

PHARMACOGNOSIS

Effects of a High Molecular Mass *Convolvulus arvensis* Extract on Tumor Growth and Angiogenesis.

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Background. Plant materials represent promising sources of anti-cancer agents. We developed and tested a novel extract from the ubiquitous plant *Convolvulus arvensis*.

Materials and Methods. *Convolvulus arvensis* components were extracted in boiling water, and small molecules were removed by high-pressure filtration. The extract's biological activity was assessed by measuring its effects on S-180 fibrosarcoma growth in Kun Ming mice and on heparin-induced angiogenesis in chick embryos. We also examined the extract's effects on lymphocytes ex vivo and tumor cell growth in vitro.

Results. The extract (primarily proteins and polysaccharides) inhibited tumor growth in a dose-dependent fashion when administered orally. At the

highest dose tested, 200 mg/kg/day, tumor growth was inhibited by roughly seventy percent. Subcutaneous or intraperitoneal administration at 50 mg/kg/day also inhibited tumor growth by over seventy percent. The extract's acute LD₅₀ in Kun Ming mice was 500 mg/kg/day when injected, indicating that tumor growth inhibition occurred at non-toxic doses. It inhibited angiogenesis in chick embryos, improved lymphocyte survival ex vivo, and enhanced yeast phagocytosis, but did not kill tumor cells in culture.

Conclusion. High molecular mass extract deserves further study as an anti-cancer agent.

Key words: Angiogenesis, Experimental therapeutics, Tumors, Plant extracts

Plant materials may be promising sources of anti-cancer agents. Ingredients in medicinal plants such as mistletoe, tea, and the Chinese herb "Sho-Ssaiko-to" have been shown to induce programmed cell death in some cancer cells (1). Other plant extracts affect tumor angiogenesis and immune cell function. Angiogenesis inhibition is a particularly interesting therapeutic strategy because tumor vascularization is essential for tumor growth and metastasis (2). Angiogenesis inhibitors derived from natural sources include flavonoids, sulphated carbohydrates, or triterpenoids (3). Plant or plant products that stimulate immune cells in ways beneficial to the

treatment of cancer include garlic, mushroom proteoglycans, and various Chinese herbs (4-6). Non-toxic plant extracts containing angiogenesis inhibiting or immune stimulating ingredients may be promising anti-cancer agents, even if they do not show efficacy in screening assays based on in vitro tumor cell growth.

After receiving an anecdotal report concerning the complete remission of a human ovarian carcinoma in a patient who consumed a tea made from the ubiquitous plant *Convolvulus arvensis*, we became interested in testing the biological effects of *Convolvulus arvensis* extracts. *Convolvulus arvensis* is known to contain alkaloids, compounds that have anti-cancer activity but may also be toxic to the host at high doses (7). We concentrated our efforts on extracting high molecular mass, water-soluble molecules from *Convolvulus arvensis* in the hopes of producing an extract that was effective against tumors but lacked the toxicity of alkaloids. This manuscript details our production and characterization of such an extract. We tested the extract's effect on tumor growth in mice and, to learn more about its mechanism of action,

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examined its effects on angiogenesis, tumor cell growth, phagocytosis, and lymphocyte growth in vitro.

Materials and Methods

Extract production and characterization. The aerial portions of *Convolvulus arvensis* were collected from land on which no pesticides or herbicides have been used for fifteen years. The fresh raw material was mixed in distilled water at a concentration of 0.16 g/mL using a commercial blender. The mixture was boiled for thirty minutes, allowed to cool, and filtered with a 100-micron sieve. The filtrate was centrifuged at 11,300 g for 15 min., at 4°C. The supernatant was filtered a 1.5 µm fiberglass and 1.2 µm nylon filters and then concentrated using a pressurized (55 psia N₂) stir cell apparatus (Model CH2, Millipore, Bedford, MA) with a 30 Kda YM-30 (Millipore) membrane. This concentrate was lyophilized (Freeze Zone 6 Freeze Drying Apparatus, Labconco Inc., Kansas City, MO) to produce the extract powder. In a preliminary effort to analyze the components of our *Convolvulus arvensis* extract, we passed it through a Superose 12 HR (Pharmacia Biotech, Piscataway, NJ) sizing column, with 0.01 M PBS containing 0.05% sodium azide, pH 7.4, at a flow rate of 0.5 mL/min. The Bicinchoninic acid protein assay (Sigma Chemical Co., St. Louis, MO) was used to estimate the extract's protein content. The assay was run according to manufactures instructions. Briefly, 13 µL extract solution (1 mg/mL) was mixed with 260 µL protein reagent (bicinchoninic acid with copper (II) sulfate) and, after thirty minutes incubation at 37 °C, the absorbance at 570 nm was determined using an Emax microplate reader (Molecular Devices, Sunnyvale, CA). The polysaccharide content of the extract was determined using the Molish reaction. Briefly, 40 µL extract solution (1 mg/mL), 40 µL 5% phenol, and 200 µL concentrated sulfuric acid were mixed in a 90 well plate and the absorbance at 450 nm was determined immediately. Absorbance was correlated with protein or polysaccharide content using a standard curve. For each parameter, the mean of four separate tests was determined, with errors given as standard deviations.

Chicken egg chorio-allantoic membrane assay. Measurements angiogenesis on allantoic membranes of chick embryos were carried out using a method similar to that detailed by Klagsbrun and coworkers (8). Briefly, one-day-old fertilized chicken eggs (Groves Farms, McPherson, Kansas) were incubated for ten days at 37°C. A 1 cm² side window was cut in the eggshell to expose the chorio-allantoic membrane (CAM). Heparin containing cellulose discs were used to induce angiogenesis in the CAM (2). Discs were prepared by mixing 100 mL methyl cellulose with 10 mg heparin (controls) or 10 mg heparin

with high molecular mass *Convolvulus arvensis* extract (50, 100, or 200 mg), dispensing a drop of the solution on a non-stick surface, and letting it dry. The resulting discs, usually 3 mm in diameter, were then gently placed onto the CAM at a site distant from large blood vessels. Six eggs were used for the control group and six for each of the treatment groups. The egg window was then re-sealed by placing tape over the opening. After four days incubation, the tape was removed, re-exposing the CAM, and the CAM image was captured using a video camera (SAC-410ND CCD Color Camera, Samsung Inc.) attached to an anatomical microscope (EMZ-TR, Meiji, Inc.). Immediately prior to image capture, 2-3 mL of dairy cream were injected under the CAM to serve as a contrast agent. CAM images were coded and given to a second scientist for 'blinded' angiogenesis scoring. Images were scored using a scale from zero (no angiogenesis) to four (capillary growth, typical of that observed using heparin containing discs).

Mouse Tumor Growth Inhibition Assays. Tumor growth inhibition experiments using subcutaneously implanted mouse sarcoma cells were performed on a contract basis by the Cancer Institute CAMS and PUMC (Beijing, China) and the Beijing Hepatitis Institute (Beijing, China). Mixed gender Kun Ming mice 3-4 wk of age, weighing 20-22 grams, were given subcutaneous injections of S-180 murine sarcoma cells (8 x 10⁵ cells in 200 µL PBS) in the left armpit. Treatments with various doses of the high molecular mass *Convolvulus arvensis* were administered orally by gavage or by intraperitoneal or subcutaneous injection (right groin). Ten animals were used for each dose. The standard treatment schedule called for extract administration to begin one day after tumor cell implantation. Two alternative treatment schedule, with treatments beginning eight days before or eleven days after tumor implantation, were also tested. In all cases, animals were treated daily for two weeks, at which point the animals were euthanized and the tumors were massed after necropsy. Tumor size and mouse mass were monitored throughout the treatment period.

In Vitro tumor growth inhibition assays. LLC-1 Lewis lung carcinoma cells and S-180 mouse sarcoma cells were seeded at concentrations of 4000 cells/well in 96 well plates and incubated for three days in the presence of up to 2 mg/mL high molecular mass *Convolvulus arvensis* extract. Surviving cell number was determined using the CFDA colorimetric assay as described previously (9).

Acute host toxicity assay. Host toxicity studies in mice were also conducted at the Cancer Institute in Beijing, China. Initially, Kun Ming mice (two per dosage) were given intraperitoneal injections of 25 to 6400 mg/kg/day high molecular mass *Convolvulus arvensis* extract and their survival over a seven day period was ascertained.

The highest dose with both mice surviving and the lowest dose that killed both mice were used as the high and low doses in the subsequent study. This was repeated twice to narrow the dose range, and then a more rigorous test was conducted using seven extract doses (256, 320, 400, 500, 625, 718, and 976 mg/kg/day) with ten mice per group. The LD₅₀ was then calculated from the mouse survival rates in this experiment using the Spearman-Kärber method, as described by the equation below:

$$\text{Log}_{10}(\text{LD}_{50}) = \text{Log}_{10}(D_7) - \frac{(\text{Log}_{10}(D_1) - \text{Log}_{10}(D_2))}{2} \sum_{i=1}^6 (P_i + P_{i+1}) \quad (1)$$

where doses D₁, D₂ and D₇ are 256, 320, and 976 mg/kg/day, respectively, and P_i represents the fraction of mice at dose "i" that died during treatment.

Ex Vivo Lymphocyte Growth. Two heparin containing vacuum tubes of human blood (roughly 16 mL) were collected venipuncture, diluted twofold in PBS, layered over Ficoll-Paque, and centrifuged at 200g for 20 minutes. The lymphocyte containing buffy layer was collected, rinsed twice in PBS, and suspended at a concentration of roughly 10⁶ peripheral blood cells per ml in AIM-V (Gibco BRL, address) supplemented with 1 ng/mL interleukin-2 (Sigma Chemical Co., St. Louis, MO) and 20 mM mercaptoethanol (Sigma Chemical Co., St. Louis, MO). High molecular mass *Convolvulus arvensis* extract was added at concentrations of 0.8, 4, 20, 100, and 500 mg/mL while two controls were set up without extract. Lymphocytes were incubated three days an atmosphere containing 95% air, 5% carbon dioxide, at 37°C, for 3 days and then counted using a Coulter Epics XL flow cytometer. The normalized cell number was determined by dividing the lymphocyte number in any given sample to the average for controls (no extract).

Ex Vivo Phagocytic Activity. To collect phagocytes, human whole blood was diluted twofold in PBS, layered over Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient solution (2.4 mL de-ionized water, 1.25 mL concentrated PBS, and 8.85 mL Percoll) and centrifuged 200 g for 20 minutes. The buffy layer was collected and rinsed twice to obtain phagocytes. These cells were suspended in growth medium and incubated five hours in the presence or absence of 125 to 500 µg/mL high molecular mass *Convolvulus arvensis* extract. Samples were then exposed to baker's yeast for one hour, stained with acridine orange, and examined by fluorescence microscopy (Nikon Microprot-FX, Nikon Inc., Garden City, NJ). The percentage of phagocytes (macrophages and polymorphonuclear cells) containing intracellular baker's yeast was determined.

Results

Using the production method described above, 200 grams of *Convolvulus arvensis* were converted into 0.8 grams lyophilized powder. This powder contained at least two peaks identified by liquid chromatography, one at a molecular mass of 20 KDa and another at the high molecular mass limit of the column (> 650 KDa). The percent composition, by mass, was determined to be 36 ± 4 % polysaccharides and 64 ± 27 % protein. We thus suspect that the high molecular mass *Convolvulus arvensis* extract is a mixture of proteins and polysaccharides, perhaps containing proteoglycans or glycoproteins.

Injections of this high molecular weight *Convolvulus arvensis* extract had a dramatic effect on the growth of S-180 tumors implanted into Kun Ming mice tumor. Specifically, a two-week regiment of 50 mg/kg/day extract given daily, starting one day after tumor cell implantation, by intraperitoneal injection inhibited tumor growth by 75 ± 8 % (four experiments, each comprising ten mice in the treatment group) compared to tumors in untreated mice. Subcutaneous injections at the same dose inhibited tumor growth by 77 ± 11 % (four experiments, each comprising ten mice in the treatment group). Moreover, the extract had remarkably low toxicity. Mice given high molecular mass *Convolvulus arvensis* after tumor cell implantation did not differ significantly in mass from controls. Doses below 400 mg/kg were completely non-toxic (100% survival), and the mouse LD₅₀ for the extract was 500 ± 27 mg/kg. Thus, the high molecular mass *Convolvulus arvensis* extract appears to inhibit tumor growth at non-toxic doses.

This high molecular mass *Convolvulus arvensis* extract was also effective when administered orally. Dose-response data for oral extract administration using three different treatment schedules are given in Figure 1. In all cases, the extract inhibited tumor growth in a dose-dependent fashion. When the treatments were started before or soon after tumor cell implantation, tumor growth was inhibited by roughly seventy percent at the highest dose tested, 200 mg/kg/day, suggesting that oral administration is not much less effective than injections. Pre-treatment with extract appeared to improve efficacy slightly, but the effect was not statistically significant. Even when the tumor was allowed to grow for eleven days prior to the onset of treatment, the extract was effective, with over fifty percent growth inhibition being achieved at 200 mg/kg/day. In these larger tumors, caliper measurements four days before and six days after the onset of treatment showed that, at the highest three doses, the extract shrunk the tumors by at least thirty percent. The shrunken tumors then resumed growth, but at roughly half the rate of untreated tumor growth.

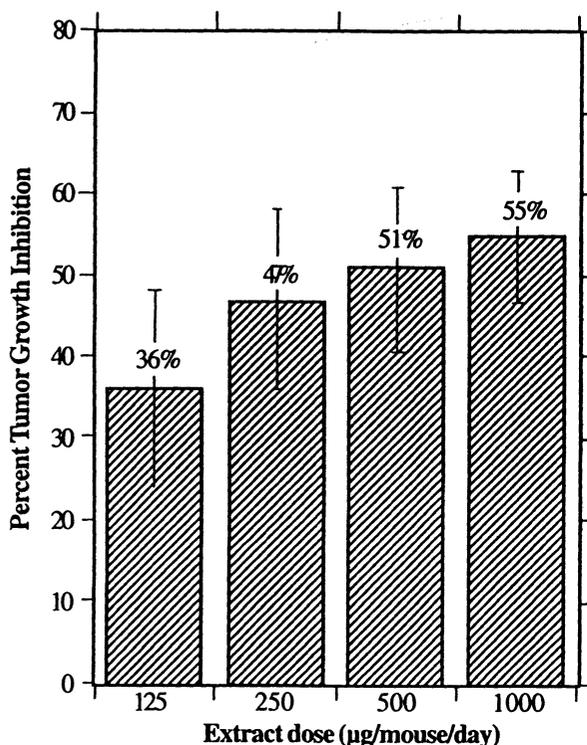
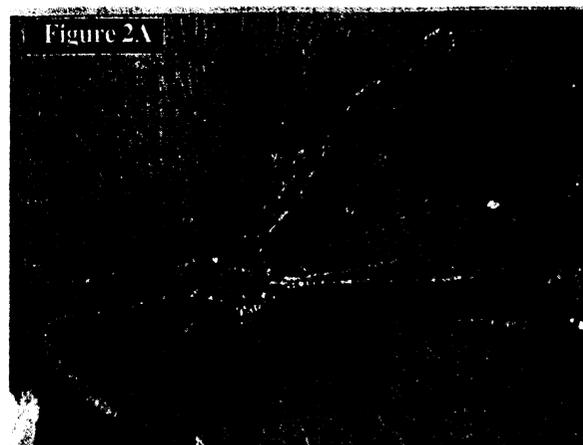


Figure 1. The percent inhibition of intradermally implanted S-180 sarcoma growth (by mass) in Kun Ming mice treated orally with a high molecular mass *Convolvulus arvensis* extract for two weeks, compared to sarcoma growth in untreated controls. Extract was administered daily starting either eight days before (white bars), one day after (black bars), or eleven days after (dotted bars) tumor cell implantation.

The mechanism of this tumor growth inhibition was examined in vitro. The high molecular mass *Convolvulus arvensis* extract did not inhibit tumor cell growth in monolayers at the concentration range tested (up to 2 mg/mL), suggesting that its mechanism of action in vivo was something other than direct cytotoxicity. The effect of the extract on angiogenesis was tested using the chick CAM assay. Examples of heparin induced vessel growth on chick CAM in the presence or absence of extract are given in Figure 2. In the absence of extract, neo-vascularization is indicated by the high vessel density, and the directional growth of small vessels toward the heparin disc. In contrast, vessel growth on the CAM was much less extensive when the heparin containing disc was impregnated with 200 mg/disc of the high molecular mass *Convolvulus arvensis* extract. The vessel density appeared to be reduced, and there was no tendency toward vessel growth in the direction of the disc. Similar results were obtained in six replicate experiments. The average angiogenesis score for the heparin controls was 2.8 ± 1.0 ,



Control: Angiogenesis score = 3.5



200 µg/disc *Convolvulus arvensis* extract: Angiogenesis score = 0.5

Figure 2. Vascular formation in chick chorio-allantoic membranes (CAM) after four days treatment with heparin containing methylcellulose discs.

(2A) A CAM for a control disc (no extract)

(2B) a disc containing 200 mg of a high molecular mass *Convolvulus arvensis* extract are shown. The location of disc is shown with arrows. Angiogenesis scores along with the high molecular mass *Convolvulus arvensis* extract doses are given in each case.

while that for CAM treated with 200 mg/ discs was 1.7 ± 0.9 , a statistically significant ($p < 0.001$) difference. Figure 3 shows the normalized angiogenesis score (compared to heparin controls) at each dose tested (50 to 200 mg extract/disc). Average angiogenesis scores were significantly below the average control score in all cases, with roughly 40% inhibition of angiogenesis, as measured by vascularization score, at the highest dose tested. Based on these data, we conclude that one or more ingredients

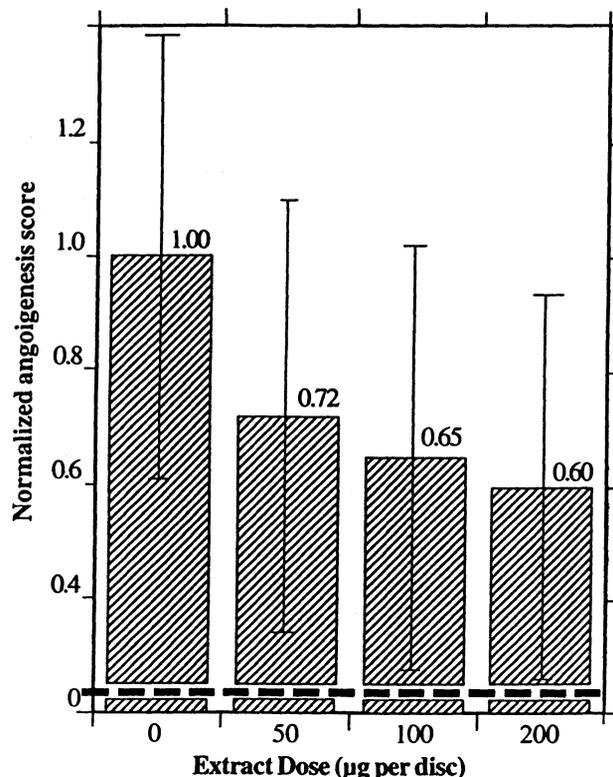


Figure 3. Normalized angiogenesis scores for chick chorioallantoic membranes (CAM) after four days treatment with heparin containing methylcellulose discs. Discs were impregnated with various amounts (50 to 200 mg /disc) of a high molecular mass *Convolvulus arvensis* extract prior to implantation on the CAM.

in the high molecular mass *Convolvulus arvensis* extract have inhibitory effects on heparin-induced angiogenesis.

High molecular mass *Convolvulus arvensis* extract may also enhance phagocytosis and improve the ex vivo survival and growth of lymphocytes. Figure 4 shows the effect of extract on lymphocyte number after three days ex vivo incubation. Concentrations between 0.8 and 100 mg/mL increased lymphocyte cell numbers compared to cells incubated without extract. The highest extract concentration tested, 500 mg/mL, actually decreased cell number. This may suggest an upper limit for toxicity, though it is difficult to directly relate ex vivo lymphocyte data to in vivo blood cell counts. High molecular mass *Convolvulus arvensis* extract also had an effect on the ability of phagocytes to digest yeasts. Without extract, the phagocytic index was 7.0 ± 1.4 %. When peripheral blood cells were pre-incubated in 250 mg/mL extract for five hours prior to yeast exposure, however, the phagocytic index was increased to 14.5 ± 0.7 %. Based on these data, we suspect that one or more ingredients in the high molecular mass *Convolvulus arvensis* extract have stimulatory effects on white blood cells.

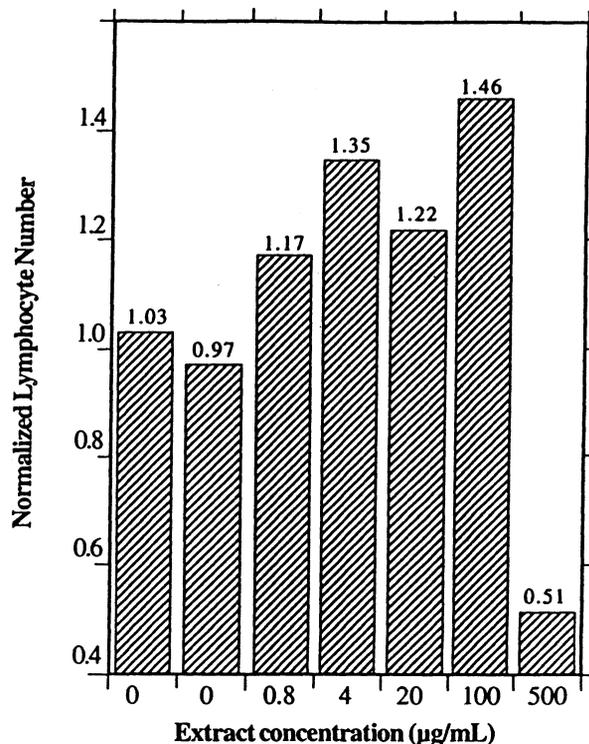


Figure 4. Normalized lymphocyte number for peripheral blood cells incubated three days in the presence or absence of a high molecular mass *Convolvulus arvensis* extract. Lymphocyte numbers, determined using forward scatter versus side scatter gating with flow cytometry, were divided by values for controls (no extract) to determine the normalized values.

Discussion

The data described above indicate that we have developed a high molecular mass, water soluble *Convolvulus arvensis* extract, containing proteins and polysaccharides that is capable of inhibiting tumor growth in mice at non toxic doses. Our data suggest that the mechanism of action for this extract is not direct tumor cell killing but may instead be related to angiogenesis inhibition or immune cell function enhancement. In this way, our high molecular mass *Convolvulus arvensis* extract is similar to plant proteoglycans and glycoproteins described in the literature. These molecules are of interest in cancer research for a variety of reasons. They are an important part of the extracellular matrix and they serve in some fashion to prevent tumor cell migration. Heparin sulfate binding proteoglycans affect the activity the tumor angiogenesis promoter FGF-2 (10), while hyaluronan-proteoglycans are thought to play a role in tumor arrest in the vascular bed (11). The 450 KDa glycoprotein Thrombospondin-1 inhibits angiogenesis and in vivo

tumor growth (12), presumably because of its ability to bind to heparin sulfate proteoglycans, growth factors, and cell surface receptors. The mechanism by which our extract inhibited vascularization in the CAM is unknown, but direct binding to and interference with heparin is a possibility. Some plant extracts also work by enhancing immune function. Mushroom proteoglycans have been shown to have an increase immune cell counts and to enhance infiltration of T-lymphocytes and dendritic cells into tumors (6). Moreover, they have been shown in clinical trials to enhance survival times of patients with a variety of cancers and to ameliorate side effects of chemotherapy (6). While preliminary, our results suggest that our extract contains ingredients that enhance lymphocyte and phagocyte function.

We conclude that the high molecular mass *Convolvulus arvensis* extract described in this manuscript deserves further study as a potential anti-cancer agent. Future *Convolvulus arvensis* extract research can take at least two approaches. One would be to isolate the most active ingredient in the extract, with the hopes of obtaining a potent anti-tumor agent. To this end, efforts are under way in our laboratory to further separate and characterize the extract's components. Another approach, based on the notion that plants contain several active ingredients that may work together synergistically, and that the combination of ingredients may in some fashion be evolutionarily optimized, is to continue biological testing of our extract along with other crude extracts from *Convolvulus arvensis* or other plants. We are presently testing such extracts in a variety of animal tumor models, with the idea of using more resistant tumor models and testing extracts in combination with other therapeutic modalities. At the same time, we do not think it would be premature to begin phase one clinical studies with our high molecular mass *Convolvulus arvensis* extract. Because of its in vivo efficacy, its low host toxicity, the simplicity of the extraction process, and the ease of cultivating the source plant, the high molecular mass *Convolvulus arvensis* extract may turn out to be a safe and inexpensive therapeutic or adjuvant agent.

Resumen

Historicamente se ha utilizado material de origen botánico como fuente de agentes para el tratamiento de cancer. Hemos desarrollado y examinado un extracto novel de una planta silvestre de amplia distribución geográfica llamada *Convolvulus arvensis*. Los componentes del *Convolvulus arvensis* se extrajeron con agua hirviendo y

además se usó filtración de alta presión para remover las moléculas mas pequeñas. La actividad biológica del extracto se evaluó midiendo sus efectos en el crecimiento del fibrosarcoma S-180 en el ratón Kun Ming y en embriones de pollo con angiogenesis inducida por heparina. También se examinó el efecto del extracto en los limfocitos ex vivo y el crecimiento de células tumorales *in vitro*. El extracto (principalmente proteínas y polisacáridos) inhibió el crecimiento tumoral en forma dependiente a la dosis al administrarse por via oral. El crecimiento tumoral se inhibió en aproximadamente un setenta por ciento (70%) en la dosis mas alta examinada (200 mg/kg/día). La administración subcutanea o intraperitoneal de 50 mg/kg/día también inhibió crecimiento tumoral por aproximadamente un setenta por ciento (70%). El LD₅₀ agudo en los ratones Kun Ming fué de 500 mg/kg/día por vía parenteral, lo que indica que hubo inhibición de crecimiento tumoral en dosis no tóxicas. Se produjo inhibición de angiogenesis en los embriones de pollo, mejoró la sobrevivencia de los limfocitos ex vivos. Y mejoró la fagocitosis de levadura, aunque no mató las células de tumor en el cultivo. Este extracto de alta masa molecular merece estudiarse mas a fondo por su posible rol terapéutico contra cancer.

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