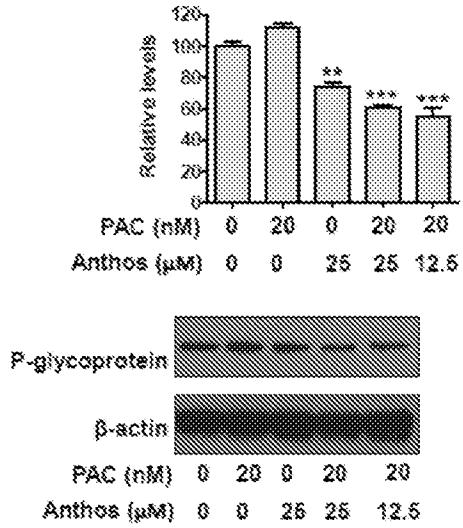


FIGURE 6

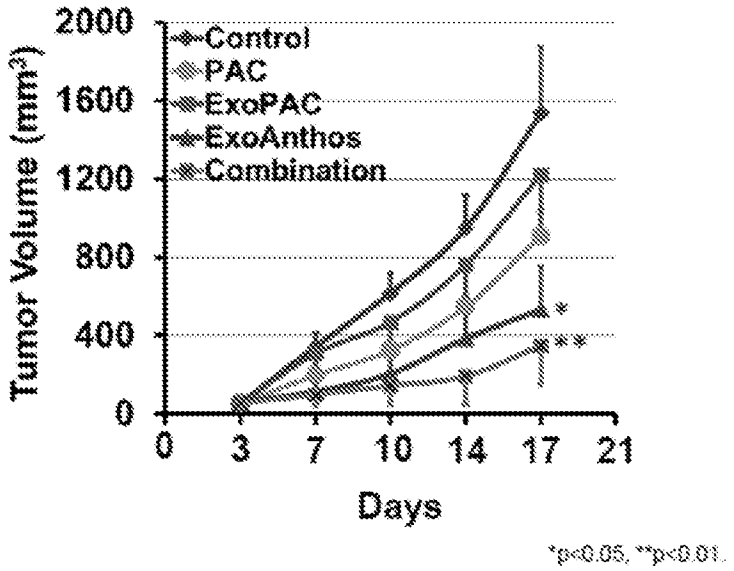


FIGURE 7

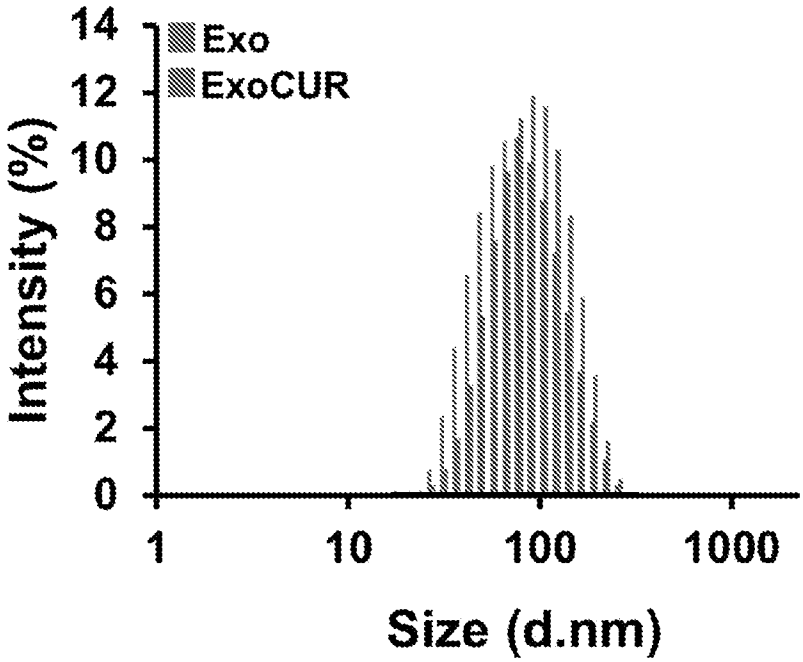


FIGURE 8

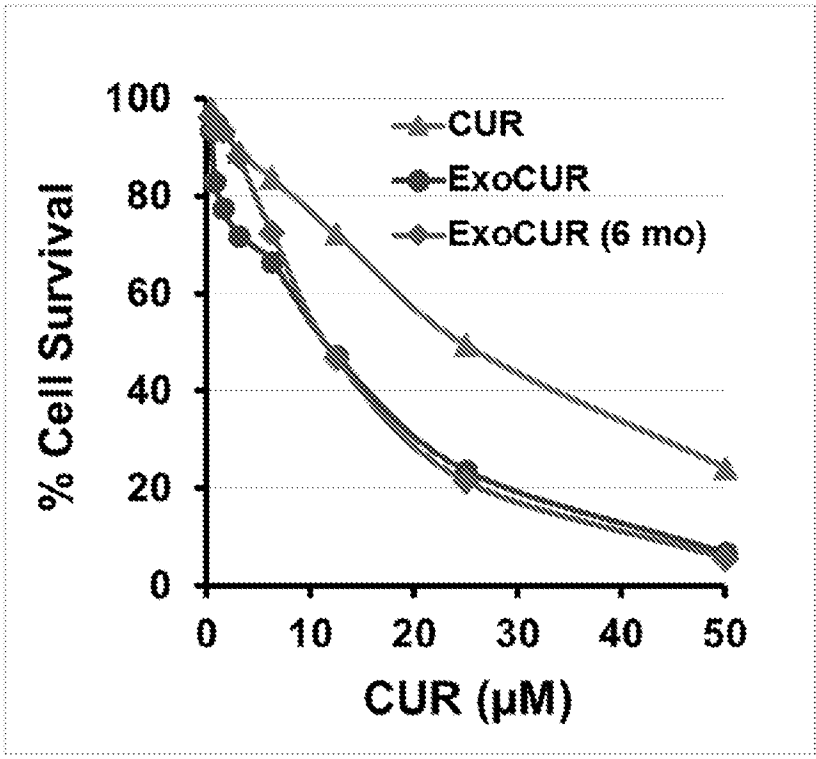


FIGURE 9

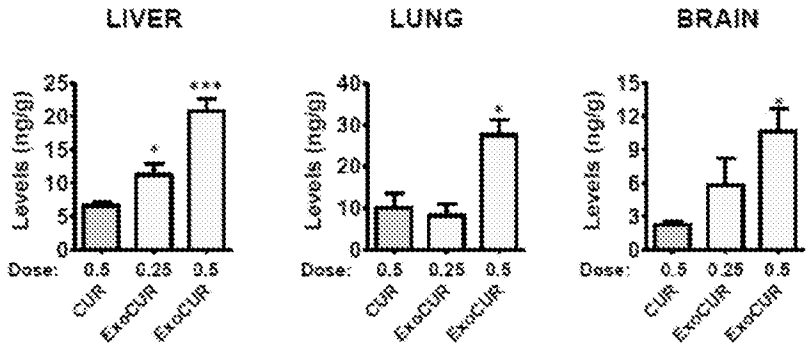


FIGURE 10

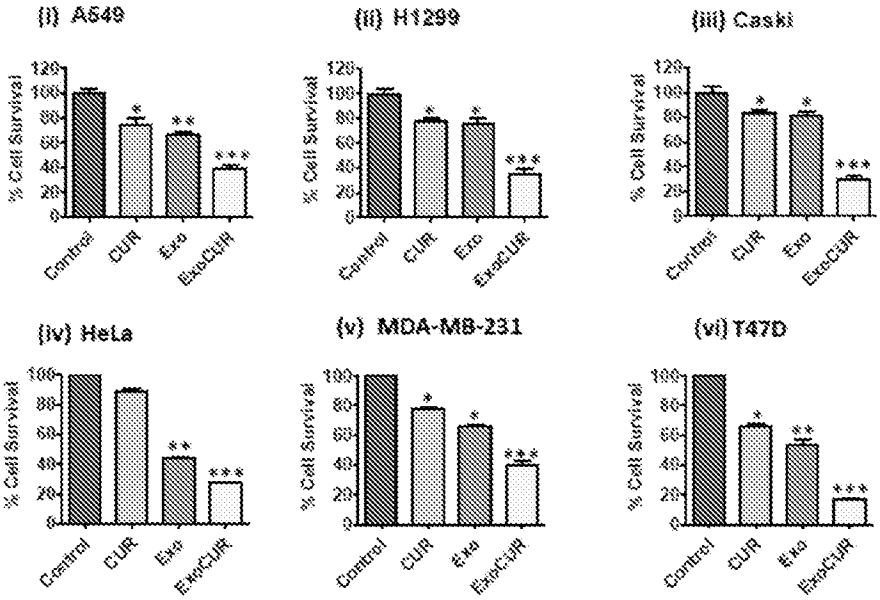


FIGURE 11

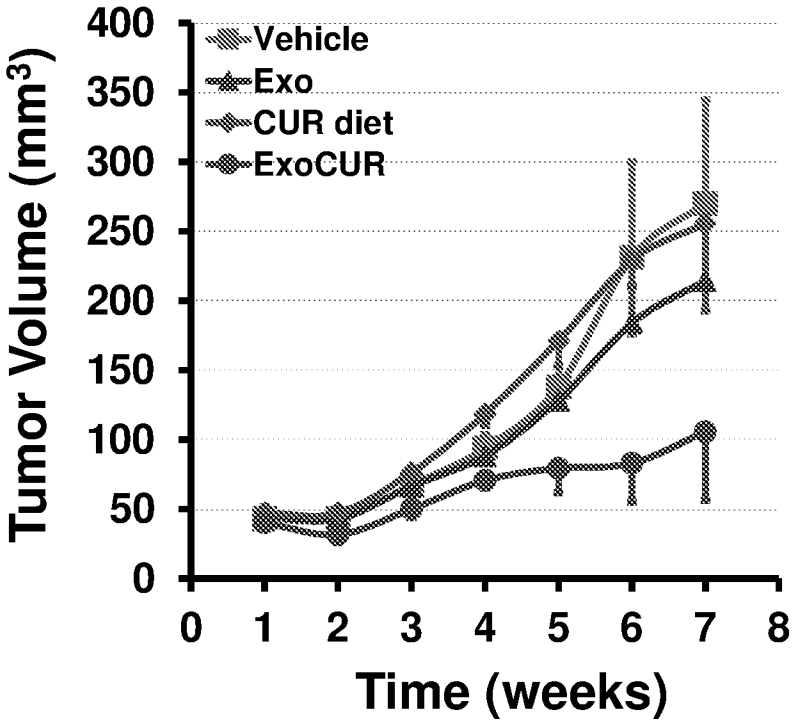


FIGURE 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2018/054450

A. CLASSIFICATION OF SUBJECT MATTER

IPC (2018.01) A61K 31/337, A61K 31/12, A61K 31/282, A61K 31/343, A61K 36/29, A61P 35/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC (2018.01) A61K 31/337, A61K 31/12, A61K 31/282, A61K 31/343, A61K 36/29, A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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

See extra sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014134132 A1 (UNIV LOUISVILLE RES FOUND [US], GUPTA RAMESH C [US] et al.); 04 Sep 2014 (2014/09/04) abstract, paragraphs [0009]-[0010], [0013], [0080], [00116], [00146] examples 4 and 10	1-3,7,8,11
Y	paragraph [0012], examples 4, 6 and 8 and figures 16, 19 and 21	4,12,13
X	Farrukh Aqil et al.:" Exosomal delivery of small molecules for the management of ovarian cancer"; Journal of Extracellular Vesicles, Vol. 6, No. Supplement 1, Page 41; 15 May 2017 (2017/05/15) entire document	1,5,6,8-10
Y	entire document	4,12,13
Y	Ajay P. Singh et al: "Cranberry proanthocyanidins are cytotoxic to human cancer cells and sensitize platinum-resistant ovarian cancer cells to paraplatin"; Phytother Res, Vol. 23, Num.8, Pages 1066–1074; 31 Aug 2009 (2009/08/31) abstract, discussion	4

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

☒ See patent family annex.

* Special categories of cited documents:

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

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 Dec 2018	13 Dec 2018

Name and mailing address of the ISA:	Authorized officer
Israel Patent Office Technology Park, Bldg.5, Malcha, Jerusalem, 9695101, Israel Facsimile No. 972-2-5651616	SHITRIT Adva Telephone No. 972-2-5651679

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US2018/054450

Patent document cited search report	Publication date	Patent family member(s)	Publication Date
WO 2014134132 A1	04 Sep 2014	WO 2014134132 A1	04 Sep 2014
		US 2016000710 A1	07 Jan 2016
		US 9943482 B2	17 Apr 2018
		US 2018185285 A1	05 Jul 2018

International application No.
PCT/US2018/054450

B. FIELDS SEARCHED:

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

Databases consulted: BLAST, Esp@cenet, Google Patents, CAPLUS, BIOSIS, EMBASE, MEDLINE, MARPAT, Google Scholar, DWPI, Derwent Innovation

Search terms used: Anthocyanidin, anthos, Anthocyanidins, Anthocyanin, Anthocyanins, Leucoanthocyanidins,curcuma, curcumin, curcuminoid, ovarian cancer, cervical cancer,exosome, milk derived exosome, colostrum derived exosome, cisplatin, platamin, paclitaxel, Taxol