Journal of Biochemistry and Molecular Biology, Vol. 32, No. 6, November 1999, pp. 585–593

JOURNAL OF
Biochemistry
and
Molecular Biology

Antiproliferative Effect of *Artemisia argyi* Extract against J774A.1 Cells and Subcellular Superoxide Dismutase (SOD) Activity Changes

Tea Eun Lee, Sie Won Park[1,*], and Tae Jin Min
Department of Chemistry, Dongguk University, Seoul 100-715, Korea
Department of Chemistry, Sangmyung University, Seoul 110-743, Korea

Received 11 June 1999, Accepted 2 September 1999

The water and methanol extracts of *Artemisia argyi* showed significant cytotoxicities against J774A.1 cells but not so much against normal leukocytes. The cytotoxicities were found to be dependent on the extract concentration and the incubation time. The concentration of water and methanol extracts inhibiting 50% of cell proliferation (IC_{50}) were estimated to be 44.2 mg/ml and 71.6 mg/ml, respectively. In the presence of *Artemisia argyi* water extract, total superoxide dismutase (CuZnSOD and MnSOD) activities of media, cytoplasmic and mitochondrial fractions of J774A.1 cells increased in accordance with cytotoxicity. MnSOD was found to be the main component of enhanced total SOD activities, particulary in the mitochondrial fraction. In contrast to SOD, catalase and glutathione peroxidase (GPx) were not found in any instance of the current investigation. In addition, substantial amount of O_2^- appeared to be generated in the mitochondrial fraction under the influence of *Artemisia argyi*. All data put together, it is postulated that *Artemisia argyi* extracts seem to stimulate O_2^- generation in mitochondria of J774A.1 cells with concomitant increases of SODs. Since H_2O_2, the reaction product of SOD on O_2^-, is known to be readily converted to very toxic OH· in the absence of catalase and/or GPx cooperation, toxicity derived from ROS such as O_2^-, H_2O_2, and OH· may be the main cause of necrosis and/or apoptosis of J774A.1 cells.

Keywords: *Artemisia argyi* extracts, Cytotoxicity, J774A.1 cells, Superoxide anion, Superoxide dismutase (SOD).

Introduction

Artemisia herba (Compositae) are medicinal plants widely used as anti-inflammatory, antimalarial, antihepatitic (Peter *et al.*, 1986; Zafrapolo *et al.*, 1991; Liu, *et al.*, 1992; Ahn *et al.*, 1997), and as moxibustion material especially in the Eastern society. In addition to such medical effects, they have been utilized as vegetable foodstuff. Recently, several investigations suggest that they exhibit more diverse activities such as antibacterial and antitumor actions (Yashphe *et al.*, 1979; Mori *et al.*, 1989; Xu *et al.*, 1989; Zheng, 1994).

Cancer has been the most malignant disease, the complete treatment of which seems still far from satisfactory in spite of the combined therapy of surgery, radiation, and oncogenic drugs (Astrow, 1994; Parker, 1996; Bailer and Gornic, 1997). Although a variety of oncogenic drugs, usually synthetic or semisynthetic, have been developed with great effort, their usage as oncogenic agents have been limited due to their toxic effects on normal cells (Keizer *et al.*, 1990; Wyllie, 1992; Hasegawa *et al.*, 1994; Bailer, 1997). On account of such toxic side effects of oncogenic drugs, many investigations have been carried out to develop nontoxic and highly effective oncogenic drugs from natural sources. Nonetheless, there is no ideal drug being used in spite of great worldwide efforts, as emphasized by the report of the National Cancer Institute that they could not get suitable anticancer agents even from 114,045 plant species and 16,196 animal sources until 1982 (Suffiness and Douros, 1982).

Along with the development of oncogenic agents, it is necessary to explore the reaction mechanism of these agents in relation to cancer pathology. At present, the most

* To whom correspondence should be adressed.
Tel: 82-2-2287-5147; Fax: 82-2-396-8758
E-mail adress: parksw@pine.sangmyung.ac.kr

reasonable theory concerning carcinogenesis suggests that the reactive oxygens species (ROS) such as O_2^-, OH·, H_2O_2 may provide the initiating event(s) for cancer induction by peroxidizing cell membrane lipids and by deteriorating DNA base structures through point mutation and strand breaks (Vuillaume, 1987; Cerutti and Trump, 1991; Feig *et al.*, 1994; Toyokuni *et al.*, 1995). In all forms of life, but mostly importantly in aerobic life, radicals and/or ROS play limited but essential roles. Aerobic cells and organisms have indeed taken advantage of all the reaction potentials of both molecular oxygen (O_2) and its reduction products (ROS) (Halliwell and Gutteridge, 1989; Harman, 1994). However, they become exposed to a major risk if the production of radicals and/or ROS increases above a critical level. Indeed, life requires a balance between the production of such potentially damaging molecular species, their scavenging, and/or their destruction. Toxicity and disease including cancer may result from a rupture in such vital balance. Against deleterious oxidative stress of ROS, superoxide dismutases (SODs) are the primary enzymatic defence mechanisms with catalase and glutathione peroxidase (GPx) (Werms and Luccesi, 1990; Pereira *et al.*, 1994). The presence of SODs in all aerobic organisms that have been examined has suggested that they may play a critical role in maintaining the normal state of cells by scavenging O_2^- (Sies, 1991; Barber, and Harris, 1994; Fridovich, 1995). Furthermore, a variety of recent elucidations concerning the dual functions of ROS both as cancer inducer and inhibitor, as evidenced by the mode of oncogenic agents, has proposed an interesting challenge to researchers from the standpont of cancer therapy in relation to ROS metabolism (Keizer *et al.*, 1990).

Along this direction, we undertook the current investigation as a preliminary study to develop a nontoxic oncogenic agent from *Artemisia aegyi* and to explore the underlying mechanism in correlation with ROS metabolism, including SOD (MnSOD and CuZnSOD) activities, using the macrophage cancer cell line J774A.1 (ATCC TIB 67) which exhibit functions similar to normal macrophage (Ralph and Nakoniz, 1977).

Materials and Methods

Materials *Artemisia argyi*, cultivated and dried over 3 years was obtained from the Kangwha Island of Korea. Mouse derived J774A.1 cell line was supplied by the Korean Cell Line Bank in Cancer Research Institute (Seoul National University, Korea). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), Primaria culture flasks and pipettes were purchased from Grand Island Biological Co. (New York, USA). Membrane filters were obtained from Sartorius Co. (Gottingen, Germany). Trypsin and trypan blue were purchased from Sigma Chemical Co. (St. Louis, USA).

Cell culture J774A.1 cell was well characterized to have the properties displayed by normal macrophages such as phagocytosis, O_2^- release, and intracellular bacteria killing (Ralph and Nakoniz, 1975). Cells were cultured in DMEM supplemented with 10% FBS in 50 ml Primaria culture flasks. All cultures were grown under a 5% CO_2 humidified atmosphere at 37°C. J774A.1 cells grew as an adherent monolayer and were passaged before reaching confluence by treatment at 37°C for 5 mins with 0.25% trypsin/0.02% EDTA. Subculture was performed at every 4 d with seed cells of 10^6/ml of media. Culture was carried out in the absence or in the presence of *Artemisia argyi* extract, and the cytotoxicity of every sample was measured with a hemocytometer. Superoxide dismutase (total SOD and MnSOD), catalase, and glutathione peroxidase (GPx) activities were also estimated in medium, cytoplasmics and mitochondrial fractions of the J774 A.1 cells.

Cytotoxicity and viability The cytotoxicity test of *Artemisia argyi* extracts was performed by counting cell numbers with a hemocytometer according to the protocol of the National Cancer Institue (NCI, 1972). The principal method for cytotoxicity assay of *Artemisia argyi* against J774A.1 cells are as follows. J774A.1 cells reaching the exponential period are distributed in 12-well plates with seed cell population of 5×10^5/ml in DMEM media with or without *Artemisia argyi* extract as necessary. After incubation for 3 d in 3 ml of media, isolated cells were collected by dissociating intercellular tissue by treating with 0.25% trypsin for 5 mins, adjusted to 3 ml with fresh media, and counted with a hemocytometer. Cytotoxicity values were calculated by comparing cell numbers (live and dead) of samples with that of corresponding controls (live and dead) and IC_{50} values were determined. Viability calculations (%) were also carried out by the trypan blue exclusion method, i.e. counting cells stained with trypan blue and comparing this cell number with total cell numbers.

Methanol and water extracts preparation of *Artemisia argyi* Water and methanol extracts of *Artemisia argyi* were prepared. Although methanol extract is usually used as the starting material to get a purified single compound, we also prepared the water extract as people traditionally take the water extract for oral administrations. For the preparation of water extract, 10 g of the dried aerial part of *Artemisia argyi* was cut into small slices with scissors, put in 300 ml of distilled water, boiled for 2 h, filtered through 8 layers of gauze, and centrifuged at 5000 × g for 10 min. The supernatant was exposed to evaporation under reduced pressure. The dried weight was 6.57 ± 1.39 g. Methanol extract was prepared as follows: 10 g of tiny slices of *Artemisia argyi* was put in 300 ml of 70% methanol and kept for 48 h at room temperature. This extraction process was repeated. Then, extracted solutions were combined and filtered through 8 layers of gauze, centrifuged, and the supernatant was evaporated under reduced pressure. The dried weight of methanol extract was 7.43 ± 1.57 g. When necessary, extracts were solubilized in distilled water and membrane filtered for usage.

Preparation of cytoplasmic, mitochondrial, and medium fractions from J774A.1 cell, and superoxide dismutase (SOD), catalase, and glutathione peroxidase(GPx) assays In an attempt to measure the antioxidant enzyme activities of subcellular fractions and medium under the cytotoxic condition by *Artemisia argyi*, three fractions from cultured J774A.1 cells

were prepared. After culture in 50 ml Primaria culture flasks, J774A.1 cells and medium were divided by centrifugation at 1000 × g for 10 min and supernatant was taken as the medium fraction. Cells were washed twice with equal volumes of phosphate saline (0.1 M, pH 7.4). To the washed cells, 1.5 ml of phosphate buffer (0.1 M, pH 7.4) was added and the cells were homogenized with an Elvejhem homogenizer for 5 s, five times at 1 min intervals, in an icebath. This cell homogenate was centrifuged at 1000 × g for 5 min to get rid of whole cells or cell debris as pellet. The supernatant was then centrifuged at 15,000 × g for 20 min to obtain the cytoplasmic fraction. The pellet containing mitochondria was ultrasonified in 1.5 ml phosphate buffer for 3 s, four times at 1 min intervals, in an icebath to expose MnSOD, known to exist in the mitochondria matrix (Bannister *et al.*, 1987; Groner *et al.*, 1992).

Total SOD and MnSOD activities of these fractions were measured by a slightly modified cytochrome *c* method (McCord and Fridovich, 1969) explained as follows in brevity. Reaction mixture in a total 3 ml contained 50 mM phosphate buffer (pH 7.8), 10^{-5} M ferricytochrome *c*, 5×10^{-5} M xanthine and enough xanthine oxidase to evoke an 0.025 absorbancy discrepancy at the very least. The reaction mixture without xanthine oxidase was kept for 10 min to adjust to a temperature of 25°C. The reaction was started by adding xanthine oxidase and absorbancy was recorded for 2 min at 550 nm. One unit of SOD activity was defined as the amount required to inhibit 50% of the absorbancy of the standard mixture. The SOD activity thus estimated shows the total activity consisting of MnSOD and CuZnSOD activities. MnSOD activity was determined in the same condition as above except under the presence of 1 mM of KCN to inhibit CuZnSOD enzyme activity.

Catalase activity was assayed according to the method of Maral *et al.* (1977), by measuring the decrease of the absorbance at 240 nm at 20°C. Reaction volume in 1 ml contained 50 mM potassium phosphate buffer (pH 7.4), 12.5 mM H_2O_2, and enzyme solution. Absorbancy was recorded for 5 min and data were expressed as nanomoles of H_2O_2 degraded per min per 10^8 cells.

GPx activity was measured by a modified method of Bjorkman and Ekholm (1995) using tertiary butylhydroperoxide as the substrate. Varying volumes of the mitochondrial and cytoplasmic solutions prepared from J774A.1 cells were added to the reaction mixture which in a final volume of 1 ml contained 5 mM EDTA, 0.134 mM NADPH, 2 mM reduced glutathione, and 1 U/ml glutathione reductase. The oxidation of NADPH was registered at 340 nm for several minutes before 0.16 mM tertiary butylhydroperoxide was added and the registration was continued at 37°C. The activity of GPx was expressed as nanomoles of H_2O_2 degraded per min per 10^8 cells.

Normal leukocytes preparation from ICR mouse strain Normal leukocytes corresponding to J774A.1 cancer cells were prepared from ICR mice according to the method of Boyum (1968). Dextran 110 was added to peripheral blood to a final concentration of 10% and incubated for 30 min at 37°C to allow most erythrocytes to sediment. Supernatant was collected and diluted 1:1 with phosphate buffered saline (PBS) and 9 ml layered onto a 6 ml Ficoll/sodium metrizoate mixture, and then centrifuged for 15 min at 400 × g. The plasma/PBSA was removed without disturbing the interface. The interface was taken carefully with a Pasteur pipette, diluted to 20 ml in serum-free medium, and centrifuged at 400 × g for 10 min. The pellet containing leukocytes was resuspended in 2 ml serum free-medium for use.

Quantitation of O_2^- generated in J774A.1 cells Release of O_2^- was measured by assaying the capacity of O_2^- to reduce ferricytochrome *c* to ferrocytochrome *c*. J774A.1 cells (10^6 cells in 300 μl PBS buffer) were placed in 24-well plates. To gain a final volume of 1 ml, 400 μl buffer and 50 μl cytochrome *c* solution (1.24 mg/ml) were added as well as 50 μl of stimulus solution (phorbol myristate acetate, 5 μ/ml) or buffer as control. After incubation for 60 min, the plates were centrifuged and aliquots (200 μl of the supernatant diluted with buffer to 1 ml) were measured in a Varian spectrophotometer at 550 nm. The reaction of ferricytochrome *c* was calculated using the molecular extinction coefficient for cytochrome *c* of 21.1 mM^{-1} cm^{-1}. Reduction of cytochrome *c* in the presence of SOD was substracted from the values without SOD. Results was calculated as nanomoles O_2^- per 10^6 cells per 60 min and further expressed as % of control for the convenience of comparison.

Data presentation All experiments were performed in duplicate sets and repeated twice. The results were represented as mean ± SD. The Student *t* test was carried out to show the confidence limit with $p < 0.05$ (*) and $p < 0.01$ (**), respectively.

Results

Cytotoxicity of *Artemisia argyi* extracts against J774A.1 cancer cell and normal leukocytes The oncogenic effect of *Artemisia argyi* was examined primarily by comparing the cytotoxicities and viabilities of water and methanol extracts against J774A.1 cancer cells and corresponding normal leukocytes. The relative cytotoxicities and viabilities of water and methanol extracts to control values were described in Table 1 and Table 2, respectively. As demonstrated in Table 1, the viability and cytotoxicity of J774A.1 cells were altered remarkably depending on the concentration of *Artemisia argyi* water extract, from 10 μg/ml to 200 μg/ml. Viability values decreased from 91.3 ± 9.5% to 58.7 ± 11.2%, suggesting that J774A.1 cells might have died extensively prior to lysis (necrosis and/or apoptosis), and cytotoxicity increased proportionally in accordance with the concentration of *Artemisia argyi*. A maximal value of 74.6 ± 10.5% was obtained with 200 μg/ml of extract showing an IC_{50} value of 48.1 μg/ml which was different from K774A.1 leukocytes which showed low cytotoxicity values from 7.7 ± 2.0 to 32.8 ± 11.7 in an identical condition.

A similar pattern of results was obtained when we used the methanol extract of *Artemisia argyi* against J774A.1 cells. As presented explicitly in Table 2, the

values of viability and cytotoxicity seemed to change in almost the same way as those of the water extract, but suggested a slightly lower cytotoxic effect with the best value being 69.5% ± 11.7% at 200 μg/ml and IC_{50} calculated to be 62.4 μg/ml. In the case of the leukocytes, the data indicates that there was not much significant cytotoxic effect of *Artemisia argyi* methanol extract, with only about 30% cytotoxicity value even at 200 μg/ml. It can be emphasized from Tables 1 and 2 together that the cytotoxic effects of the *Artemisia argyi* extracts are substantial against J774A.1 cells whereas far less against normal leukocyte and, therefore, it seems appropriate to expect nontoxic oncogenic agents from *Artemisia argyi*.

In order to confirm the oncogenic effect of the *Artemisia argyi* water extract, which showed more cytotoxicity against J774A.1 cells than the methanol extract, cytotoxicity and viability assays were again performed, this time changing the incubation period from 1 to 7 d. Data described in Table 3 indicates that the viability and cytotoxicity effects of *Artemisia argyi* water extract against J774A.1 cells changed significantly according to the incubation period in both cases of 50 μg/ml and 100 μg/ml of extract. Viability decreased considerably in proportion to the incubation period even to 20–35% at 7 d leaving only a few nonstained viable cells. Cytotoxicity also showed remarkable alterations from a slightly stimulatory effect (−5 ~ −7%) at day 1 to significant cytotoxicity over 90% at 7 d of incubation with 100 μg/ml sample. Hence, we could conclude that the cytotoxic effect of *Artemisia argyi* extracts are dose- and time-dependent.

Table 1. The effect of *Artemisia argyi* water extract on the cytotoxicity and viability of J774A.1 cells and normal leukocytes according to the concentration of extract. Incubation was carried out for 3 d.

Artemisia concentration (μg/ml)	J774A.1 cell		Leukocytes	
	Cytoxicity (%)	Viability (%)	Cytotoxicity (%)	Viability (%)
0 (control)	0	91.3 ± 5.5	0	96.6 ± 5.7
10	19.9 ± 1.9**	87.5 ± 8.3	7.7 ± 2.6*	91.4 ± 8.1
20	38.5 ± 8.5**	71.7 ± 6.7*	11.9 ± 3.2**	87.6 ± 11.2
50	54.8 ± 8.4**	69.3 ± 10.3*	16.4 ± 3.8**	81.6 ± 13.2
100	69.9 ± 11.7**	61.4 ± 13.9*	23.5 ± 5.3**	76.3 ± 9.3*
200	74.8 ± 10.5**	58.7 ± 11.2**	32.8 ± 11.7**	67.9 ± 13.5*

Table 2. The effect of *Artemisia argyi* methanol extract on the cytotoxicity and viability of J774A.1 cells and normal leukocytes according to the concentration of extract. Incubation of extract was carried out for 3 d.

Artemisia concentration (μg/ml)	J774A.1 cell		Leukocytes	
	Cytoxicity (%)	Viability (%)	Cytotoxicity (%)	Viability (%)
0 (control)	0	92.5 ± 9.7	0	95.4 ± 5.6
10	14.6 ± 2.7**	89.5 ± 9.5	11.8 ± 3.5**	96,8 ± 11.4
20	34.8 ± 2.6**	72.3 ± 11.7*	21.4 ± 4.2**	98.5 ± 9.3
50	50.8 ± 8.3**	66.4 ± 8.9*	26.9 ± 6.9**	83,5 ± 12.5
100	63.1 ± 13.5**	62.7 ± 10.5**	2.9 ± 7.6**	76,9 ± 9.7*
200	69.5 ± 11.7**	57.4 ± 12.5*	35.7 ± 8.8**	65.2 ± 15.4*

Table 3. The effect of *Artemisia argyi* water extract on the cytotoxicity and viability of J774A.1 cells according to the incubation period. The concentrations of *Artemisia argyi* were 50 μg/ml and 100 μg/ml.

Incubation period (days)	Cytotoxicity (%)		Viability (%)	
	50 μg/ml	100 μg/ml	50 μg/ml	100 μg/ml
1	−5.2 ± 1.4**	−7.5 ± 1.3**	89.7 ± 9.9	87.2 ± 13.2
2	18.3 ± 0.9**	17.5 ± 1.5*	83.5 ± 19.2	85.2 ± 21.3
3	49.4 ± 9.4**	68.5 ± 8.2**	71.5 ± 9.9**	65.3 ± 12.6**
4	67.3 ± 18.3**	72.8 ± 21.3**	69.3 ± 14.8**	61.8 ± 11.2**
5	72.5 ± 16.4**	78.9 ± 16.2**	63.8 ± 13.7**	52.4 ± 15.8**
6	81.6 ± 17.3**	92.4 ± 15.7**	54.8 ± 9.3**	38.5 ± 11.5**
7	88.6 ± 11.4**	92.5 ± 5.2**	35.7 ± 6.4**	20.7 ± 4.3**

Generation of O_2^- in J774A.1 with addition of *Artemisia argyi* Since it was shown that J774A.1 cell death occured with the addition of *Artemisia argyi* in a dose- and time-dependent manner, we tried to find any relation of ROS with this cell death phenomena by measuring O_2^-. The results in Table 4 indicate that the generation of O_2^- in J774A.1 cells was increased steadily from 150% to over 500% of the control value at concentrations of *Artemisia argyi* from 10 to 100 μg/ml. Such a remarkably enhanced quantity of O_2^- proposes a role of O_2^- in the course of J774A.1 cancer cell death under the influence of *Artemisia argyi*. Very naturally, we became interested in the metabolic pathway of O_2^- and its related enzymes such as SOD, catalase, and glutathione peroxidase (GPx).

Subcellular superoxide dismutase (SOD) activity changes according to the *Artemisia argyi* water extract concentration and the incubation period As suggested above, we tried to explore the relation between the cytotoxic effect of *Artemisia argyi* and O_2^--related events, the antioxidant enzyme activities. We determined the activity changes of SOD (total SOD and MnSOD) in subcellular fractions as MnSOD and CuZnSOD are known to be localized in the mitochondria and cytoplasm of eukaryote cell, respectively. Activities of total SOD and MnSOD of the cytoplasmic fraction changed significantly according to the concentration of *Artemisia argyi* in both cases of 3 and 5 d of incubation, as presented in Table 5. Total SOD consisting of CuZnSOD and MnSOD displayed activity increases steadily from 1.6 ± 0.5 units/ml in control sample to 5.1 ± 1.6 units/ml at 200 μg/ml concentration after 3 d of incubation. When incubation was carried out for 5 d, there was a very similar pattern of activity change showing substantial increase of total SOD and MnSOD activities depending on the *Artemisia argyi* concentration and incubation time. In either case, MnSOD appeared to comprise about 30~40% of the total SOD value and we could assume that MnSOD had migrated to the cytoplasm in the course of J774A.1 cell death.

The subsequent experiment was carried out to confirm whether MnSOD was induced virtually in mitochondria by the *Artemisia argyi* water extract, by measuring the total SOD and MnSOD activities of the mitochondrial fraction. As presented in Table 6, we observed prominent activity changes of MnSOD as well as total SOD in the mitochondrial fraction. This tendency was particularly significant in 5 d of incubation with 3~4 folds enhanced activities of total SOD and MnSOD. Furthermore, MnSOD activity appeared to comprise more than 90% of total SOD activity and this result indicated that MnSOD was clearly induced in the mitochondrial under the cytotoxic condition of *Artemisia argyi*.

Along a similar line of investigation, we conducted an experiment regarding the changes of total SOD and MnSOD activities of the media fraction to pursue SOD's migration during J774A.1 cell death. The results of Table 7 show considerable activities of MnSOD as well as total SOD in either incubation times of sample, accounted for by the release of SOD enzymes through the mitochondrial and cytoplasmic membranes of dying cells or derived from already lysed cells.

Catalase and glutathione peroxidase activities Since the reaction product of SOD is H_2O_2, there must be accompanying cooperative induction of catalase or GPx for SOD enzymes to accomplish their cellular protective action by degrading $O_2^-O_2^-$ completely to nontoxic H_2O. Otherwise, H_2O_2 would either attack the cell itself because it is one of the toxic ROSs or it would be readily converted to OH·, the most toxic species of ROS. To investigate the metabolic pathway of H_2O_2 produced by SODs in J774A.1 cells, the activities of catalase and GPx were assayed in an identical condition as the SOD assay system. The data described in Table 8 present some interesting results with respect to the metabolic pathway of H_2O_2. From the data, we can recognize instantly that there is no further metabolic pathway of H_2O_2 in J774A.1 cells except to OH· or accumulation of H_2O_2 itself since there is no detectable activities of catalase or GPx to convert H_2O_2 to H_2O.

Table 4. The generation of superoxide anion (O_2^-) in J774A.1 cells according to the concentration of *Artemisia argyi* extract. Culture was carried out for 3 and 5 d.

Artemisia concentration (μg/ml)	O_2^- generated (% of control) 3 d	5 d
0 (control)	100 ± 0.0	100 ± 0.0
10	148.6 ± 7.5**	280.6 ± 8.7**
20	266.9 ± 8.3**	327.3 ± 11.5**
30	316.4 ± 8.4**	424.8 ± 16.2**
50	337.9 ± 21.3**	449.3 ± 11.4**
100	421.5 ± 27.5**	519.4 ± 27.8**

Table 5. Total SOD and MnSOD activities changes of cytoplasmic fraction of J774A.1 cells according to the *Artemisia argyi* concentration. Incubation was carried out for 3 and 5 d.

Artemisia concentration (μg/ml)	SOD activities (units/10^8 cells)			
	3 d		5 d	
	Total SOD	MnSOD	Total SOD	MnSOD
0 (control)	1.6 ± 0.5	0.4 ± 0.2	2.1 ± 0.7	0.3 ± 0.2
10	2.3 ± 1.1	0.8 ± 0.4	2.7 ± 1.3	0.7 ± 0.4
20	3.1 ± 0.9*	0.7 ± 0.3	3.9 ± 0.8*	1.8 ± 0.6**
50	3.9 ± 1.5*	1.3 ± 0.3**	4.8 ± 1.5*	2.1 ± 1.3*
100	4.2 ± 1.8*	1.6 ± 0.6*	5.5 ± 1.9*	1.8 ± 1.5
200	5.1 ± 1.6*	1.9 ± 0.8*	5.9 ± 2.1*	2.6 ± 0.4**

Table 6. Total SOD and MnSOD activities of mitochondrial fraction of J774A. 1 cells according to the concentration of *Artemisia argyi*. Incubation was carried out for 3 and 5 d.

Artemisia concentration (μg/ml)	SOD activities (units/10^8 cells)			
	3 d		5 d	
	Total SOD	MnSOD	Total SOD	MnSOD
0 (control)	1.7 ± 0.6	1.9 ± 0.4	1.4 ± 0.5	1.1 ± 0.5
10	2.3 ± 0.5	1.8 ± 0.7	2.8 ± 0.7*	2.5 ± 0.8*
20	3.1 ± 1.2	2.3 ± 0.7	4.1 ± 1.8*	3.3 ± 0.9*
50	3.7 ± 1.5*	2.8 ± 0.6	4.6 ± 1.3**	3.2 ± 0.6**
100	4.3 ± 1.7*	3.6 ± 1.3*	5.3 ± 1.9**	5.0 ± 1.8*
200	5.1 ± 1.4**	3.9 ± 1.5*	4.8 ± 1.5**	4.3 ± 1.6*

Table 7. Total SOD and MnSOD activities of media fraction according to the concentration of *Artemisia argyi*. Incubation was carried out for 3 and 5 d.

Artemisia concentration (μg/ml)	SOD activities (units/ml)			
	3 d		5 d	
	Total SOD	MnSOD	Total SOD	MnSOD
0 (control)	1.2 ± 0.4	0.2 ± 0.1	0.9 ± 0.3	0.4 ± 0.2
10	1.2 ± 0.5	0.3 ± 0.2	2.1 ± 0.6	0.8 ± 0.5
20	1.4 ± 0.3	0.8 ± 0.5	2.0 ± 0.3**	1.2 ± 0.3*
50	1.8 ± 0.5	0.7 ± 0.2*	3.1 ± 0.5**	1.5 ± 0.5*
100	2.4 ± 0.6*	0.8 ± 0.6	3.7 ± 0.8**	2.3 ± 0.6**
200	2.5 ± 0.8*	0.8 ± 0.4*	2.8 ± 1.2*	2.5 ± 0.4**

Table 8. The changes of catalase and glutathione peroxidase (GPx) activities in the cytoplasmic and mitochondrial fractions of J774A.1 cells according to the *Artemisia* extract concentration.

Artemisia concentration (μg/ml)	Cytoplasm		Mitochondria	
	Catalase	GPx	Catalase	GPx
	(nM/10^8/min)		(nM/10^8/min)	
0 (control)	0.01 ± 0.01	0.02 ± 0.03	ND	0.01 ± 0.02
10	ND	0.01 ± 0.02	0.02 ± 0.01	ND
20	0.02 ± 0.01	ND	ND	ND
50	ND	ND	ND	ND
100	ND	0.03 ± 0.02	ND	ND

Discussion

In order to develop the nontoxic oncogenic agent from natural sources and explore the underlying mechanism, the cytotoxic effects of *Artemisia argyi* extracts against J774A.1 cancer cells and changes of antioxidant enzyme activities such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) were investigated. Firstly, the water and methanol extracts of *Artemisia argyi* were both found to exhibit relatively strong cytotoxicities against J774A.1 cells with concomitant decrease in viability (Tables 1, 2, and 3). In contrast to the cancer cell, normal leukocytes showed far less cytotoxicity with addition of *Artemisia argyi*. These results seem to be enough to put forward *Artemisia argyi* for further study as the source of a nontoxic oncogenic agent.

When we measured the O_2^- generated in J774A.1 cells under the presence of *Artemisia argyi*, we found predominantly enhanced amounts of O_2^- depending on *Artemisia argyi* concentration and incubation time (Table 4). Enhanced amounts of O_2^- must give rise to the induction of its degrading enzyme for the cell to overcome the toxicity of O_2^-, and so we measured the activity changes of subcellular SODs in the course of cytotoxicity with *Artemisia argyi* as presented in Tables 5, 6, and 7. Common patterns of results on the whole were obtained showing significant changes of CuZnSOD and MnSOD activities in the cytoplasmic, mitochondrial and even media fractions of J774A.1 cells according to the *Artemisia argyi* extracts concentration and incubation time. In particular, MnSOD activity was found to comprise more than 90% of total SOD activities in the mitochondrial fraction. Thus, we could draw one conclusion that *Artemisia argyi* induces the generation of O_2^- with concomitant induction of SODs, especially MnSOD in mitochondria and CuZnSOD in cytoplasm during the course of J774A.1 cell death. In fact, such mitochondrial induction of O_2^- has been regarded to be virtually the mechanism of various oncogenic agents for apoptosis and/or necrosis (Hickman, 1992; Fluda, 1997).

Recently, there have been some reports on the paradoxical action of SODs that produce the OH· radical in addition to the conventional action of SOD to protect cells by scavenging O_2^- (Eum *et al.*, 1998). As suggested also in various papers, SODs are apt to fail in protecting cells without the cooperative actions of catalase and/or GPx because there would be no way of H_2O_2 metabolism except to generate the very toxic OH· spontaneously by the Fenton reaction (Halliwell, 1994; Evans *et al.*, 1997) or H_2O_2 accumulation, both compounds of which are known to be deleterious to cells.

OH· is so reactive that no enzyme systems involving it as a substrate exists (Southorn and Powis, 1988). This deleterious OH· has been assured to play a central role to cause major tissue injury together with other ROS species in a number of diseases including cancer, AIDS, diabetes, atherosclerosis, acute inflammation, aging, Alzheimer's disease, and neurodegenerative diseases (Halliwell, 1994; Sen and Packer, 1996; Evans *et al.*, 1997). Therefore, it can easily be postulated that O_2^- and OH· generated extensively by the addition of *Artemisia argyi* would have provided a continual onslaught of oxidative stress to J774A.1, and finally evoked necrosis and/or apoptosis resulting in membrane structure degradation because these ROSs can attack virtually any molecule found in its path, especially membrane components.

Against such a devastating attack of OH· and other ROSs, the antioxidant enzyme SOD is certainly a major defence mechanism that combats oxygen toxicity. However, the efficacy of SOD as an antioxidant relies on its cooperation with accompanying enzymes, i.e. catalase and GPx, which metabolize the dismutation product of O_2^-, H_2O_2, to water and O_2. As we could not detect catalase or GPx activity in the J774A.1 cells (Table 8) despite prominent increases of SOD activities in the course of the cytotoxic process, it appears that the death of J774A.1 cells by *Artemisia argyi* extracts might have been accomplished by the oxidative stress of ROS including O_2^-, H_2O_2, and OH·.

With respect to the role of *Artemisia argyi* in the cytotoxic process against J774A.1, other postulations are also available including the cytokines mimic action. For instance, tumor necrosis factor (TNF) was first described as a tumoricidal agent and thereafter recognized as a mediator of cytotoxicity through superoxide generation (Tilden *et al.*, 1987; Xu *et al.*, 1989; Laudanna *et al.*, 1990). On the other hand, the cytotoxic and acute inflammatory cytokines such as Inteleukin-1, Interleukin-6, or transforming growth factor can also be considered to exert their actions in a similar way as *Artemisia argyi*, primarily by inducing O_2^- with concomitant augmentation of SOD activities as reviewed by many reports (Visner *et al.*, 1990). On this basis, it is suggested that *Artmisia argyi* may contain a novel kind of cytokine inducing necrosis and/or apoptosis of J774A.1 cells *via* oxidant and antioxidant systems.

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