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# Extract of lotus root (*Nelumbo nucifera* rhizome) causes necrotic damage to human colorectal cancer cells in culture

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## ABSTRACT

Lotus root (a rhizome of *Nelumbo nucifera*) is known to contain various bioactive phytochemicals, and traditionally used for medical treatment in East Asia. However, this plant is mainly used as a food material, and the joint part of this plant is dumped as an agricultural waste in Japan. Then, to use it as medicinal resources, the aqueous extracts were prepared from the edible and joint parts of lotus root, and the direct effects of these extracts on human colorectal cancer cells were examined. The joint part extract reduced the viabilities of HT29 and HCT116 cells by causing the oxidative damage to these cells. The aqueous extracts prepared from both parts of lotus root contained large amounts of polyphenolic compounds, and their concentrations in the joint part extract were higher as compared with the edible part extract. Furthermore, the joint part extract was shown to liberate hydrogen peroxide in the culture medium, which might be derived from polyphenolic compounds contained in the extract, thus suggesting that the joint part extract may directly act on the cells as a pro-oxidant, resulting in the oxidative damage to the cells, which might be unexpectedly mediated through the necrotic rather than the apoptotic pathway.



## KEYWORDS

Lotus root extract;
Polyphenolic compounds;
Pro-oxidant activity;
Necrotic cell death;
Human colorectal cancer cells.

## INTRODUCTION

Colorectal cancer is a serious health problem in the Western world, and acknowledged as one of the leading causes of cancer-related death[1]. Recently, the epidemiological study has shown that there is a possible correlation between diet and colon cancer[2], and suggested that the intake of fruits and vegetables in large quantities may be effective for reducing the risk of carcinogenesis[3]. Therefore, many kinds of phytochemicals contained in fruits, vegetables, herbs and medicinal plants have been generally considered to possess the properties of preventing carcinogenesis[4]. Among of these phytochemicals, polyphenolic compounds, such

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as catechins, bioflavonoids and cinnamic acid derivatives, have previously been reported to be well endowed with the anti-cancer activities, which can be expressed in their abilities to prevent both carcinogenesis and cancer cell proliferation as well as to protect the normal cells against the oxidative damage[5].

In general, polyphenol compounds are known to exert not only their anti-oxidant but also their pro-oxidant action on the cells, and reported to show their pro-oxidant activities particularly at higher concentrations or in the presence of transition metals[6]. Previous studies using cultured cancer cells have shown that several flavonoid compounds reveal the anti-cancer properties based on their capabilities to interact with cellular peroxidases and/or thiols, thereby producing highly reactive phenoxyl radicals, resulting in the formation of glutathione-conjugates or the oxidization of glutathione to the disulfide form[7]. Polyphenols have also been shown to generate reactive oxygen species (ROS) via several indirect pathways which may be located the downstream of surface receptors and associated with an NAD(P)H-dependent ROS production. On the other hand, phenoxyl radicals derived from polyphenols have been reported to disturb the mitochondrial function by collapsing the transmembrane potential, thereby generating ROS to a considerable extent[7,8].

Lotus root (rhizome of *Nelumbo nucifera*) is widely used as a traditional folk medicine in East Asia, and this medicinal plant is known to have a variety of pharmacological activities, such as hypolipemic[9], antiviral[10], antipyretic[11], antiinflammatory[12] and antioxidative[13] effects, thus commonly applying to the treatment of diarrhea, gastritis, insomnia, and nervous prostration in Korea, China and India[13]. On the other hand, the extracts of lotus root have been extensively analyzed, and shown to contain many phytochemicals including isoliensinine, kaempferol and procyanidins[14,15] as the effective components. In addition, lotus root has been shown to reveal hypoglycemic[16], antidiarrheal[17], antipyretic[18], and immunomodulatory[19] effects, and moreover suggested to have a potential activity improving learning and memory functions[20]. However, this plant is uncommonly used as a medicinal plant in Japan, and popularly utilized as a food material for Japanese cuisine. In contrast to the edible part of lotus root, the joint part, which is the part connecting the edible parts each other, is too hard to eat, and therefore dumped as agricultural wastes at great cost, thereby becoming an earnest desire to establish a way to utilize the inedible part of lotus root. Since this plant has been effectively used as a folk medicine in other Asian countries, we presumed the joint part of lotus root to have biological actions, which can be expected to practically apply to medical treatment. Recently, the cytotoxic effects of various chemicals and natural substances on malignant cancer cells in culture have been investigated by preference as a primary screening for their potential anticancer activities. Then, to evaluate the anti-cancer effect of aqueous extract prepared from the joint part of lotus root, the effect of joint part extract on the growth of malignant cancer cells was examined using human colon carcinoma HCT116 cells as an *in vitro* screening system.

## EXPERIMENTAL PROCEDURES

### Cell lines and chemicals

Human colorectal carcinoma cells (HCT116 and HT29 cells) were purchased from the American Type Culture Collection (Rockville, MD, USA). Neutral red solution, reduced-form glutathione (GSH), *N*-acetylcysteine (NAC) and propidium iodide (PI) were obtained from Sigma Chemical Co. (St Louis, MO, USA). TACS Annexin V-FITC Apoptosis Detection Kit was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Catalase was obtained from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Other chemicals used were commercially available reagent grade or ultra pure grade.

### Preparation of lotus root extracts

Lotus root (the rhizome of *Nelumbo nucifera*) was kindly donated by the Naruto agricultural cooperative in Tokushima (Naruto, Japan). To prepare the aqueous extract, the lotus root was cleaned by washing with water, and the joint and edible parts were sliced in an approximately 1 cm thickness. The slices were dehydrated by exposing them to wind stream for several weeks, and then ground into coarse powder. The powder was soaked in distilled water at the concentration of 4% (w/v), and kept at 4°C for 1 hr. Then, the insoluble materials were removed by centrifuging at 6,000

x *g* for 20 min at 4°C. The aqueous extract was filtered through a 0.2 μm-pore filter, and aliquots of the extract were stored at -20 °C until use.

**Cell culture**

HCT116 cells and HT29 cells were maintained on a 60-mm culture dish in 5 mL of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum, 50 units/mL of penicillin, 50 μg/mL of streptomycin and 50 μg/mL of gentamycin sulfate at 37 °C in a humidified incubator containing a 95% air–5% $CO_2$ atmosphere.

**Determination of cell viability**

Cells were plated on a 24-well cluster plate at a density of $5 \times 10^4$ cells/well, and maintained for 24 h to allow them to attach to the bottom of the vessel before starting the experiment. The cells were exposed to the lotus root extracts at various concentrations for different time periods. In some case, the cells were exposed to 5 mM glutathione, 5 mM NAC, or 5 units/mL of catalase for 30 min before adding the extracts. The cell viability was determined by measuring the amounts of neutral red taken up into the cells as described previously[21,22]. Briefly, the cells were washed with saline solution, and incubated in 0.5 mL of DMEM containing neutral red (50 μg/mL) for 2 h in a humidified incubator. The cells were washed twice with saline solution, and then extracted with acidified ethanol solution (50% ethanol - 1% acetic acid) for 20 min at room temperature with constant gentle shaking. The amount of neutral red taken up into the cells was determined by measuring the optical density at 540 nm.

**Determination of hydrogen peroxide generation**

Cells were plated on a half of a 24-well plate at a density of $5\times 10^4$ cells/well, and the same volume of growth medium was added to the other half. The cell culture and the medium alone were incubated with different concentrations of the extract for 1 h, and the medium of both group was collected, and then centrifuged at 20,000*g* for 20 min to remove the floating cells and cell fragments. The concentration of hydrogen peroxide in the supernatant fraction was measured using a PeroXOquant quantitative peroxide assay kit as reported previously[23].

**Determination of total phenolic compound concentration**

The contents of phenolic compounds in the aqueous extracts of lotus root were measured according to the Folin-Ciocalteau method as described previously[26] with modification. Briefly, 50 μl of the extract was mixed with 50 μl of 50% Folin-Ciocalteau reagent, and 1 mL of 2% $Na_2CO_3$ solution was added to the mixture at 3 min after starting the reaction, and then left to stand for 60 min in the dark. The concentration of total phenolic compounds in the extract was determined by measuring the absorbance at 750 nm using gallic acid as a standard, and expressed as gallic acid equivalents (GAEs).

As an alternative way for the determination of total polyphenols in the extracts, the contents of tannin in the aqueous extracts were measured using a classic method of Lowenthal permanganate titration[24], and expressed as tannic acid equivalents.

**Characterization of cytotoxic effect**

For a flow cytometric analysis, the cells were plated on a 35-mm culture dish at a density of $1 \times 10^5$ cells/dish, and exposed to the extract for 24 h, and then harvested by trypsinization. The cells were collected and rinsed with phosphate-buffered saline (PBS), and then stained with both PI and FITC-labeled annexin V using TACS Annexin V-FITC Apoptosis Detection Kit according to the manufacture's instruction. The analysis was carried out using an EPICS XL-MCL System II flow cytometer (Beckman Coulter, Inc., Fullerton, CA, USA) as described previously[25].

To assess the damage to the plasma membrane, the cells were plated on a 24-well cluster plate at a density of $5 \times 10^4$ cells/well, and then exposed to the extract for 24 h. Lactate dehydrogenase (LDH) activities in both the culture medium and the cell lysate were determined according to the method reported previously[26] with some modification. The leakage of LDH from the cells was expressed as the percentage of total enzyme activity released into the medium.

**Data analysis**

The results were presented as the mean ± SE, and analyzed using a one-way analysis of variance (ANOVA) followed by Fisher's post hoc test. The probability lev-

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els less than 0.05 were accepted as a statistically significant difference.

## RESULTS AND DISCUSSION

Lotus root is widely used as a folk medicine, and applied to the treatment of several disorders, such as diarrhea, gastritis, insomnia, and nervous prostration in East Asia[13]. On the other hand, this plant is a popular food material, and the joint part, which is connecting the edible part of the root, is usually dumped as agricultural waste in Japan. To obtain a clue to developing a way to use this inedible part effectively, we examined the direct effect of the aqueous extract prepared from the joint part of lotus root on the viabilities of human colorectal cancer cells, thereby trying to evaluate its potential anti-cancer effect on colon cancers, which are a serious health problem in the Western world[1].

Human colorectal cancer cells were exposed to the aqueous extracts of either the joint part or the edible part of lotus root at the concentration of 10% for 48 h, and the effects of these extracts on the cell viabilities were determined by measuring the uptake of neutral red into these cells. As shown in Figure 1, the uptake of neutral red into HCT116 and HT29 cells was reduced by exposing them to the aqueous extract prepared from the joint part, but the extract from the edible part failed to cause any significant reduction of the dye uptake into these cells. The joint part extract caused the reducing effect on the viabilities of HCT116 cells in a manner dependent on its concentration. Actually, the extract caused no significant change in the cell viability at the concentration of 2%, and caused a significant reduction of the viability at the concentration of 5%. An approximately 40% reduction of the cell viability was obtained by exposing to the extract at the concentration of 10% for 48 h (Figure 2), and this reduction was observed up to at least for 72 h after that time (data not shown). These results indicated that the aqueous extract prepared from the joint part of lotus root, but not prepared from the edible part, reduced the viabilities of human colorectal cancer cells, thus suggesting that the joint part of lotus root might contain larger amounts of active substance(s) causing the toxic damage to human colorectal cancer cells.

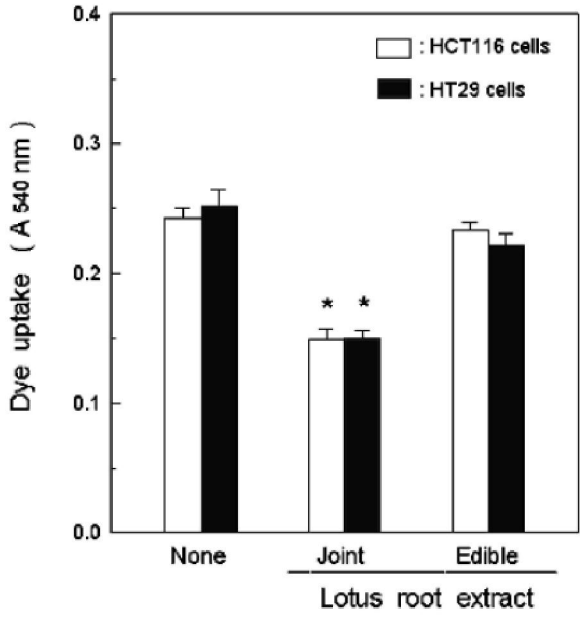


**Figure 1 : Effects of lotus root extracts on the viabilities of human colorectal cancer cells. HCT116 and HT29 cells were exposed to 10% of the aqueous extracts prepared from the joint and edible parts of lotus root for 48 h, and the cell viabilities were determined by measuring the dye uptake into the cells as described in the text. Results were expressed as the absorbance at 540 nm. Values are the mean ± SE (*$P$ < 0.05, n = 6)**

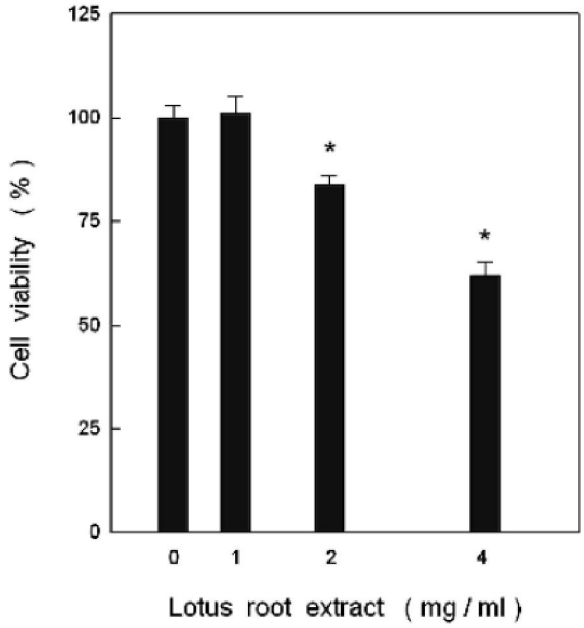


**Figure 2 : Concentration-dependent effect of the joint part extract on the cell viability. HCT116 cells were exposed to different concentrations of the aqueous extract prepared from the joint part of lotus root for 48 h, and the dye uptake into the cells was then determined as described in the text. Results were expressed as the cell viability. Values are the mean ± SE (n=6, *$P$<0.05).**

In general, the cytotoxic effects of various chemicals and natural substances have previously been reported to be mostly connected with the oxidative damage to the cells, and hence it seemed possible to assume that oxidative stress and reactive oxygen species might be connected with the toxic effect of the lotus root extract on HCT116 cells observed here. To verify this assumption, the reducing effect of the joint part extract on the cell viability was assessed again in the presence of hydroperoxide scavengers, and the reduction of the cell viability caused by the joint part extract was inhibited by adding GSH (Figure 3), and other different-type scavengers, such as catalase and NAC, also completely inhibited the cell death caused by the extract (data not shown). Next, the question of whether the joint part extract might be able to generate hydrogen peroxide during the exposure to the cells was addressed. As shown in Figure 4, the concentration of hydrogen peroxide in the culture medium was increasing in a concentration-dependent manner, and the substantial amount of hydrogen peroxide was generated even by incubating the extract in the culture medium without the cells. Therefore, it seemed reasonable to consider that cytotoxic effect of the joint part extract might be closely connected with the oxidative damage to the cells through the generation of hydrogen peroxide, thus proposing the possibility that some substances, such as polyphenolic compounds, contained in the joint part extract was capable of directly acting on cancer cells as a pro-oxidant, which might be responsible for the toxic effect of the joint part extract observed here.

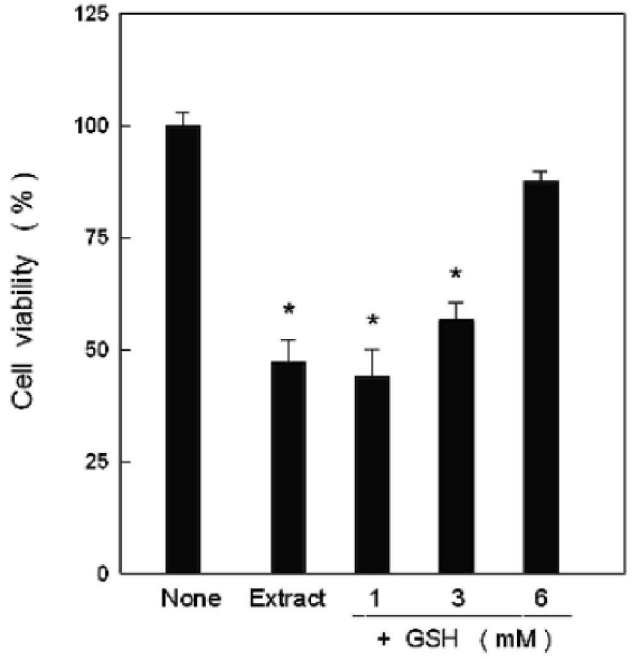


**Figure 3 : Effect of antioxidant on the joint part extract-induced damage to cancer cells. HCT116 cells were exposed to10% of the aqueous extract prepared from the joint part of lotus root for 48 h in the presence of different concentrations of GSH, and cell viability was determined as described in the text. Values are the mean ± SE (*$P$<0.05, n=6).**

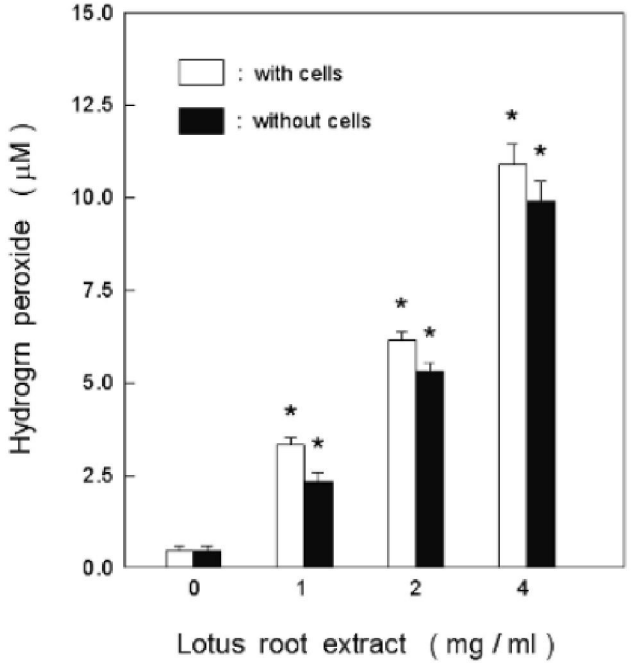


**Figure 4 : Liberation of hydrogen peroxide from the aqueous extract prepared from the joint part of lotus root. Various concentrations of the joint part extract were maintained for 1 h in the culture medium with or without HCT116 cells, and the amount of hydrogen peroxide in the medium was determined as describe d in the text. Values are the mean ± SE (*$P$<0.05, n=6).**

Polyphenolic compounds in various vegetables, fruits and herbs have previously been reported to cause the damage to various human cancer cells, resulting in the growth suppression and/or the cell death[5,30]. Since the joint part extract was more toxic to human colorectal cancer cells as compared with the edible part extract, the concentrations of total phenolic compounds contained in these extracts were then determined to answer the question of whether polyphenol compounds in these extracts might be related to their toxic effects on HCT116 cells. As shown in Figure 5A, the concentrations of total polyphenolic compounds in the extracts prepared from the joint and

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edible parts were 760 and 335 μg/mL, respectively, and the polyphenol concentration in the joint part extract was actually shown to be approximately 2.3-fold higher than that in the edible part extract. As an alternative estimation of polyphenolic compounds contained in these extracts, the contents of tannin in the extracts were determined using the classic oxidation-reduction titration method, and the obvious difference in total polyphenol contents between the joint and edible part extracts was confirmed (Figure 5B). These results suggested that polyphenolic compounds contained in the joint part extract might be responsible for its toxic effect on human colorectal cancer cells.

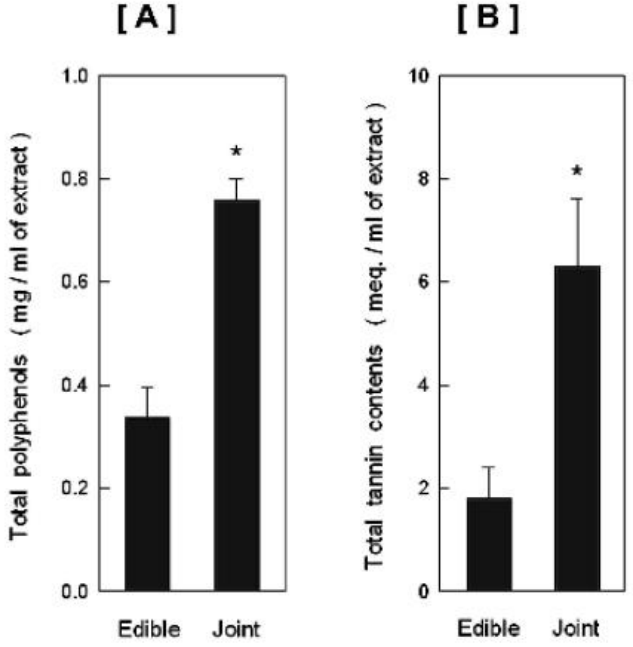


**Figure 5 : Total polyphenolic compounds contained in the aqueous extracts prepared from the joint and edible parts of lotus root. The contents of total polyphenols [A] and tannin [B] in the joint and edible part extracts were then determined as described in the text. Values are the mean ± SE (*$P$<0.05, n=6).**

Polyphenolic compounds contained in vegetables and medicinal plants are known to show not only an anti-oxidant but also a pro-oxidant effect, depending upon the conditions interacting with the cells[7,8,31]. Also, these compounds have been shown to cause the cell death through their pro-oxidant actions, thereby exerting their toxic effects on cancer cells[5,30]. Moreover, various kinds of polyphenolic compounds, such as kaempferol, procyanidins and catechins, have recently been shown to be contained in lotus leaves, seedpods and root[14,15,32]. Taking these previous findings into consideration, it seems reasonable to consider that polyphenolic compounds in the joint part extract may be acting as a pro-oxidant during the exposure periods, thereby causing the toxic damage to cancer cells under the conditions used in this work.

The cytotoxic effect of the joint part extract on HCT116 cells was characterized by analyzing the cells stained with both PI and annexin V-FITC using a flow cytometric technique. As shown in Figure 6A, an increase in the populations of the cells positively stained with PI was observed by exposing them to the joint part extract for 24 h, but notable change in the cell populations stained with annexin V was not detected. Both PI-positive and annexin V-negative cells appeared at 3 h after exposing the cells to the joint part extract (data not shown). Furthermore, the fluorostaining of the cells with PI and acridine orange showed that chromatin condensation, a typical marker of apoptotic cell damage, was scarcely observed in the nuclei of the cells exposed to the joint part extract (data not shown). These results strongly suggested that the cytotoxic effect of the joint part extract observed here might be necrotic rather than apoptotic. Then, the leakage of LDH from the cells exposed to the joint part extract was determined as an additional marker of the necrotic cell damage, and the joint part extract caused the LDH leakage from the cells into the culture medium, and more than a half of the cellular enzyme was shown to leak out of the cells by the extract at the concentration of 10% (Figure 6B). Thus, it seems reasonable to conclude that the aqueous extract of the joint part of lotus root may cause the necrotic rather than apoptotic damage to the cells, and suggest that a potential activity of the joint part extract to cause the necrotic cell damage could be directly responsible for its cytotoxic effect on human colorectal cancer cells. On the other hand, the pro-oxidants are generally considered to cause the apoptotic cell damage as a consequence of generating hydrogen peroxide, but the observations presented here suggest that the generation of hydrogen peroxide from the joint part extract may cause the necrotic damage to the cells, which is contradictory to the general concept of pro-oxidant action. However, the recent study has shown that marine toxin may cause the toxic effect on human colorectal cancer Caco-2 cells without

the consistently detectable expression of both apoptotic and necrotic markers[32]. Therefore, it seems still necessary to more precisely investigate the characteristics of the necrotic cell damage caused by the joint part extract of lotus root.

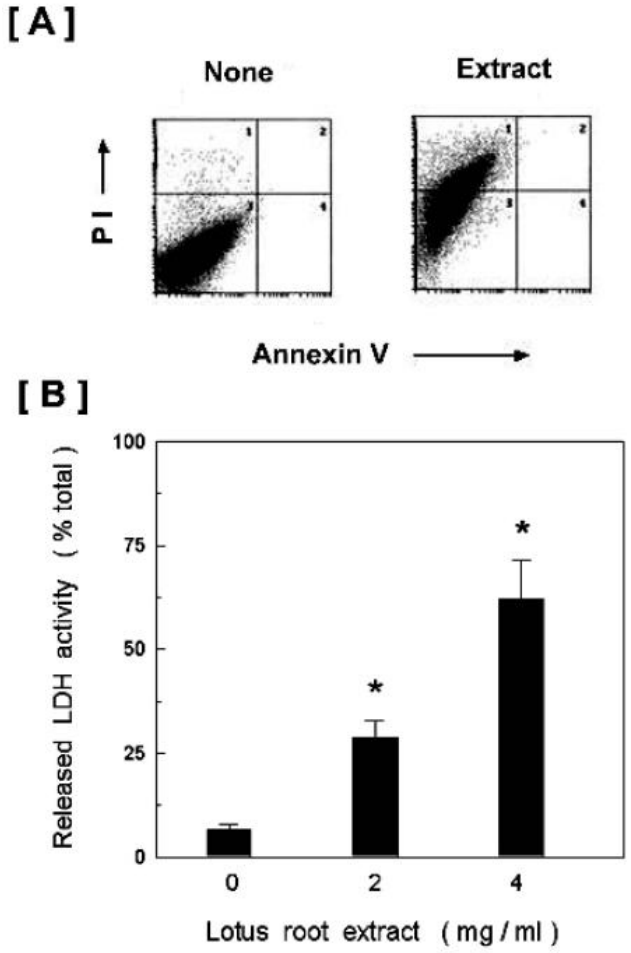


**Figure 6 : Characteristics of the joint part extract-induced damage to cancer cells. HCT116 cells were exposed to 10% of the aqueous extract prepared from the joint part of the rhizome for 24 h, and the flow cytometric analysis [A] and LDH assay [B] were then carried out as described in the text. Values are the mean ± SE (n=6, *$P$<0.05).**

Polyphenolic compounds are generally known to reveal their pro-oxidant activities when they are present with the high concentrations of transition metals and when interacting with the cellular components, such as peroxidases, thiols, and surface receptors[6-8]. However, the present study indicated that a considerable amount of hydrogen peroxide was generated by incubating the joint part extract with or without the cells in the culture medium. Therefore, it seems unlikely that the interaction of polyphenols contained in the joint part extract with the cellular components may be essential for the generation of hydrogen peroxide in the cell culture, but seems possible that some constituents of the culture medium may be involved in the liberation of hydrogen peroxide from the joint part extract. On the other hand, the contents of catechins in the lotus root and its knot have previously been shown to be 0.0025% and 0.011%, respectively[33], thus indicating that the catechin contents in the joint part of lotus root is higher than that in the edible part. In agreement with this previous finding, we indicated in this work that the joint part of lotus root might contain larger quantities of phytochemicals as compared with the edible part, and therefore the joint part is considered to be more attractive as the objective of our research to use their biological activities for preventing various diseases including cancers. However, the effective substances contained in the joint part extract are still unidentified, and the mechanism underlying the cytotoxic effect of the joint part extract is poorly understood, and hence seems necessary to be further investigated. Moreover, the *in vitro* cytotoxic effects of phytochemicals are not always relevant as a positive index of the *in vivo* carcinostatic action (25), and therefore proposing here the *in vivo* additional experiments to confirm the anti-cancer activity of the extract prepared from the joint part of lotus root.

## CONCLUSION

The present study proposes the possibility that the aqueous extract of lotus root may be able to prevent the growth of colorectal cancers as a consequence of inducing the necrotic damage to cancer cells through the pro-oxidant action. Practically, biologically active substances extracted from lotus root have not yet been identified, but it seems most likely that the anti-cancer activity of lotus root may be attributed to the pro-oxidant activities of polyphenolic compounds contained in this plant.

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