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Cytotoxic Effect of Urushiol on Human Ovarian Cancer Cells

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Abstract Urushiol, a natural pro-electrophilic quinone compound, has potential structural characteristics as antitumor chemotherapeutic agents. However, urushiol's use as an antitumor drug has some problems, because it is hardly miscible with an aqueous solution. Purified urushiol is highly viscous and soluble only in strong solvents. For this study, we prepared an urushiol-ethanol micro-emulsion with a unimodal size distribution by high-speed homogenization. This generated effective delivery of urushiol to its action sites, so that we could investigate its cytotoxic activity against cancer cells. Using a colony-forming assay, we were able to show that urushiol selectively inhibited the growth of the ovarian cancer cells PA-1 and 2774 at a concentration of 10^{-6} M, whereas it had only a negligible effect on normal CHO cells at the same concentration. The data suggest that urushiol may have potential as an effective antitumor agent in the treatment of ovarian cancer. In addition, we addressed the question of whether the specific cytotoxic effect of urushiol is linked to apoptosis, by DNA fragmentation and DAPI staining assays. The inhibitory effects of urushiol on the growth of ovarian cancer cells was found to be associated with DNA fragmentation and the fragmented nuclei formation, both of which represent markers for the induction of apoptosis. Therefore, the results suggested that urushiol affected its profound cytotoxicity by triggering apoptosis in ovarian cancer cells.

Key words: Urushiol-ethanol micro-emulsion, cytotoxicity, ovarian cells

Urushiol has first been recognized as a contact allergen, since most cases of poison ivy/oak contact dermatitis arise following an exposure to the alkylcatechols, collectively known as urushiols [27]. The allergic contact dermatitis induced by the urushiol is known to be mediated by T lymphocytes that specifically recognize the hapten urushiol [18]. Production of cytokines, chemotactic factors, and adhesion molecules increases in response to contact with allergen exposure. In turn, this amplifies generation as well as migration of tumor cell specific immune cells and inflammatory cells [18, 25, 26]. Baldwin *et al.* found that a tumor immune response could be developed by administering the urushiols as highly potent hypersensitizers which localized in tumor cell membranes of animals [4]. Moreover, a recent study has revealed that the antigen-specific effector cells in allergenic contact dermatitis are very diverse and that this diversity might explain the continued reaction to some contact allergens in the late phase of HIV diseases [29]. These studies suggested that the urushiols could be used as immunostimulants in both cancer and AIDS patients.

Besides the immunostimulating effect, it is probable that urushiols have several characteristics of an antitumor chemotherapeutic agent. In oxidative metabolism, urushiols could be converted into reactive electrophilic quinone intermediates by oxidative enzymes as well as by reactive oxygen species such as oxygen biradicals, hydroxyl radicals, and superoxide, if the reductive components were provided by either specific or nonspecific reductases, glutathione, NAD(P)H, FADH, *etc.* [27, 8]. These reactive quinone intermediates share a common chemical structure with vitamin K, which has an antitumor activity against various human cancer cells [32]. Furthermore, the inside and around the malignant cells are abound with large concentrations of redox enzymes which can produce urushiol intermediates and free radicals directly akylated by these intermediates [12]. Alkylation of cellular nucleophiles by the intermediates and the formation of toxic oxygen species, especially

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through the redox cycling of o-quinones, may contribute to the cytotoxic property of the parent compound urushiol [8]. These lines of evidence suggest that the urushiols with quinone structure could play a prominent role in cancer chemotherapy [32].

The objective of this study was to examine the cytotoxic effect of the urushiol against ovarian cancer cells, and the mechanisms by which urushiol inhibited ovarian cancer cell growth. We prepared an urushiol-ethanol micro-emulsion in order to enhance the affinity of the urushiol for an aqueous medium and to facilitate its penetration through the cell membrane. We evaluated the cytotoxicity of the urushiol by a colony-forming assay. In addition, we used DNA fragmentation and DAPI staining assays to investigate whether the growth inhibition of ovarian cancer cells by the urushiol was due to apoptosis [7, 24]. We report here that the urushiol had a selective cytotoxic effect against ovarian cancer cells and this cytotoxicity was correlated to the induction of apoptosis.

MATERIALS AND METHODS

Reagents and Preparation of the Compound Studied

All chemicals were purchased from Aldrich and Sigma Chemical Co. unless otherwise stated. Urushiol, a major component of the sap of Oriental lacquer (*Rhus vernicifera*), is a mixture of 3-substituted catechols with pentadecyl, 8-pentadecenyl, 8,11-pentadecadienyl, and 8,11,13-pentadecatrienyl groups, and was obtained from an ethanol extract of the sap derived from a Korean lacquer tree of Wonju, Korea after the ethanol was evaporated. The urushiol, after being stored in a desiccator for a day, was then used without further separation of isomers [33] for the preparation of the urushiol-ethanol micro-emulsions. The urushiol-ethanol solution was prepared by dissolving 4 g of urushiol in 8 g of ethanol. The resulting 30% (wt/wt) of urusiol-ethanol solution was mixed with 200 ml of deionized water by a homogenizer (Kinematica AG PT 3100 Polytron, U.S.A.) at 15,000 rpm for 10 min to form a water/oil emulsion. The emulsion was stirred for 24 h at 500 rpm, and left for 18 h in order to separate the supernatant, after which the micro-emulsions were obtained by filtration through a 0.5 μm PTFE filter (Millex LCR, Millipore, U.S.A.), and concentrated by centrifugation at 300 ×g. Dilutions of the urushiol-ethanol micro-emulsion to be used in experiments were prepared with phosphate-buffered saline (PBS) immediately before use. Average size and size distribution of the urushiol-ethanol micro-emulsion were measured by a dynamic light scattering (DLS) spectrometer (ELS-800, Photal, Otsuka Electronics Co. Ltd., Japan) equipped with a He-Ne laser beam at a wavelength of 633 nm at 20°C. The intensity of the scattered light was detected at an angle of 90° to the incident beam. By analyzing the DLS data using the cumulative method, information about the particle size was obtained.

Cis-diamminodichloroplatinum (*cis*-platin), known as one of the most widely prescribed and extensively studied chemotherapeutic agents, was used to examine the relative cytotoxicity and urushiol's efficacy as an anti-tumor agent.

Cell Cultures and Treatments

Three human ovarian cancer cell lines (2774, SK-OV-3, and PA-1) and a normal Chinese hamster ovarian cell line (CHO) were used in this study, since we are interested in investigating the specific role of antitumor agents in ovarian cancer. All cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, U.S.A.) and maintained as monolayers in RPMI 1640 which was supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (250 units/ml), and streptomycin (250 units/ml) (GIBCO Laboratories, Grand Island, NY, U.S.A.). Cells for monolayer cultures were plated at a density of 2×10^5 cells/2 ml in six-well culture dishes, and incubated in a 5% CO_2 incubator at 37°C. Subconfluent exponentially growing cultures were treated with either urushiol or *cis*-platin and the treated cells were subsequently incubated for 24 h at 37°C.

Determination of Cell Viability by a Colony-Forming Assay

Monolayer cultures of normal and cancer cells were initiated at a density of 3×10^2 per 60 mm culture dish and cultured for 24 h. The cells were then treated with either urushiol (0, 10^{-6}, 10^{-5}, and 10^{-4} M) or *cis*-platin (0, 10^{-6}, 10^{-5}, and 10^{-4} M) for seven days. After colonies had clearly been formed in the untreated control dish, the cells were washed with PBS and fixed with methanol and acetic acid (3:1) solution for 3 min. The fixed cells were washed with PBS and stained with 0.4% Trypan blue in order to count the number of viable colonies formed after being exposed to either urushiol or *cis*-platin. The cell viability was calculated from the total number of colonies formed after being exposed to urushiol. The value was expressed as a percentage of the total number of colonies in the untreated control dish. All values are means of three determinations.

Measurement of DNA Fragmentation

DNA fragmentation was assayed to evaluate apoptosis by agarose gel electrophoresis as described previously [2, 3]. Briefly, after 24 h of incubation, normal and cancer cells were treated with 10^{-4} M urushiol for various lengths of time (0, 24, and 48 h). Each culture of the cells was then harvested, washed with PBS, and lysed in 0.5 ml of lysis solution (0.5% SDS, 25 mM EDTA, and 100 mM NaCl) containing 100 μg/ml of proteinase K for 3 h at 55°C. The denatured proteins were extracted with phenol-chloroform (1:2), and the nucleic acids were precipitated with ethanol.

The pellet was dissolved in 50 µl TE buffer at 37°C for 1 h. The DNA samples were electrophoresed on a 1.5% agarose gel at 5 V/cm and stained with ethidium bromide to visualize DNA fragments.

TUNEL Assay and DAPI Staining of Apoptotic Features in Ovarian Cancer Cells Treated with Urushiol

To further assess apoptotic features, a TUNEL assay and DAPI staining were performed. A TUNEL assay was carried out according to the manufacturer's instruction using the *In Situ* Cell Death Kit (Roche). For DAPI staining, PA-1 ovarian cancer cells were grown on coverslips and treated with 10^{-5} or 10^{-4} M urushiol for 24 h. Each culture of the cells was then submerged in 70% ethanol for 5 min at 4°C to fix and be permeable to the cells. Following two washes with PBS, the coverslips with the cells were incubated in extraction buffer (0.2 M Na_2HPO_4, pH 7.8, and 4 mM citric acid) for 5 min and washed with PBS. DAPI staining was performed in DNA staining buffer [10 mM Pipes, pH 6.8, 2 mM $MgCl_2$, 100 mM NaCl, 0.1% (v/v) Triton X-100] containing 2 µg/ml 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI) for 30 min at room temperature. Images of the stained nuclei were visualized for apoptotic changes in the chromatin by fluorescence microscopy (Olympus 70AX, Japan) under 460–500 nm light.

RESULTS AND DISCUSSION

Size Distribution of the Urushiol-Ethanol Micro-Emulsion

Unlike other types of chemotherapeutic agents, the urushiol is barely miscible with an aqueous media, and the purified urushiol is highly viscous and only soluble in strong solvents. However, micro-emulsification of urushiol using ethanol seemed to be one of the easiest and the most suitable methods to deliver the urushiol to various desired sites of action. In addition, unimodal size distribution of the urushiol-ethanol micro-emulsion, which plays an important role in drug delivery, could be achieved easily by this method. Figure 1 presents the size distribution of the urushiol-ethanol micro-emulsion under different preparatory conditions. The particle sizes of the urushiol-ethanol micro-emulsion

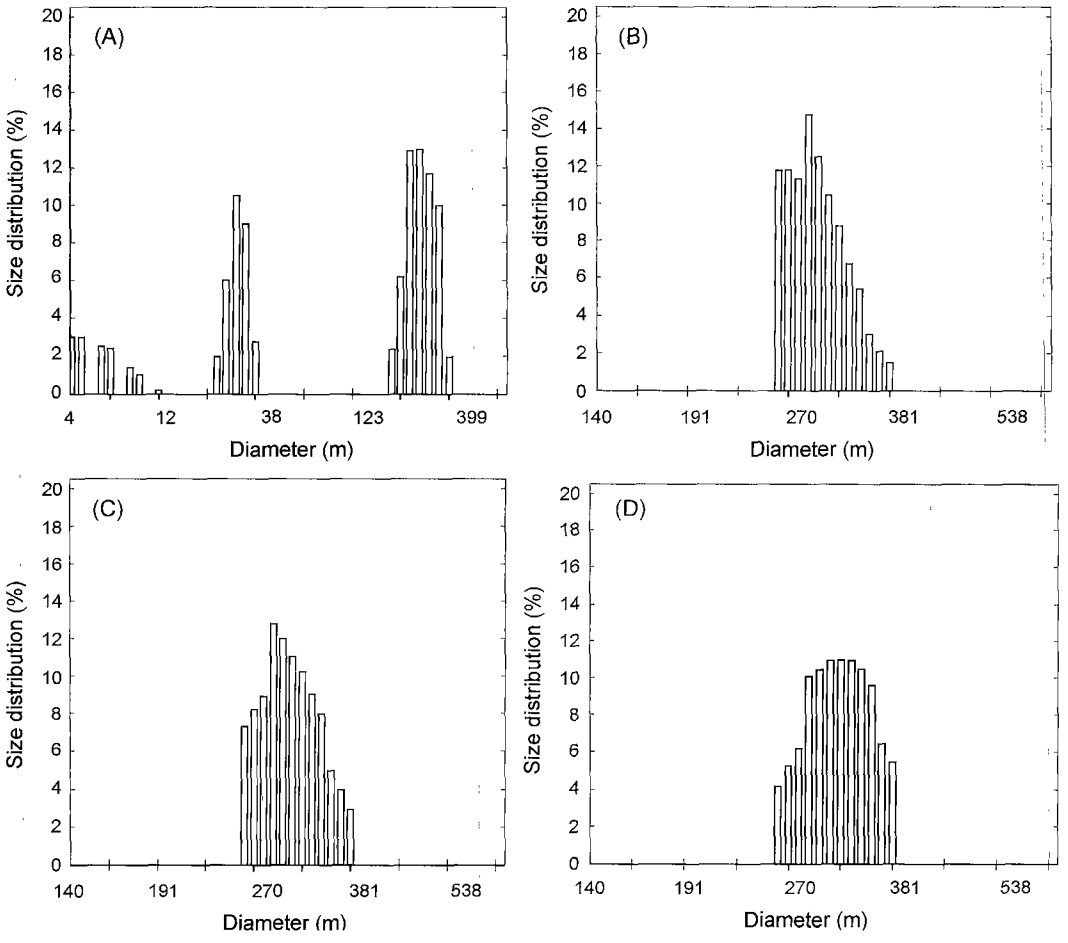

Fig. 1. Size distribution of urushiol-ethanol micro-emulsions.
(A) 7,000 rpm, (B) 10,000 rpm, (C) 13,000 rpm, (D) 15,000 rpm; average diameter of (B), (C), and (D) is ~280 nm.

were randomly distributed at 7,000 rpm (Fig. 1A). In contrast, the micro-emulsion shown in Figs. 1B, 1C, and 1D had a more unimodal distribution with average diameter of 280 nm, indicating that the urushiol-ethanol micro-emulsion with uniform diameters could be obtained by high-speed homogenization at 13,000 and 15,000 rpm.

Effect of Urushiol on the Growth of Ovarian Cancer Cells

In order to elucidate the specific effect of the urushiol, a natural electrophilic quinone compound, on the growth of cancer cells, we compared the viabilities of normal and ovarian cancer cells after treatment with various concentrations of urushiol (Figs. 2A and 2B). In this study, a colony-forming assay was used, since it is more reliable to assess viable cell numbers than colorimetric assays, such as an MTT assay [10, 20]. The inhibitory effect of 10^{-5} M urushiol on the growth of both normal CHO and SK-OV-3 ovarian cancer cells was hardly detected, while cells of the PA-1 and 2774 ovarian cancer cell lines exhibited over 50% of growth inhibition under the same conditions. Normal CHO cells indicated only insignificant growth inhibition in the presence of an even higher urushiol concentration (10^{-4} M). It was likely that a large amount of redox enzymes to produce urushiol intermediates existed inside and around the cancer cells [12]. In line with previous results [15], the data demonstrate that the urushiol has a selective cytotoxic effect on several types of cancer cells. We also observed that the SK-OV-3 cells were resistant to urushiol in the same manner as these cells reacted to the tumor necrosis factor as well as several types of cytotoxic drugs such as diphtheria toxin, *cis*-platin, and adriamycin [6, 20, 28]. In contrast, the cytotoxic effect of the urushiol on 2774 and PA-1 cells was strong and occurred in a dose-dependent manner (Fig. 2B). The PA-1 ovarian cancer cell line seemed to be the one most susceptible to growth inhibition by the urushiol, with an IC_{50} of approximately 4.5 μM.

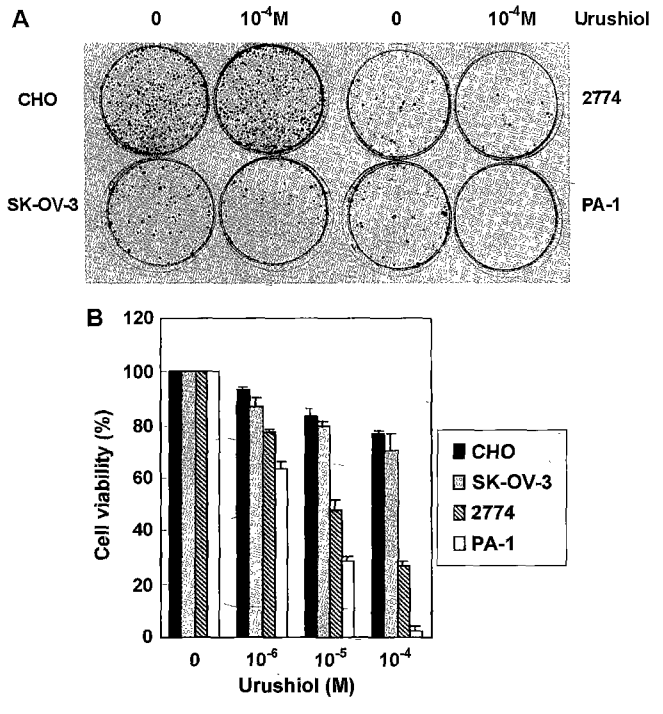

Fig. 2. Comparison of cell viabilities between normal and ovarian cancer cell lines after treatment with urushiol.
Cells were treated with the indicated concentrations of urushiol for seven days. Cell viability was determined by counting the number of colonies formed after being exposed to urushiol. These experiments were performed at three times for each drug concentration. (A), representative result of a colony forming assay. (B), relative cell viabilities of various kinds of ovarian cancer cells after being treated with urushiol. Values are expressed as the percentage relative to untreated cells. Means were derived from three independent replicates; bars, SD.

To confirm the relative cytotoxic effects and urushiol's efficacy, *cis*-platin was tested under the same experimental condition as urushiol. *Cis*-platin is one of the most widely prescribed and extensively studied antitumor agents known today. It induces apoptosis of various cancer cells and has the ability to create covalent bonds between adjacent guanosine residues in DNA, thus blocking DNA replication [10, 11, 23, 31]. The normal CHO cells exhibited a *cis*-platin sensitivity that was an intermediate between the three transformed cancer cell lines SKOV-3 (known to be resistant to *cis*-platin), PA-1, and 2774 (Fig. 3). PA-1, the most sensitive to the growth inhibition by urushiol, responded similarly to *cis*-platin. Consequently, the data suggest that urushiol may have the potential to be used as an effective antitumor agent in the treatment of ovarian cancer.

Induction of Apoptosis by Urushiol in Ovarian Cancer Cells

Numerous studies, performed previously with various chemotherapeutic agents, indicate that alkylating agents [9, 21], *cis*-platin [1, 5, 17], and taxol [14, 16] promote apoptosis of normally proliferating as well as malignant cells. Apoptosis, a physiologically programmed cell death, is a crucial component of the regulation of normal development and cellular function in multicellular organisms, but also to tumor growth where it plays a role in maintaining a balance between cell proliferation and cell death [19]. Apoptosis is characterized by cell shrinkage and DNA fragmentation due to the activation of a caspase-dependent DNA endonuclease which cleaves the genomic DNA into nucleosome-sized units [13, 22]. In order to evaluate whether the growth inhibition by urushiol treatment is linked to apoptosis, cells of each cell line were treated with 10^{-4} M urushiol for 24 or 48 h (Fig. 4), and the induction of apoptosis was identified by DNA fragmentation patterns in mutiples of 180–200 bp of oligonucleosomal DNA fragments, using agarose gel electrophoresis [13, 30]. In line with the cell viability assays

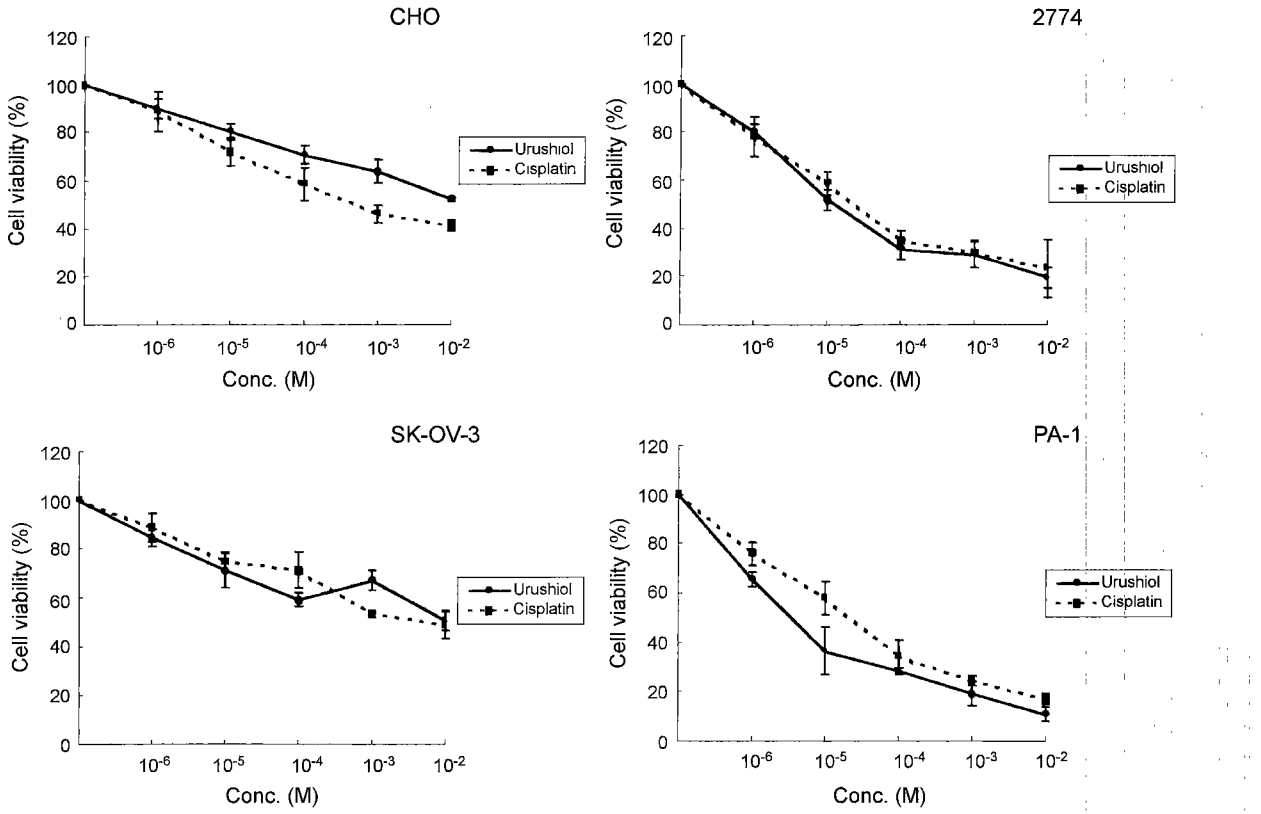

Fig. 3. Comparison of cell viabilities after treatment with *cis*-platin or urushiol.
Cells were treated with various concentrations (as indicated) of *cis*-platin or urushiol for seven days. Cell viability was determined by counting the number of colonies formed after being exposed to *cis*-platin or urushiol. The determinations were performed at three times for each drug concentration. Values are expressed as a percentage relative to untreated cells. Means were derived from three independent replicates; bars, SD.

shown in Fig. 2, normal CHO cells exhibited no DNA fragmentation even after treatment of up to 48 h with urushiol. A clear multiple-unit ladder pattern of apoptotic DNA was observed for the cancer cells 2774 and PA-1, and a considerable increase in signal intensity was observed with prolonged treatment. Thus, the data indicate that urushiol inhibited growth of 2774 and PA-1 cells by inducing apoptosis, which is a mechanism also observed with other typical antitumor agents [1, 14, 17].

To further substantiate apoptosis, a TUNEL assay and DAPI staining were performed: Nucleosomal DNA fragmentation was directly visualized by staining the nuclei of cells with DAPI for the morphological consequences of exposure to urushiol. The untreated PA-1 cells exhibited no morphological alterations after 48 h of culture in the absence of urushiol (Fig. 5). In contrast to the untreated control cell culture, among the PA-1 cells treated with urushiol (Fig. 5), typical apoptotic morphological changes such as small, typical apoptotic cells, namely shrunken cells with fragmented nuclei (apoptotic bodies), were observed by staining the nuclei of cells with DAPI under fluorescence microscopy. After 48 h of exposure, they showed a significantly greater incidence of chromatin condensation and fragmentation characteristic of apoptosis than the untreated cells. In addition, cells stained positively by a TUNEL assay, which was identified by labeling DNA strand breaks, were observed for the cancer cell lines 2774 and PA-1. Consequently, we concluded that the cytotoxic effect of urushiol might be attributable to its apoptosis-inducing activity. Although the signal transduction pathway that was set in motion by urushiol has not yet been identified, the formation of DNA fragments and apoptotic bodies suggested some involvement of the caspase-activated DNA endonuclease [13, 22].

Conclusions derived from this study are: First, a urushiol-ethanol micro-emulsions with a uniform size distribution can be prepared by high-speed homogenization. Second, ovarian cancer cells are significantly sensitive to urushiol, whereas urushiol is almost nontoxic to normal ovarian cells at concentrations which are lethal to cancer cells. Third, the growth inhibition of cancer cells evoked by urushiol is linked to apoptosis. Finally, urushiol may have some potential to be developed as an effective antitumor agent for the treatment of ovarian cancers.

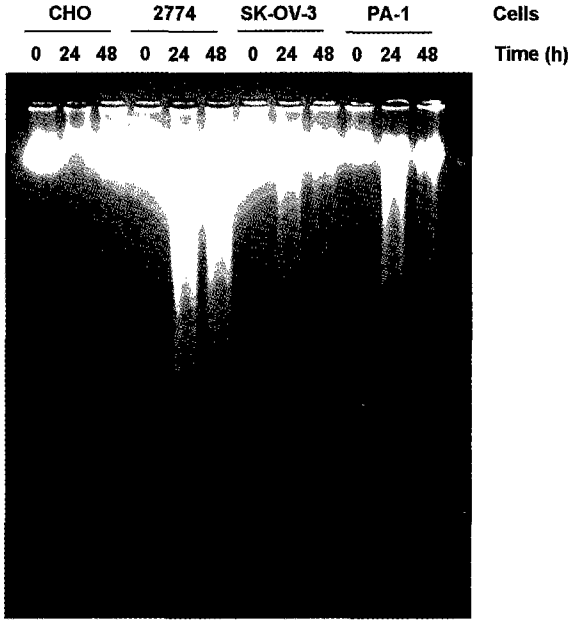

Fig. 4. Detection of apoptosis by DNA fragmentation upon treatment with urushiol.
Normal CHO cells and cells of two ovarian cancer cell lines were incubated without (0.05% ethanol) or with 10^{-4} M urushiol for various lengths of time as indicated. DNA was isolated and subjected to electrophoresis through a 1.5% agarose gel as described under Materials and Methods. No apoptosis was observed when normal CHO cells were treated with urushiol. Urushiol clearly induced DNA fragmentation in both ovarian cancer cell lines (2774 andPA-1).

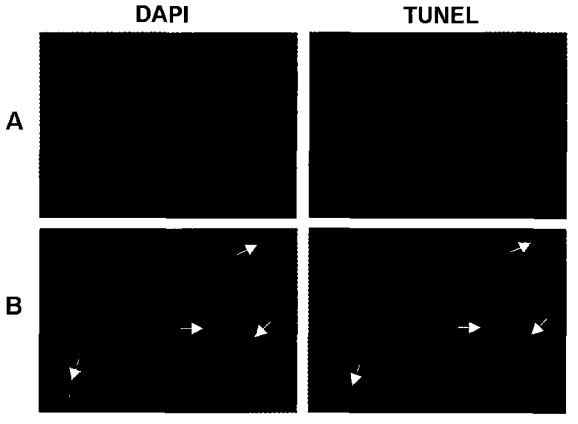

Fig. 5. Detection of apoptosis by a TUNEL assay and DAPI staining.
PA-1 cells, either untreated (A) or treated (B) with 10^{-4} M of urushiol for 24 h, were fixed and stained with DAPI (blue) or labeled with fluorescein-dUTP in a TUNEL assay (green) as described in Materials and Methods. The fragmented nuclei are indicated by arrows.

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