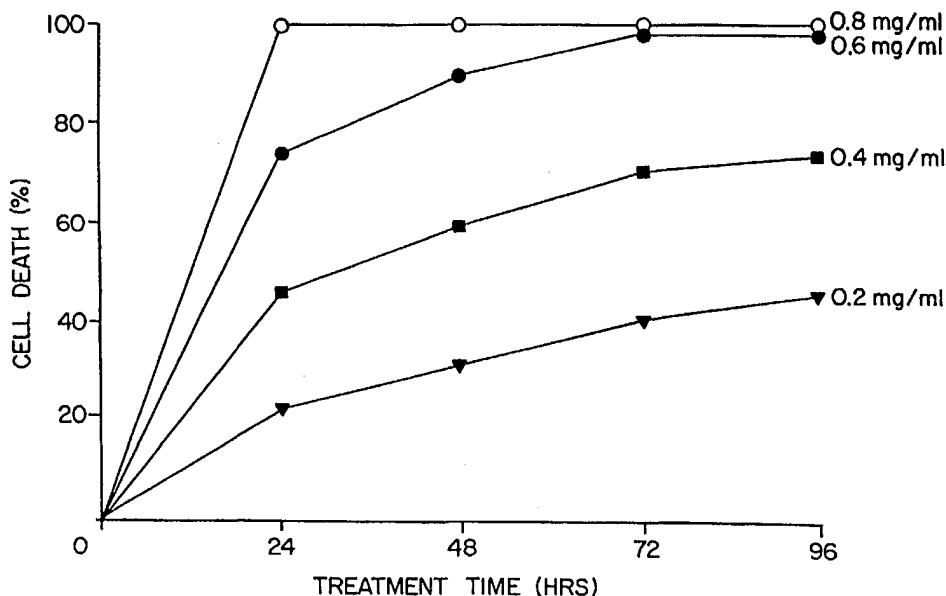




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(21) International Application Number: PCT/CA97/00873 (22) International Filing Date: 21 November 1997 (21.11.97) (30) Priority Data: 2,191,923 3 December 1996 (03.12.96) CA (71) Applicant: EASTWOOD BIOMEDICAL RESEARCH INC. [CA/CA]; Suite 1701, 1111 West Georgia Street, Vancouver, British Columbia V6E 4M3 (CA). (72) Inventors: YOON, Ji-Woon; 206 Edgeview Drive N.W., Calgary, Alberta T3A 4W9 (CA). HAM, Seung, Shi; 1919 University Drive N.W. #J104, Calgary, Alberta T2N 4L2 (CA). JUN, Hee, Sook; 3031 Hospital Drive N.W. #1309, Calgary, Alberta T2N 2T8 (CA). (74) Agents: SECHLEY, Konrad, A. et al.; Gowling, Strathy & Henderson, Suite 2600, 160 Elgin Street, Ottawa, Ontario K1P 1C3 (CA).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published With international search report.

(54) Title: SPECIFIC INHIBITION OF TUMOR CELL GROWTH BY *PORTULACA OLERACEA*

(57) Abstract

Portulaca oleracea has been used throughout history for many different medicinal purposes. This invention is directed to the novel use of *P. oleracea* for the treatment of cancer. More specifically it is disclosed that *P. oleracea* has a specific and distinct effect on the inhibition and/or suppression of gastric tumor cell growth *in vitro* and *in vivo*. An aqueous extract of *P. oleracea* showed a tumoricidal activity against KATO III (human gastric carcinoma cell line) and COLO 320 HSR cells (human colon adenoma cell line) in a dose-dependent and time-dependent manner, but not against the non-tumorous cell lines, L929 (murine lung connective tissue) and WI38 (human lung diploid cell) cells. Subcutaneous injection of six week old CD1 nude mice with COLO 320 HSR cells and subsequently *Portulaca oleracea* extract showed a clear inhibition of tumor growth as compared to the control nude mice which received only COLO 320 HSR cells.

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SPECIFIC INHIBITION OF TUMOR CELL GROWTH BY *PORTULACA OLERACEA*

The present invention relates to the tumoricidal activity of *Portulaca oleracea* extracts. More specifically this invention relates to the suppression of tumor cell growth *in vitro* and *in vivo*.

BACKGROUND OF THE INVENTION

Full citations for references appear at the end of the examples section.

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Portulaca oleracea is found ubiquitously in habitats with warm climate (Habtemariam et al., 1993). It has been used as an edible plant by humans since the prehistoric age. In China, the Philippines, Southeast Asia, and Africa, this wild plant is still consumed as food in various ways (Herklots, 1972). *P. oleracea* is recognized as a quite nutritious vegetable. It includes carbohydrates, amino acids, omega-3 fatty acids (Simopoulos et al., 1992, Mohamed and Hussein, 1994), several vitamins and minerals and many others (Wattenbergs, 1993) (Table 1).

Table 1. Composition of *Portulaca oleracea*

20

	1. Noradrenaline	15. Oxalic acid
	2. Dopa	16. Glutamic acid
	3. Dopamine	17. Asparaginic acid
25	4. Cardiac-glycoside	18. Malic acid
	5. Anthraquinone-glycoside	19. Tannic acid
	6. Glucose	20. Vitamins A, B ₁ , B ₂ , C
	7. Sucrose	21. Catechol
30	8. Fructose	22. Urea
	9. Coumarin	23. Wax
	10. Amino acids	24. Batacyanin
	11. Saponins	25. K-salt (KCl, K ₂ SO ₄ , KNO ₃)
	12. Alkaloids	26. K ₂ O
35	13. Flavonoids	27. Fe ^{**}
	14. Citric acid	

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In some countries, *P. oleracea* has been well known for ages as a medicinal herb. It has been used in the treatment of cardiovascular disease in Jamaica and in the topical application for swellings, bruises, abscesses and boils in Nigeria. In addition, it is known to be effective as an expectorant to treat cough (Vemla et al., 1982). It was also used as a "heart-tonic" and a diuretic. There are reports about the analysis of its composition (Tulloch, 1975, Simopoulos et al., 1992, Mohamed and Hussein, 1994), its influence on muscle (Parry et al., 1987, Parry et al., 1993), its effect on antifertility (Verma et al., 1982), and its efficacy in treating blood sugar levels in diabetics (Kin Y, JP 63,208,531, published August 30, 1988).

Several plant products are known for their potential for the prevention and treatment of cancer (Retsas, 1986, Huang et al, 1994, Lau et al 1994). However, the influence of *P. oleracea* on cancer has not been reported.

SUMMARY OF THE INVENTION

The present invention relates to the tumoricidal activity of an extract obtained from *P. oleracea*.

According to the present invention there is provided a composition comprising an aqueous extract obtained from *P. oleracea* with tumoricidal activity. This extract may also be obtained following solvent extraction of the plant, preferably leaf tissue.

This invention further relates to a bio-active ingredient from *P. oleracea* having tumoricidal activity.

This invention also provides for a method for the preparation of a tumoricidal extract obtained from *P. oleracea* involving homogenizing the plant in at least one solvent and retaining the aqueous fraction.

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This invention also relates to a method of preventing tumor cell growth, preferably stomach tumor cells through the administration of a composition comprising an extract obtained from *P. oleracea*.

5 This invention also relates to a method of treating tumor cells, preferably stomach tumor cells through the administration of a composition comprising an extract obtained from *P. oleracea*.

10 This invention also provides for pharmaceutical compositions comprising an aqueous extract obtained from *P. oleracea*, and a suitable carrier.

Although the present invention is exemplified by the treatment of stomach cancer cells, in practice any tumor cell can be treated with the extract of this invention.

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BRIEF DESCRIPTION OF THE DRAWINGS

20 These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

25 **FIGURE 1** is the procedure for extraction of *P. oleracea* extract. Whole plant was sliced into small pieces and homogenized in 70% ethanol (100 w/v). The homogenate was concentrated and dissolved in a solvent containing diethyl ether ethanol and water (10:1:9). The aqueous layer was further extracted progressively through chloroform ethylacetate and butanol. The final aqueous layer, the aqueous extract, was freeze-dried, dissolved in PBS prior to use.

30 **FIGURE 2** shows the cytotoxic effect of *P. oleracea* extract on KATO III cells.

KATO III cells (2.5×10^4) in 100 μ l of media in 24 well plate were cultured

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for 48 hours *P. oleracea* extract was added and incubated for 24 hours.

Live cells were evaluated by a trypan blue incorporation assay. Figure 2A:

P. oleracea extract-treated, Figure 2B: no extract-treated control.

5 **FIGURE 3** is the dose and time-dependent cytotoxic effect of *P. oleracea* extract on KATO III cells. KATO III cells (2.5×10^4) in 100 μ l of media in 24 well plate were cultured for 48 hours. *P. oleracea* extract at different concentrations was added and incubated for 24, 48, 72 and 96 hours and the percent of cell death calculated.

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FIGURE 4 is the cell-specificity of cytotoxic effect of *P. oleracea* extract. KATO III, COLO 320 HSR, L929, or WI38 cells (2.5×10^4) in 100 μ l of media in 24 well plate were cultured for 48 hours. *P. oleracea* extract (0.4 mg/ml) was added and incubated for 24, 48, 72, and 96 hours and the percent of cell death calculated.

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FIGURE 5 is the total protein analysis of KATO III cells after treatment of *P. oleracea* extract. KATO III cells (1×10^6) were collected 16 hours after incubation with *P. oleracea* extract at various concentrations (0.4, 0.6, 0.8, 1.6, 3.2 mg/ml). Lysates were prepared and separated on 10% SDS-PAGE. The proteins were stained with Coomassie Blue. Lane M: molecular weight standard in KD, Lane 1: no serum, Lane 2: no extract, Lane 3: 0.2 mg/ml, Lane 4: 0.4 mg/ml, Lane 5: 0.8 mg/ml, Lane 6: 1.6 mg/ml, Lane 7: 3.2 mg/ml of *P. oleracea* extract-treated. Arrows indicate the protein bands that were decreased by the treatment of *P. oleracea* extract.

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FIGURE 6 shows DNA fragmentation analysis of KATO III cells after treatment of *P. oleracea* extract. KATO III cells (1×10^6) were collected 16 hours after incubation with various concentrations of *P. oleracea* extract. The genomic DNA was isolated and electrophoresed on 1.5% of agarose gel. DNA was stained with ethidium bromide. Lane M: molecular weight standard in Kb,

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Lane 1: no extract treated control, Lane 2: 0.2 mg/ml, Lane 3: 0.4 mg/ml, Lane 4: 0.8 mg/ml, Lane 5: 1.6 mg/ml of *P. oleracea* extract-treated.

FIGURE 7 is an electron microscopic picture of KATO III and COLO 320 HSR cells after treatment of *P. oleracea* extract. KATO III (Figure 7A) or COLO 320 HSR cells (Figure 7B) were cultured for 24 hours with *P. oleracea* extract (0.6 mg/ml) and fixed. Magnification 18000x.

FIGURE 8 is an electron microscopic picture of L929 (Figure 8A) or WI38 (Figure 8B) cells after treatment of *P. oleracea* extract. L929 or WI38 cells were incubated for 24 hours with *P. oleracea* extract (0.6 mg/ml) and fixed. Magnification 15000x.

FIGURE 9 is the inhibition of tumor growth after treatment of *P. oleracea* extract in CD1 nude mice induced by COLO 320 HSR cells. Six-week-old CD1 nude mice received COLO 320 HSR cells (1×10^8) and *P. oleracea* extract (5mg/day/mouse; 0.05 mg/mg/day) for 10 days (right side). As a control, the mice received COLO 320 HSR cells (1×10^7) and same volume of 10% ethanol without *P. oleracea* extract (left side). The picture was taken at 30 days after tumor cell injection.

FIGURE 10 is the picture of isolated tumor after treatment of *P. oleracea* in CD1 nude mice induced by COLO 320 HSR cells. The tumor was isolated from tumor-induced CD1 nude mice which was treated with *P. oleracea* extract and the picture was taken as described in the legend to Figure 9. Figure 10A, left side: no extract-treated control, right side: extract-treated with 0.05 mg/gm/day aqueous extract. Figure 10B, left side: no extract-treated control, right side: extract-treated with 0.25 mg/gm/day of the aqueous extract.

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FIGURE 11 is an outline of the experimental protocol of the *in vivo* treatment of a stomach cancer patient treated with *P. olearcea* extract.

FIGURE 12 is a light micrograph of stained stomach cells obtained from a stomach cancer patient before treatment (**FIGURE 12A**) or after 8-12 months treatment (**FIGURE 12B**) with 0.007-0.015 mg/gm/day *P. oleracea* extract.

FIGURE 13 is a gastroscopic photograph obtained of the atrium of the stomach of a stomach cancer patient before treatment (**FIGURE 13A**) and after 8-12 months treatment (**FIGURE 13B**) with 0.007-0.015 mg/gm/day *P. oleracea* extract.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention is directed to the tumoricidal effect of *P. oleracea* extracts. This invention also provides for pharmaceutical compositions and related methods for the use of the extract from *P. oleracea* as an anti-cancer agent.

According to the present invention, by "aqueous extract" or "extract" it is meant the aqueous fraction following the extraction of *P. oleracea*. Such an aqueous fraction may be obtained following one or more solvent extraction steps of plant or leaf tissue.

By "tumoricidal activity" it is meant the selective cytotoxic effect of the *P. oleracea* aqueous extract on tumor cells, exemplified by gastric carcinoma and colon adenoma cell lines, but not non-tumor cells, exemplified by lung connective tissue, and lung diploid cell lines. Based on the action of the oncogenes involved in the cancerous development in these cells, the aqueous extract would also have an effect on tumoricidal activity in tumors associated with liver and kidney cells as well.

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It is widely known that *P. oleracea* is a very valuable plant for many different medicinal purposes and as a supply of various nutrients (Tulloch, 1975, Verma et al., 1982. Parry et al., 1987, Simopoulos et al., 1992, Parry et al., 1993). In our search for therapeutic agents from natural sources with a potential for the treatment of cancer, we investigated anti-tumor activities of *P. oleracea*. We found that *P. oleracea* extract has an anti-tumor activity on tumor cell lines such as COLO 320 HSR and KATO III. *P. oleracea* extract showed dose and time-dependent cytotoxic activity on these cells (Figure 3). However, *P. oleracea* extract did not have cytotoxic effect on normal fibroblast cell lines such as L929 and WI38 (Figure 4). This result suggests that *P. oleracea* extract has a specific cytotoxic effect on tumor cells, but not normal cells. When the cytotoxic activity of *P. oleracea* extract was compared with other plant extracts, *P. oleracea* showed the strongest tumoricidal activity among 5 plant extracts that were tested (see Example 1).

15 Mechanism of cell death

Apoptosis is a distinct form of cell death (William et al., 1991, Kerr et al., 1994) and a variety of anti-cancer drugs have been shown to induce extensive apoptosis (Hickman, 1992). In order to study the precise mechanism of anti-tumor effect of *P. oleracea* extract, inter-nucleosomal DNA fragmentation of KATO III cells, after treatment with the extract, was examined since DNA fragmentation is one of the hallmarks of apoptosis (Anderson et al, 1994). However, no fragmentation of genomic DNA was shown (Figure 6). We also observed the morphology of extracted-treated cells and found that the cells showed cytoplasmic vacuoles with disintegrated cell membrane, which is different from the morphology of apoptosis.

The morphological changes were shown to be similar to the effect of dopamine on lymphoid tumor cell lines, which revealed cytoplasmic swelling and vacuolization and nuclear pycnosis (Braesch-Anderson et al., 1992). *P. oleracea* contains dopamine as a major constituent. It may also be possible that dopamine

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plays a role as an active compound for the anti-tumor effect. Thus we further examined the mechanism of cytotoxic effect of this plant extract. When we analyzed protein synthesis of KATO III cells after treatment of *P. oleracea* extract, total protein synthesis was generally decreased. Specifically, three proteins with a
5 molecular weight of 85,000 (P85), 64,000 (P64), and 40,000 (P40) daltons were decreased in amount (Figure 5), and likely to be inhibited in a dose dependent manner of *P. oleracea* extract. This result imply that these proteins might be involved in tumor cell proliferation.

10 Based on these observations, and without wishing to be bound by theory, it appears that *P. oleracea* has a specific cytotoxic effect on tumor cells *in vitro* and *in vivo*. The mechanism of action appears to be due to a cell-specific cytotoxic effect.

In vivo Studies

15 Whether *P. oleracea* extract could also inhibit tumor growth *in vivo* was also examined. Nude mice were induced to form tumors with COLO 320 HSR cells. In mice treated with COLO 320 HSR cells and with a *P. oleracea* extract, tumor growth was clearly inhibited (Table 2). The treatment with 5 mg of *P. oleracea*
20 extract per day for 10 days depressed the size of tumor in the range of 0.1 cm at 30 days after the tumor cell inoculation. In contrast, the tumor size of untreated animals was in the range of 1.4 cm. The treatment with 1 mg per day showed less effect on tumor regression. This result shows that the greater the amount of *P. oleracea* extract, the greater the inhibition of tumor growth *in vivo*.

25 The bio-active ingredient of the water-extract obtained from *P. oleracea* can be administered to effect treatment of tumor cells. Such a bio-active ingredient would be formulated as a pharmaceutical composition in the presence of a suitable carrier such as those used for the preparation of, but not limited to, solid,
30 particulate or suspension formulations for tablets, capsules, elixir, parenteral, suppository, transdermal or topical administration. Suitable carriers include, but

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are not limited to magnesium stearate, starch, lactose, sucrose and cellulose for the formulation of tablets; aqueous gums, celluloses, silicates or oils and the dispersion or suspension of bio-active ingredient into gelatin capsules; liquid carriers including syrups, glycerin, peanut or olive oil, ethanol, saline and water in the case of elixirs; 5 parenteral compositions could be formulated with a solution or suspension of the bio-active ingredient in a sterile aqueous carrier or parenterally acceptable oil such as polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil, sesame oil or the like; binding, and /or lubricating agents, for example polymeric glycols, gelatins or coca butter or other low melting vegetable or synthetic waxes or fats in the case of 10 suppository compositions; aqueous or non-aqueous vehicles such as creams, ointments, liposome preparations, lotions, paste or medicated plaster, patch or membrane or the like for typical transdermal applications; and, for topical administration, pharmaceutical typically formulations include solutions, suspensions, ointments and solid inserts for example water, water miscible solvent 15 mixtures including lower alkanols, or vegetable oils, emulsifying, preserving wetting and bodying agents such as polyethylene glycols, and antibacterial components. Furthermore, the bio-active ingredient may be administered in a timed release dosage unit form to permit sustained release of the bio-active ingredient. Such carriers may include glyceryl monostearate or glyceryl distearate, alone or in 20 combination with a wax.

The pharmacokinetic properties of the bio-active component must be determined to formulate dosing regimes that induce tumoricidal activity without significant adverse side effects. The bio-active ingredient may also be administered 25 singly or in combination with other bio-active agents of interest.

Suitable dosages of the bio-active ingredient of *P. oleracea* in a suitable pharmaceutical carrier for use in the treatment of tumorous cell growth in mammals including humans, according to the present invention, should not exceed about 1.0 30 mg/gm body weight. Preferably the dosage is in the range of 0.005-0.5 mg/gm body weight. It will be appreciated that the actual preferred dosages of the bio-

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active ingredient being administered will vary according to the particular composition formulated, the site and host being treated, and the mode of administration.

5 **Examples**

Animals

10 Six week-old CD1 nude mice (nu/nu) were purchased from Charles River, St-Constant, Quebec, Canada and maintained in a specific pathogen free (SPF) facility at the University of Calgary.

Extraction of *P. oleracea*

15 Whole plants were washed, sliced into small pieces, and extracted as shown in Figure 1. Briefly, the sliced plant was homogenized in 75% ethanol at a ratio of 10% (w/v). The homogenates were filtered and concentrated using a rotatory evaporator. The concentrated extract was dissolved in a solvent containing diethyl ether, ethanol and water at the ratio of 10:1:9, and then partitioned between diethyl
20 ether and water. The aqueous layer was further extracted using chloroform, ethylacetate, and butanol in an orderly fashion. The separated final aqueous layer was freeze-dried. This aqueous extract of *P. oleracea* was dissolved in phosphate buffered saline (PBS) and filtered through a 0.22 μ m membrane filter.

25 **Cell Culture**

 KATO III (human gastric carcinoma cell line), COLO 320 HSR (human colon adenocarcinoma cell line), WI38 (human lung diploid cells), and L929 (murine lung connective tissue) were obtained from the American Type Culture
30 Collection (ATCC). The cells were grown in RPMI 1640 (Gibco BRL,

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Gaithersberg, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained in a 5% CO₂ incubator at 37°C.

Cytotoxicity Assay

5

Growth of tumor cells was evaluated by a trypan blue incorporation assay. In brief, KATO III cells in 100 µl of media (2.5x10⁵ cells/ml) were placed in a 24 well microtest plate (Falcon no:3047, Beckton Dickinson Co., Lincoln Park, NJ) and cultured in a 5% CO₂ incubator at 37°C for 48 hours. Extracts of *P. oleracea*,
 10 *Taraxacum platicarpium*, *Fagopyrum esculentum*, *Prunella asiatica*, *Veratrum album* were added to the cell suspension and incubated the cells for 1 to 4 days. The cells were centrifuged and the cell pellet was suspended in 100 ul of RPMI medium and 0.2% trypan blue solution. Untreated cells were used as a control. The number of dead cells was counted under a microscope and calculated using the
 15 following equation:

$$\% \text{ of cell death} = \frac{\text{No. of stained cells}}{\text{No. of total examined cells}} \times 100$$

20 Electron Microscopy

To examine morphological changes, the cells treated with or without *P. oleracea* extract were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4°C for 1 hour and washed 2 to 3 times with the same buffer to remove excess aldehyde prior to second fixation. One percent of osmium tetroxide (OsO₄) in 0.1
 25 M cacodylate buffer was used for post fixation at 4°C for 1 hour. These fixed cells were dehydrated using a series of concentrations of ethanol to remove all free water, then embedded in Epon epoxy resin, and polymerized at 45°C in an oven for 18 to 24 hours. Ultrathin sections were made by cutting with an ultramicrotome (Riechert-Jung, Austria). Sample sections were stained with uranyl acetate and lead
 30 citrate, and examined with a Hitachi H-7000 electron microscope (Hitachi LTD, Kaka works, Katsuda city, Japan).

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Protein Analysis

KATO III cells (1×10^6 cells) were harvested 16 hours after incubation with *P. oleracea* extract at various concentrations (0.2, 0.4, 0.8, 1.6, 3.2 mg/ml). After washing with PBS, the cells were pelleted and dissolved in 40 μ l of lysis buffer (4% SDS, 73 mM Tris-Cl, pH 6.8, 10 mM DTT). The cell lysates were centrifuged at 55,000 rpm (Ti 100, Beckmann) for 1 hour at 4°C. After taking out the supernatant, protein concentration was measured using BioRad protein assay kit (BioRad, Richmond, CA, USA). The protein samples were mixed with dye solution (0.25% bromophenol blue, 25% Ficoll®), boiled for 2 mins. and loaded on a 10% SDS-polyacrylamide gel. After gel electrophoresis, protein bands were visualized by staining with a 0.1% Coomassie Blue solution.

DNA Fragmentation Analysis

KATO III cells (1×10^6) were collected 16 hours after treatment of various concentrations of *P. oleracea* extract (0.2, 0.4, 0.8, 1.6 mg/ml). The cells were washed with PBS, centrifuged at 200xg for 10 mins. and lysed in a lysis buffer (0.5% sodium lauryl sarcosinate, 0.5 mg/ml proteinase K, 10 mM EDTA and 50 mM Tris-Cl, pH 7.5). The samples were extracted once with phenol/chloroform and then chloroform. Genomic DNA was precipitated with 2 volumes of ethanol in the presence of 3M sodium acetate (pH 5.2) and washed with 70% ethanol. The pellet was dried in a vacuum drier and dissolved in TE (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). RNA was removed by RNase (20 μ g/ml) treatment at 37°C for 1 hour and 3 μ g of DNA was loaded on a 1.5% (w/v) agarose gel. Electrophoresis was carried out and DNA was visualized by ethidium bromide staining.

In Vivo Treatment using *P. oleracea* Extract

CD1 nude mice (5 mice/group) were injected subcutaneously in the neck with a single dose of COLO 320 HSR cells (1×10^7 cells). The extract of *P.*

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oleracea (1 mg or 5 mg in 100 μ l of 10% ethanol) was given intraparenteraly from the same day for 10 days consecutively. Control mice (n=5) were injected with the same volume of 10% ethanol. The size of tumors was measured on the day of 10, 20, 25, and 30 after tumor cell injection.

5

Example 1: Cytotoxic Effect of *P. oleracea* Extract on Tumor Cells *in vitro*

To determine whether or not a *P. oleracea* extract had any effect on cultured tumor cells, cytotoxic activity of *P. oleracea* extract against KATO III cells was
10 examined at different incubation hours after treatment. *P. oleracea* extract contains a tumoricidal activity against KATO III cells (Figure 2 and 3). Most of the extract-treated cells were dead (Figure 2B) compare to non-treated cells (Figure 2A). The incubation of these cells with *P. oleracea* extract caused cell death in a dose-dependent and time-dependent manner (Figure 3). The extract at the
15 concentration of 0.6 mg/ml for 24 hours of incubation killed 74% of KATO III cells for 48 hours 92% and 72 hours 100%. The higher concentration of the extract showed higher cytotoxic activity. To compare the cytotoxic activity of *P. oleracea* with other plant extracts we tested the cytotoxic effect of several different kinds of plant extract on KATO III cells. *P. oleracea* extract showed the highest inhibitory
20 activity against KATO III cells showing 100% of cell killing after 24 hours of incubation. Under the same condition, *Taraxacum platicarpurn*, *Fagopyrum esculentum*, *Prunella asiatica*, and *Veratrum album* showed 0, 8, 18, 32% of cell death, respectively. We also examined the specificity of the cytotoxic effect of *P. oleracea* extract on different tumor cells. *P. oleracea* extract killed KATO III cells
25 (70%) and COLO 320 HSR cells (80%), but did not greatly affect much L929 and WI38 cells showing less than 20% of killing (Figure 4).

Example 2: Mechanism of Action

30 To see whether *P. oleracea* extract has any effect on the specific inhibition of protein synthesis of tumor cells which may lead to cell death, we examined the

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pattern of protein synthesis after the treatment of KATO III cells with the extract (Figure 5). In general, the protein synthesis was significantly decreased at the increased dosage of *P. oleracea* extract. Three specific proteins i.e. P40, P64, and P85 were apparently inhibited at dosages of 0.8 and 1.6 mg/ml of the extract. This result suggests that these proteins may be involved in the proliferation of tumor cells.

To see whether *P. oleracea* extract-induced cell death is due to apoptosis, we examined the fragmentation of genomic DNA of KATO III cells after the treatment of the extract since DNA fragmentation is one of the hallmarks of apoptosis. We found that there is no fragmentation of genomic DNA regardless of duration of the treatment and dosage of the extract (Figure 6). Without wishing to be bound by theory, this result indicates that KATO III cell death may not be due to apoptosis but probably due to cell-specific cytotoxic effects.

The cell-specific cytotoxicity was confirmed by electron microscopic examination. The extract-treated KATO III cells revealed numerous cytoplasmic vacuoles of various sizes with disintegrated membrane (Figure 7), while the same extract-treated L929 or WI38 cells did not show any morphological changes (Figure 8).

Example 3: Inhibition of Tumor Growth by *P. oleracea* extract *in vivo*

To examine the anti-cancer effect of *P. oleracea* extract *in vivo*, six week-old CD1 nude mice (nu/nu) were injected subcutaneously in the neck with COLO 320 HSR cells and subsequently *P. oleracea* extract (5 mg/day) daily for 10 days. We found that the mice showed only minimum sign of tumors (0.02cm at 10 days, 0.05cm at 20 days, 0.1cm at 30 days after injection) (Figures 9 and 10 and Table 2). In contrast, the control mice which received COLO 320 HSR, but not treated *P. oleracea* extract, developed distinct tumors by the 10th day which continued growing. The mean size of tumor was 0.53 cm at 10 days. 1.24 cm at 20

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days, and 1.45 cm at 30 days after injection. This result indicates that *P. oleracea* extract clearly inhibits tumor growth *in vivo*.

Table 2***Effect of Portulaca oleracea on a tumor growth***

Days after tumor cell injection	Tumor Size (cm)			
	Untreated positive control mice	1 mg-treated mice (0.05mg/gm)	5 mg-treated mice (0.25 mg/gm)	10 mg-treated mice (0.5 mg/gm)
10	0.53	0.12	0.02	0.0
20	1.24	0.31	0.05	0.0
30	1.45	0.50	0.10	0.0

Nude mice (5 mice/group) were injected subcutaneously in the neck with COLO 320 HSR cells (1×10^8). The value is the mean of the tumor sizes of five samples/group.

Example 4: Treatment of Human Stomach Tumor Growth using *P. oleracea* extract.

A stomach cancer patient at the terminal stage of illness was administered *P. oleracea* extract for 8-12 months (see protocol, Figure 11). The dosage of *P. oleracea* administered during this treatment period was between 500-1000 mg/day (0.007 - 0.015 mg/gm body weight/day).

Histological examination of biopsied tissues obtained from cancerous lesions of the stomach prior to treatment with *P. oleracea* revealed abortive granular cells composed of large hyperchromatic nuclei with clear eosinophilic cytoplasm. Furthermore, these cells floated in abundant extracellular mucous see Figure 12a. Following the treatment period, histological examination revealed normal stomach cells (Figure 12b).

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Gastroscopic examination of the stomach of the cancer patient prior to treatment with *P. oleracea* indicated irregular, marginated ulcerating and partial infiltrating lesions in the atrium (Figure 13a). Following treatment with *P. oleracea* (Figure 13b) the atrium appears normal.

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This result demonstrates the efficacy of *in vivo* tumor cell treatment using *P. oleracea* extract.

10 All scientific publications and patent documents are incorporated herein by reference.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a
15 number of variations and modifications can be made without departing from the scope of the invention as described in the following claims.

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- 20 -

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A bio-active ingredient form *Portulaca oleracea* having tumoricidal activity.
2. A composition comprising the bio-active ingredient of claim 1 as an aqueous extract obtained from *Portulaca oleracea*.
3. The composition of claim 2 wherein the aqueous extract is obtained following homogenization in an alcohol and extraction by at least one solvent.
4. The composition of claim 2 wherein the tumoricidal activity is observed in gastric tumor cells.
5. A method for the preparation of the bio-active ingredient of claim 1 involving extracting *P. oleracea* in a solvent and retaining the aqueous fraction.
6. The method of claim 5 wherein the solvent is ethanol and the retained aqueous fraction is further extracted with diethyl ether, chloroform, ethylacetate or butanol or a combination of thereof.
7. A pharmaceutical composition comprising the bio-active ingredient of claim 1 and a suitable carrier.
8. A method for the prevention of tumour cell development by administering a suitable amount of the bio-active ingredient of claim 1 in such a manner that the bio-active ingredient can exert tumoricidal activity on developing tumor cells.

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9. The method of claim 8 wherein the developing tumor cells are stomach tumor cells.
10. The method of claim 8 wherein the developing tumor cells are human tumor cells.
11. A method for the prevention of tumour cell development by administering a suitable amount of the bio-active ingredient of claim 7 in such a manner that the bio-active ingredient can exert tumoricidal activity on developing tumor cells.
12. The method of claim 11 wherein the tumor cells are developing stomach tumor cells.
13. The method of claim 11 wherein the developing tumor cells are human tumor cells.
14. A method for the treatment of tumour cells by administering a suitable amount of the composition of claim 7 to a patient in need thereof.
15. The method of claim 14 wherein the pharmaceutical composition is administered at a range from about 0.005 mg/gm body weight to about 0.5 mg/gm body weight.
16. The method of claim 15 wherein the pharmaceutical composition is administered at a dose of about 0.007mg/gm body weight to about 0.015 mg/gm body weight.
17. A method for the treatment of tumour cells by administering a suitable amount of the bioactive ingredient of claim 1 to a patient in need thereof.

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18. The method of claim 17 wherein the bioactive ingredient is administered at a range from about 0.005 mg/gm body weight to about 0.5 mg/gm body weight.
19. The method of claim 18 wherein the bioactive ingredient is administered at a dose of about 0.007 mg/gm body weight to about 0.015 mg/gm body weight.
20. The composition of claim 3 wherein the alcohol is ethanol, and the solvent comprises either diethyl ether, chloroform, ethylacetate, or butanol, or a combination thereof, and the aqueous extract is filtered before use.

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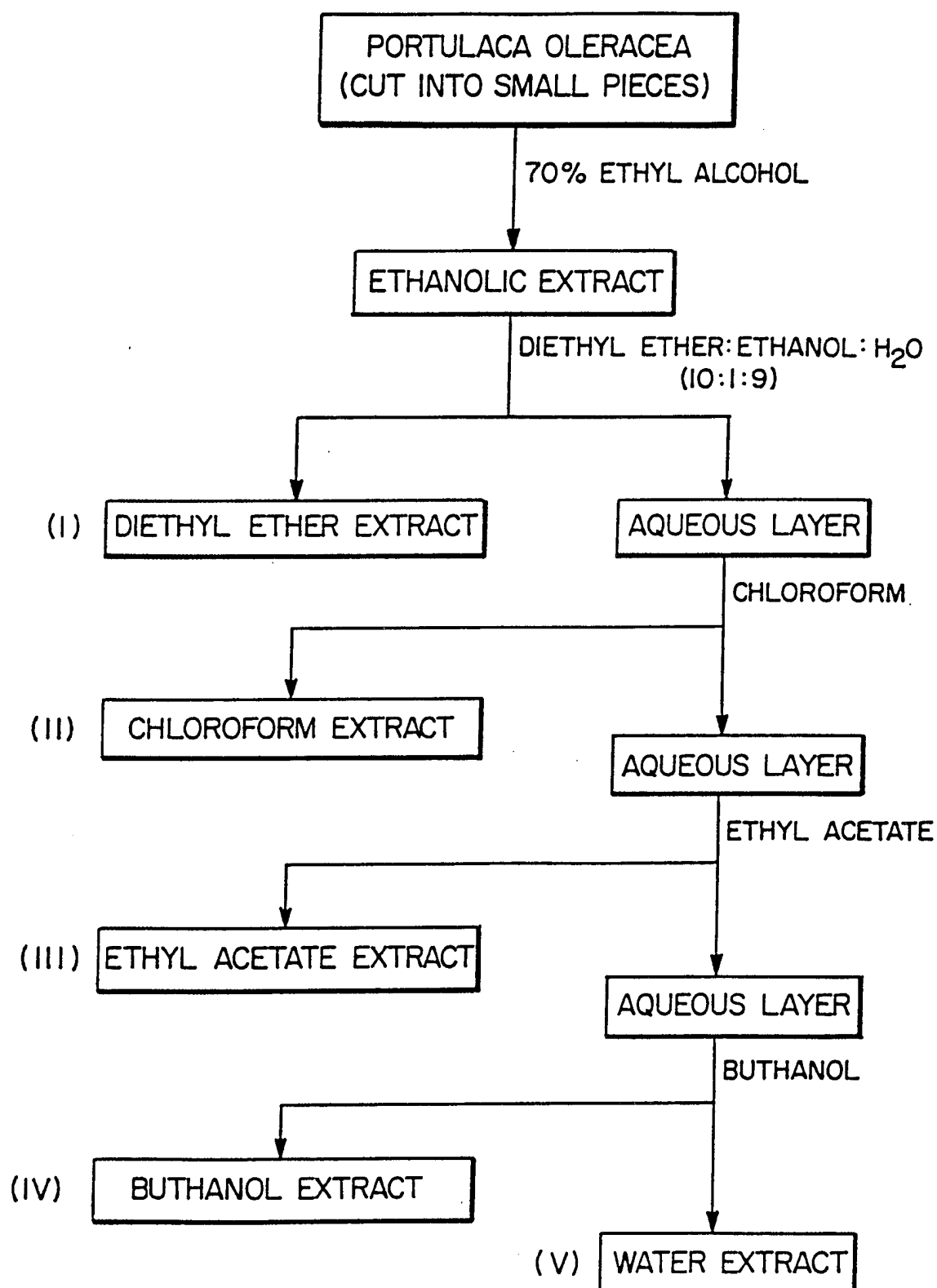


FIG. I

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Portulaca oleracea -treated stomach tumor cells

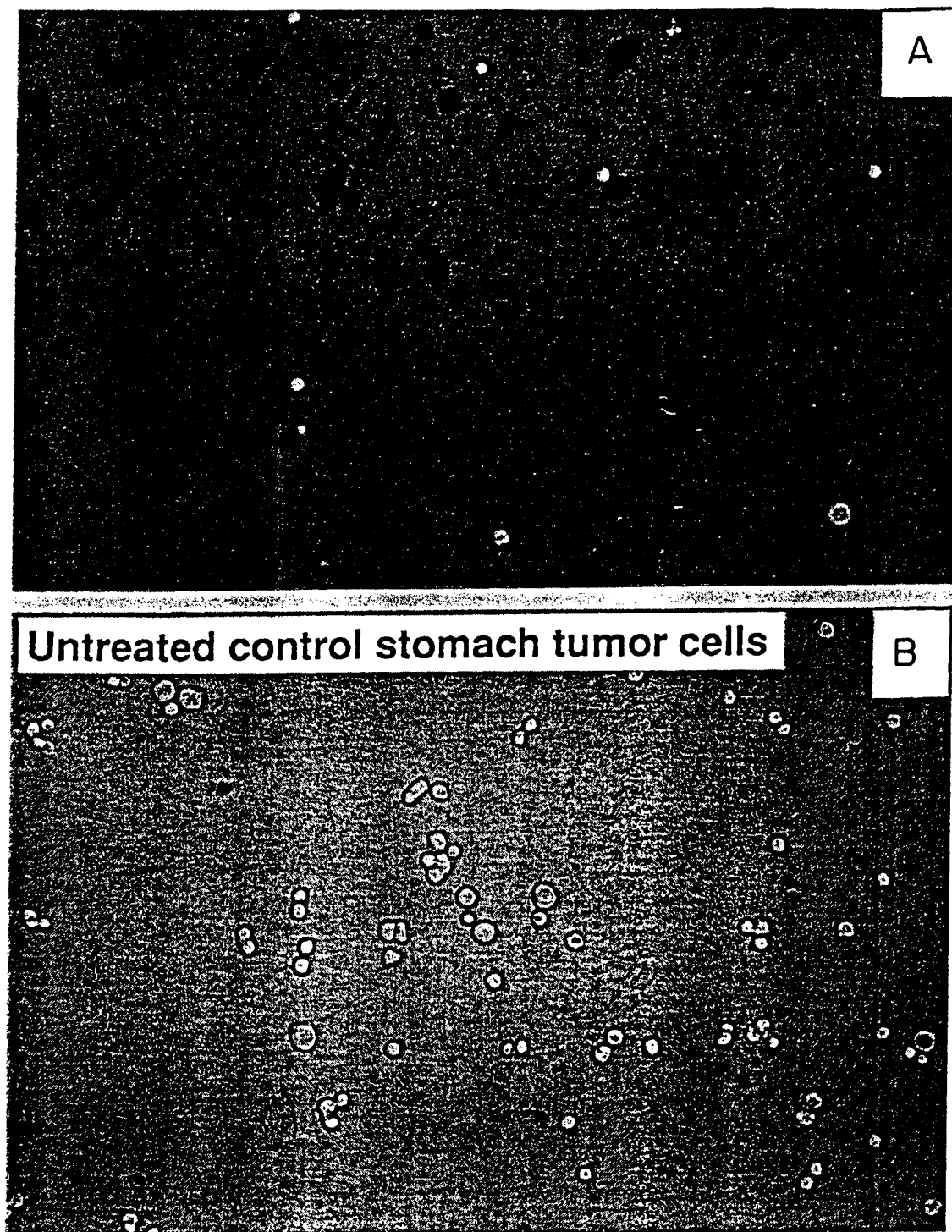


FIG. 2

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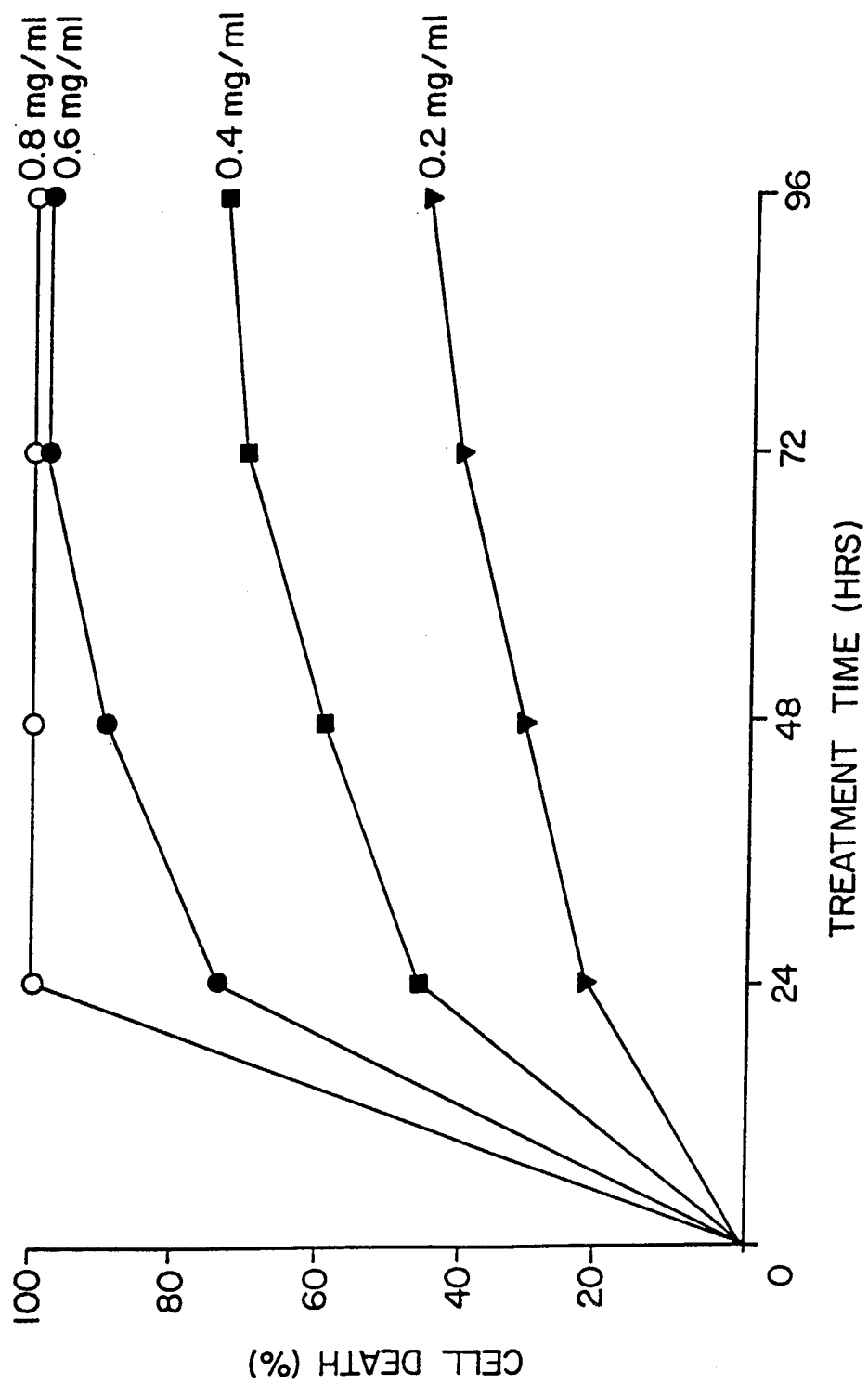


FIG. 3

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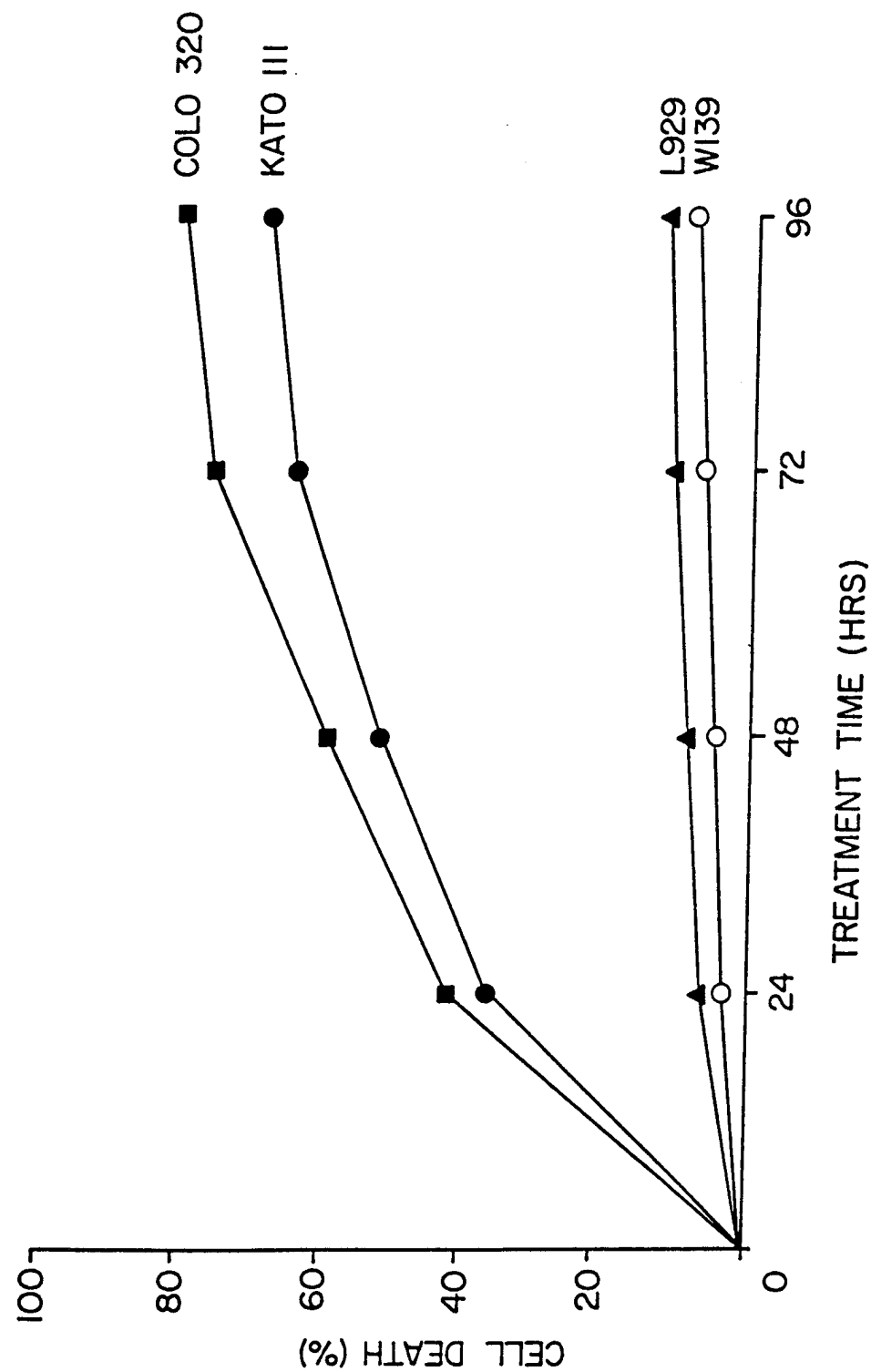


FIG. 4

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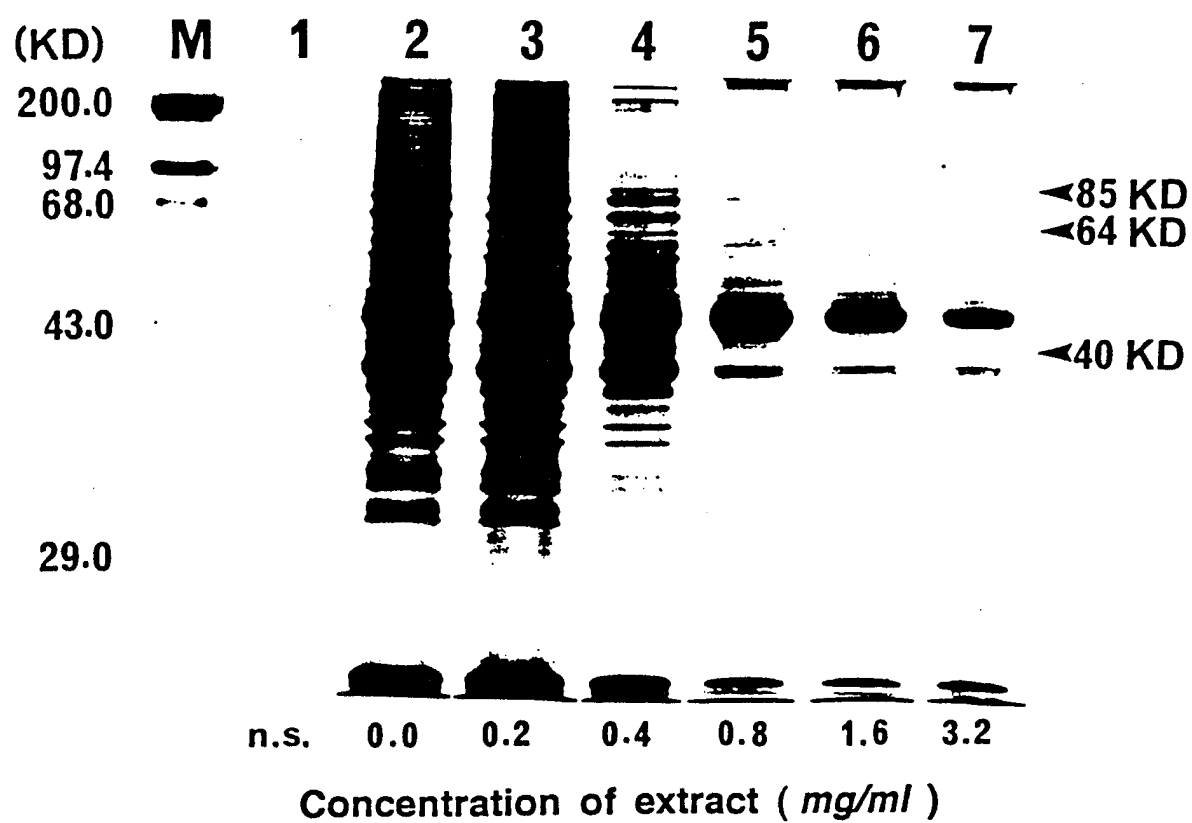


FIG. 5

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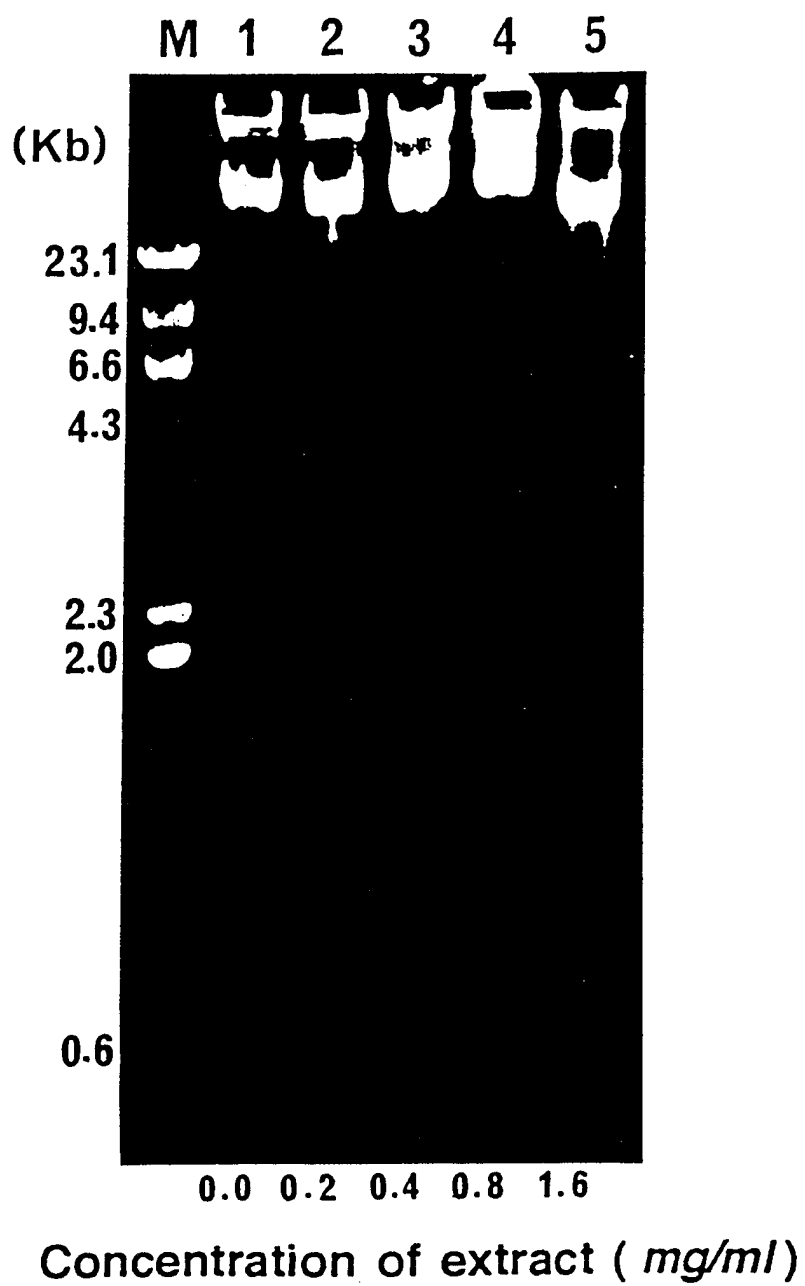


FIG. 6

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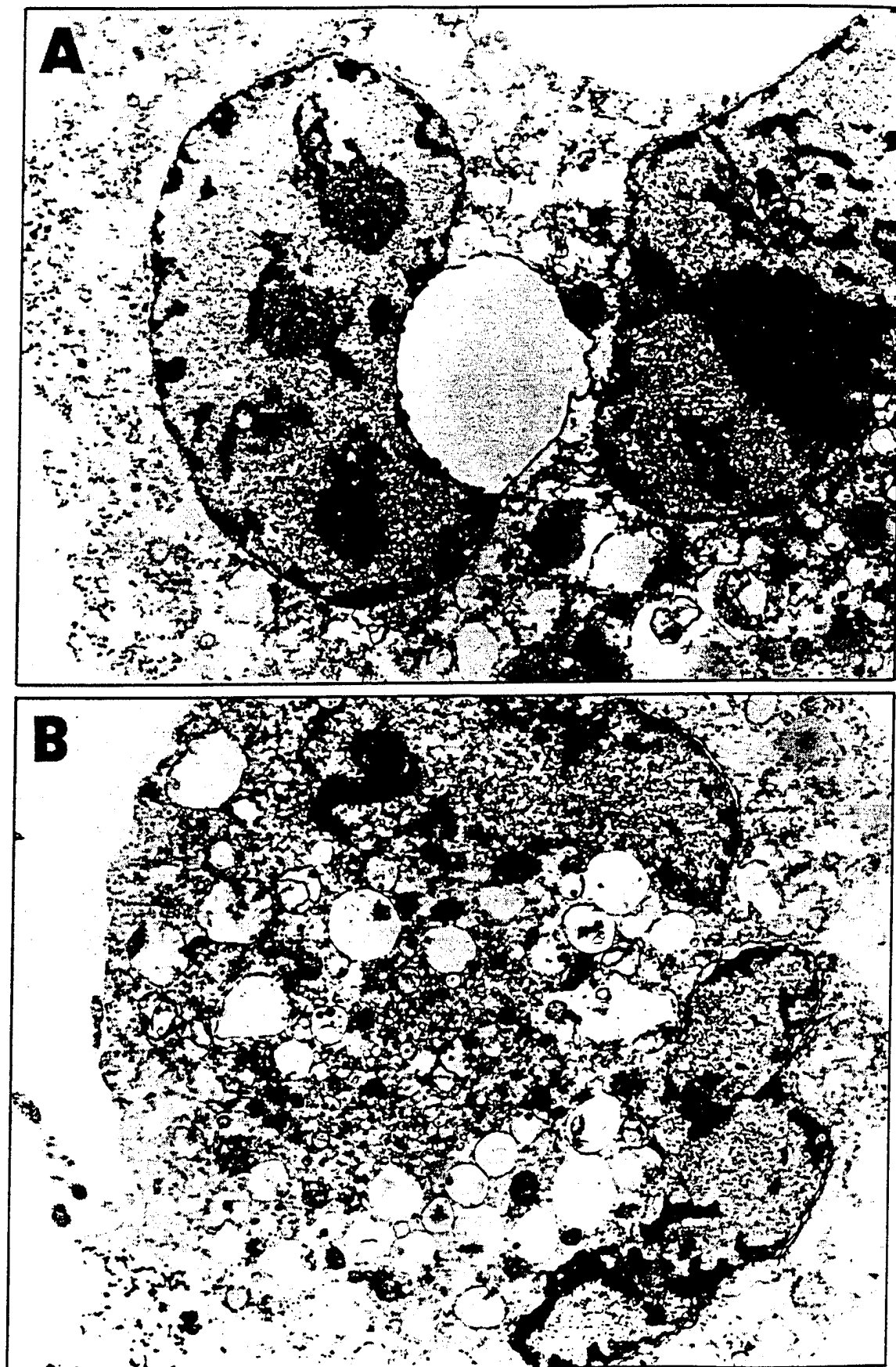


FIG. 7

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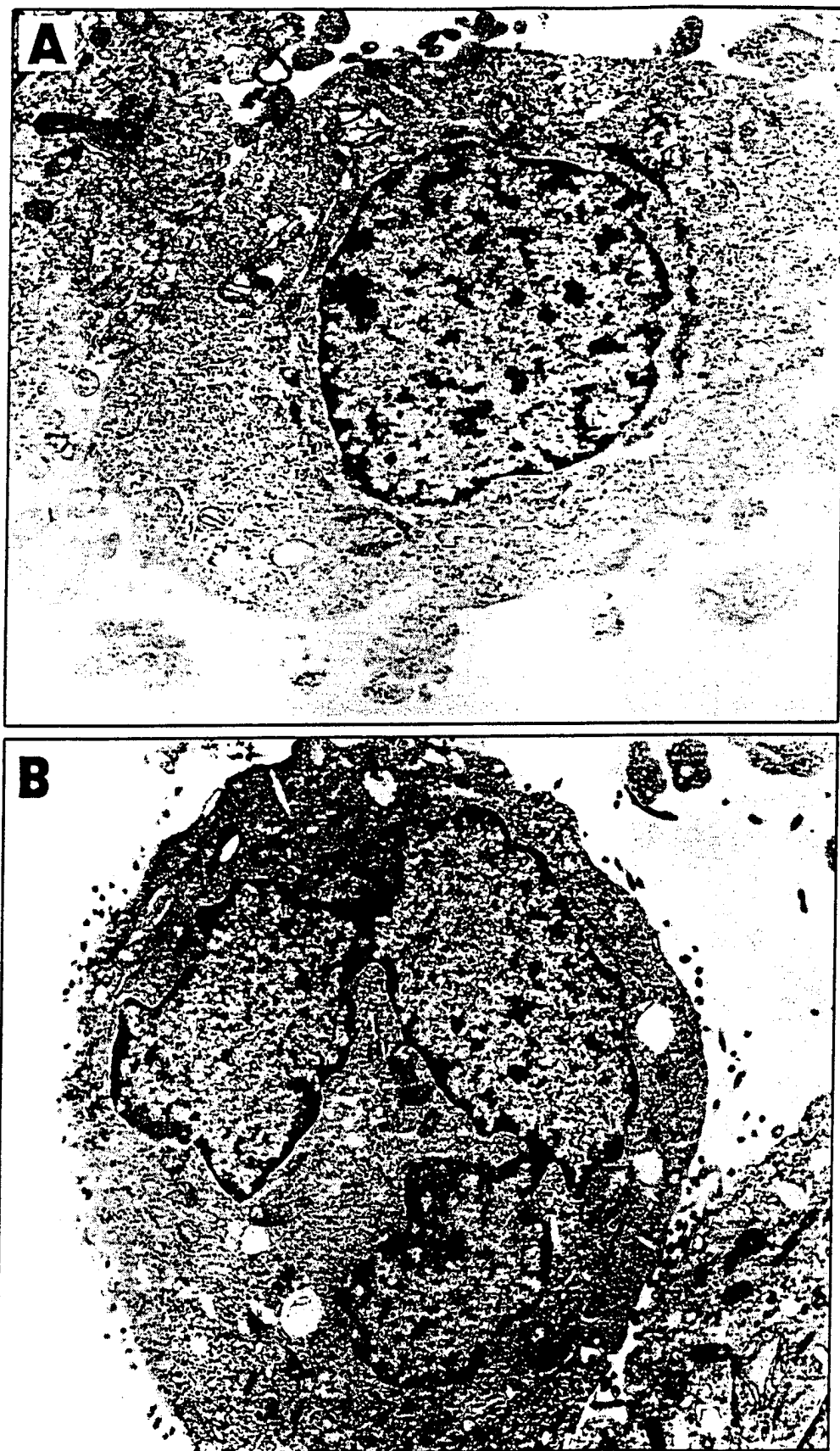


FIG. 8

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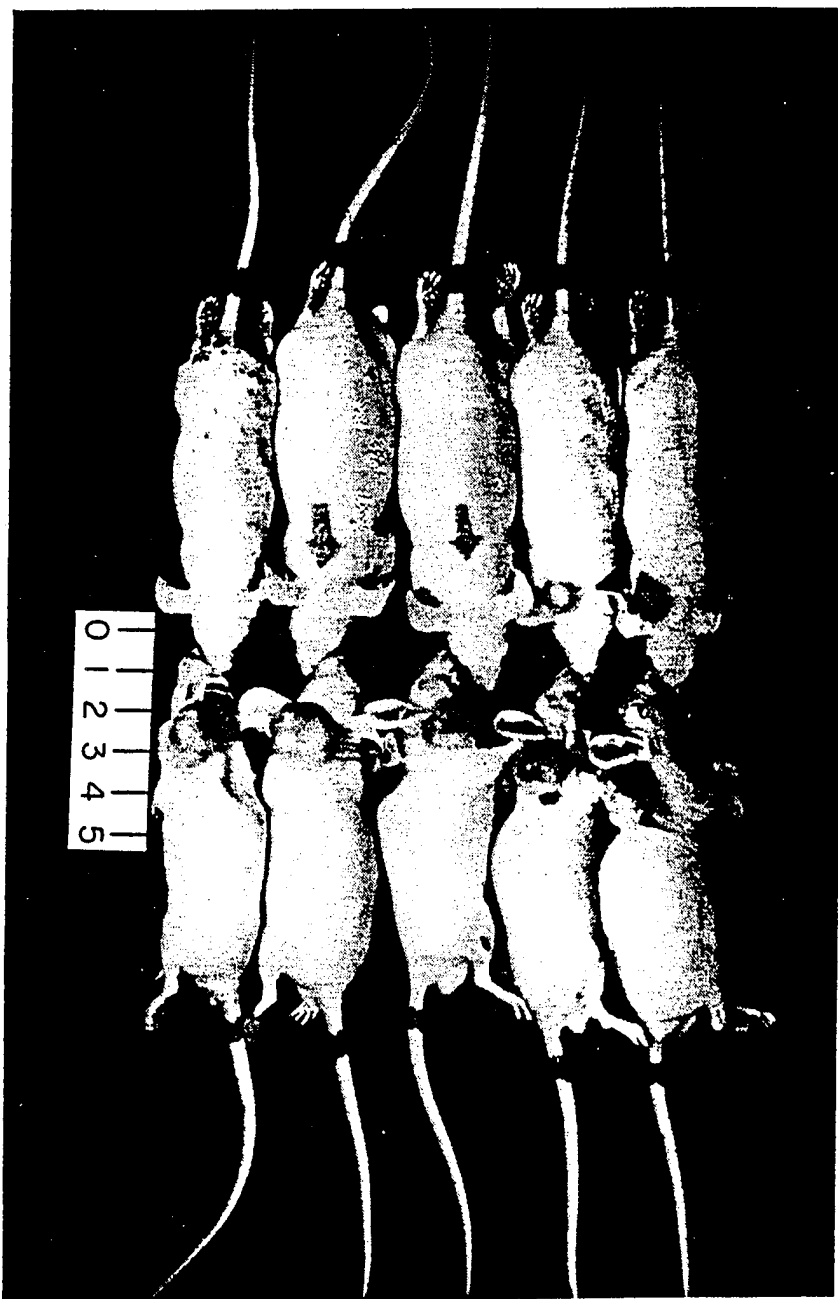


FIG. 9

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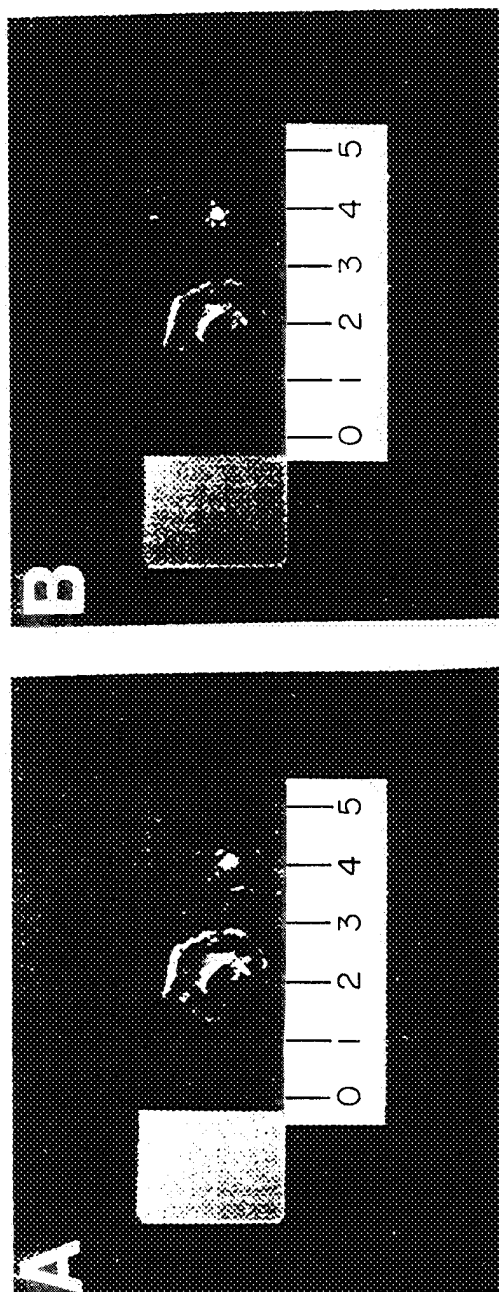


FIG. 10

EFFECT OF PORTULACA OLERACEA
ON STOMACH CANCER OF HUMAN

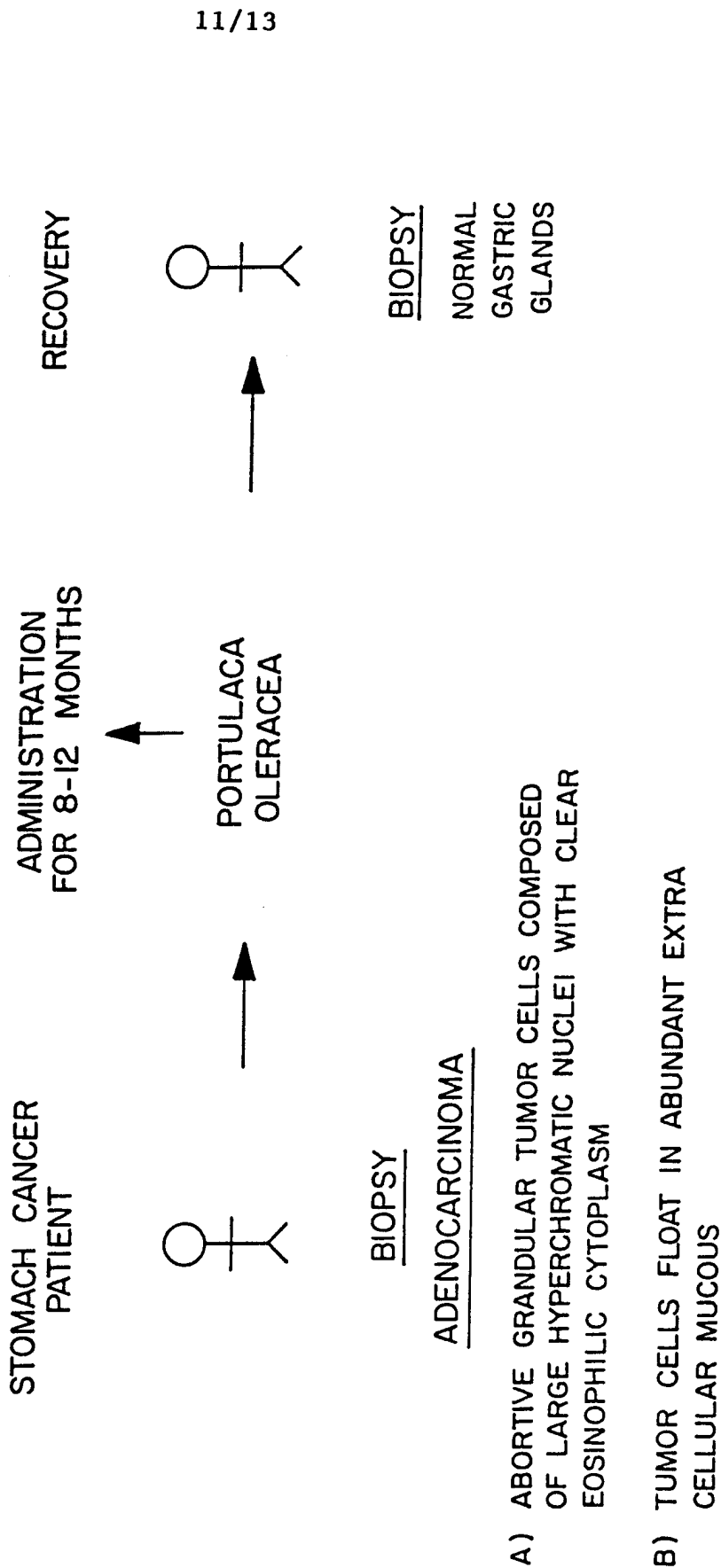


FIG. 11

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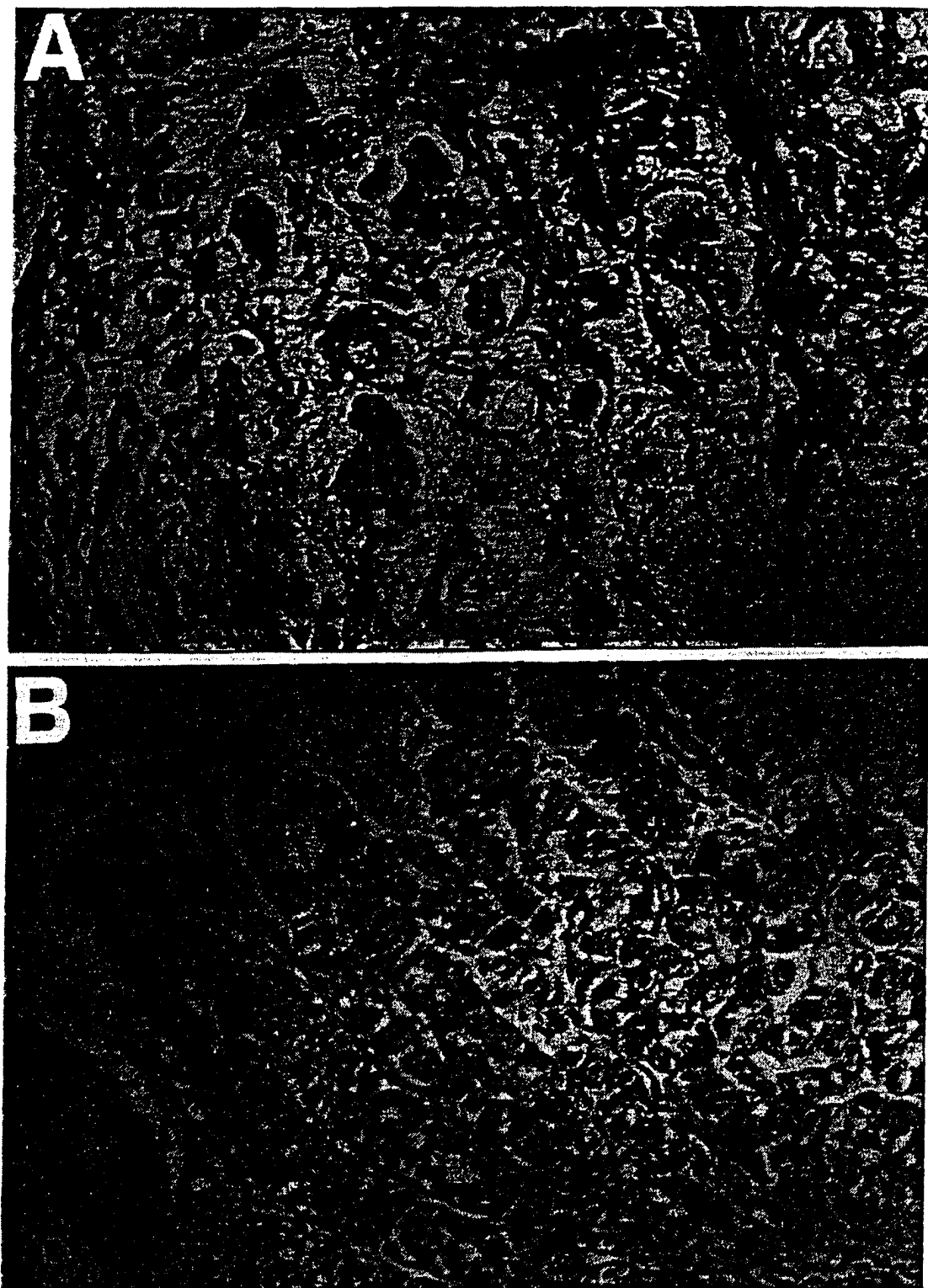


FIG. 12

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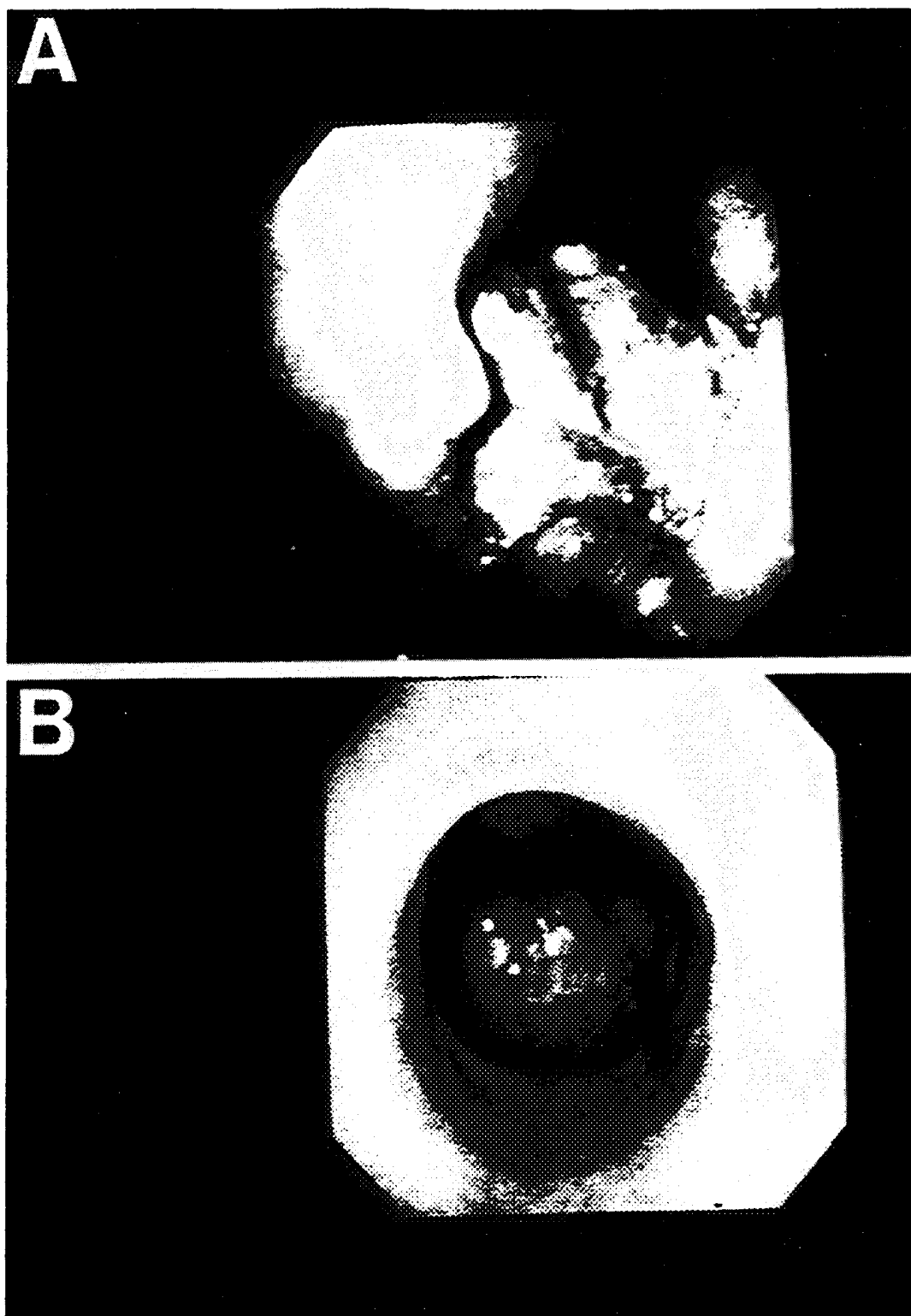


FIG. 13

INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 97/00873

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K35/78

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 6 A61K

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 practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 018, no. 291 (C-1208), 3 June 1994 & JP 06 056861 A (TETSUO IKEGAWA;OTHERS: 01), 1 March 1994, see abstract	1-20
A	GB 2 213 373 A (POON THEODORE MAN) 16 August 1989	

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

☒ Patent family members are listed in 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 document 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

13 March 1998

Date of mailing of the international search report

25.03.1998

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Rempp, G

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA 97/00873

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
see FURTHER INFORMATION sheet PCT/ISA/210
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 8-19

because they relate to subject matter not required to be searched by this Authority, namely:

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

Remark : Although claims 8-19 are directed to a method of treatment of the human/animal body , the search has been carried out and based on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 97/00873

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
GB 2213373 A	16-08-89	HK 53791 A	19-07-91
