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Protective Activities of Fractions of Water Extract Obtained from *Artemisia iwayomogi* Kitamura against Oxidative Stress-induced Mutagenesity: Correlation with Their Reactive Oxygen Scavenging Activity

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Abstract Water extracts of *injinssuk* (*Artemisia iwayomogi* Kitamura) (WE) were obtained from the dried and ground leaves and stems of *injinssuk*. The WE was further fractionated into crude polysaccharide (C-PS) and nonpolysaccharide fractions (N-PS). The protective activities against the *tert*-butyl hydro peroxide induced mutangenecity on *Escherichia coli* PQ37 and reactive oxygen species scavenging activity of the WE, C-PS, and N-PS were studied. The WE obtained from leaves showed a significantly higher inhibitory effect on the mutagenicity than WE from stem. The WE obtained from the leaves having higher crude polysaccharide content but lower content of total carbohydrates had significantly higher antimutagenicity than that from the leaves with lower crude polysaccharide but higher total carbohydrate contents. Further study showed that C-PS fraction showed markedly stronger antimutagenic effect than N-PS. C-PS was also more effective than N-PS for hydroxyl radical scavenging activity, but was similar to N-PS in superoxide radical scavenging activity.

Keywords: antimutagenic, antioxidative, *Artemisia iwayomogi* Kitamura, free radical, SOS chromotest

Introduction

Reactive oxygen species generated by sunlight, ionizing radiation, chemical reactions, and metabolic processes may lead to a variety of pathological consequences such as DNA damage, carcinogenesis, cellular degeneration related to aging, and damage to the immune system (1). Hydroxyl radical and superoxide anion radical are the two most representative reactive oxygen species. The reactive oxygen radical scavengers present in natural plant sources or in human diet are of great interest as possible protective agents against oxidative damage (2). A variety of compounds in natural plants have been reported to possess strong active oxygen radical scavenging and antimutagenic activities (3). The Oriental medicinal plants '*injinho*' (*Artemisia capillaris* THUNB) and '*injinssuk*' (*Artemisia iwayomogi* Kitamura) have been traditionally used for treating liver disease without special distinction because of the similarity of species, but '*injinssuk*' is mainly used in Korea. The *injinssuks* are traditionally collected at 2 different seasons (May and September) for the medicinal purpose. However, the differences in their biological effectiveness among the *injinssuk* collected at different seasons has not been previously studied. The active components in the *A. iwayomogi* have been reported as low molecular compounds including esculetin 6-methylether, esculetin 7-methylether (scopoletin), scopolin, β-sitosterol, chlorogenic acid, essential oil, fatty acid, sesquiterpene lactones, eudesmanolides, and flavonoids (4). Song (5) reported, however, that polysaccharide extract of *injinssuk* (*A. iwayomogi* Kitamura) showed especially strong antihepatotoxic activities. Koo *et al.* (6) reported that polysaccharide fraction obtained from *injinssuk* showed immunomodulation and anti-tumor activity in mice. These activities of polysaccharide fraction of *injinssuk* might be related, at least to some extent, to its antimutagenic and/or reactive oxygen scavenging activity. Ahn *et al.* (7) reported recently that the ethanol insoluble part among the water extracts of *injinssuk* had the strong inhibitory effect against the mutagenicity of benzo[a]pyrene conversed by a mixed-function oxidase system to an ultimate metabolite (7). However, benzo[a]pyrene, the authors used in the study, was not a direct oxidative stress-inducing mutagenic compound. Protective activity of the polysaccharide extract of *Artemisia* species against oxidative stress-inducing mutagencity has never been previously reported. Biological action of *tert*-butyl hydroperoxide (*t*-BOOH) was considered to be relevant for the study of oxidation-related mutagenecity due to its oxygen radical generating properties under physiological condition (8). Thus, the objectives of this research were to study the inhibitory activity of crude water extract, polysaccharide and non-polysaccharide fractions of *injinssuk* on mutagenicity in SOS chromotest by a direct oxidative inducing mutagen (*t*-BOOH), and to determine their hydroxyl radical and superoxide anion radical scavenging activities.

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Materials and Methods

Materials Samples of *injinssuk* (*Artemisia iwayomogi* Kitamura) were obtained from field in Korea (Jinan, Jeonbuk) in May 25 and September 25, 2007. *tert*-Butyl hydroperoxide, ascorbic acid, glutathione, nitro blue tetrazolium (NBT), riboflavin, cytochrome *c*, 1,1-diphenyl-2-picrylhydrazyl, *O*-nitrophenyl-D-galactopyranoside (ONPG), and *p*-nitrophenyl phosphate disodium (PNPP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). *Escherichia coli* PQ37 was kindly provided by Drs. P. Quillardet and M. Hofnung (the Institute Pasteur, Paris, France).

Extraction and fractionation of *injinssuk* Extraction of polysaccharide from the leaves of *injinssuk* was performed according to the previous reported method (5). Dried and ground leaves of *injinssuk* were first extracted with 99% ethanol for 24 hr at 37°C for the elimination of low molecular weight components, and the ethanol portion was discarded. The remained portion was dried at 45°C, and then extracted with hot water for 5 hr at 70°C. The water extraction was repeated one more time. The extract was combined and was freeze-dried. The obtained water extract (WE) powder were soaked in 75% ethanol solution for 12 hr at 4°C and centrifuged at 5,000×g for 20 min. The supernatant was filtered with a Whatman No. 2 filter paper, and evaporated *under vaccuo*, and then freeze-dried to obtain non-polysaccharide (N-PS) portion. Crude polysaccharide (C-PS) portion was obtained by freeze-drying the residue. This procedure is shown in Fig. 1.

SOS Chromotest Measurement of antimutagenicity was performed according to the method of Quillardet and Hofnung (9) with a slight modification. *E. coli* PQ37 overnight culture was diluted 1:50(v/v) with L medium (bacto trytone 10 g, bacto yeast extract 5 g, NaCl 6 g/L) and incubated for 2 hr at 37°C and 1 mL of the culture was diluted with 9 mL of L medium. Then, 0.6 mL portions of this dilution were distributed into tubes containing 3.33 mM of *t*-BOOH and 10 μL of sample solution. These mixtures were incubated for 2 hr at 37°C with shaking. In order to prevent measurement problems due to the absorbance of water extract itself, centrifugation step (4,500×g, 15 min) was followed. The remaining bacterial pellets were resuspended in 0.6 mL of L medium. Subsequently, 0.2 mL portions were drawn into fresh tubes in order to make 2 sets of tubes. To determine the induced β-galactosidase activity, 1.8 mL of B buffer [$Na_2HPO_4 \cdot 12\ H_2O$ 40.6 g, $NaH_2PO_4 \cdot 2\ H_2O$ 6.22 g, $MgSO_4 \cdot 2\ H_2O$ 0.25 g, sodium dodecyl sulfate (SDS) 1.0 g, β-mercaptoethanol 2.7 mL/L, pH 7.0] and 0.4 mL of 0.4% ONPG solution were added to each test tube of 1 series. The mixtures were incubated for 15 min at 37°C in water bath. The incubation was stopped by adding 1.4 mL of 1 M sodium carbonate. The absorbance at 415 nm was read against a blank containing all ingredients except the bacteria. Determination of alkaline phosphatase activity was performed in a manner similar to the β-galactosidase assay except that B buffer was replaced the P buffer (trisaminomethane 121.0 g, SDS 1.0 g/L, pH 8.8) and ONPG solution replaced the 0.4% PNPP solution. The conversion of PNPP was stopped by adding 0.7 mL of 3.75 M hydrochloride and after 10 min, 0.7 mL of 2 M tris(hydroxymethyl)aminomethane solution was added. The absorbance was read at 415 nm. Positive controls were carried out as the above procedures except that 10 μL of distilled water replaced the sample solution.

The enzyme units, R value, and induction factor (IF) were calculated as described by Quillardet and Hofnung (9). The inhibition rate (%)=[IF(o)−IF(s)/IF(o)]×100. Here IF(o) represented induction factor in presence of mutagen only and IF(s) was induction factor in presence of mutagen and sample.

Hydroxyl radical scavenging activity The hydroxyl radicals were generated in a L-ascorbic acid-$CuSO_4$ system by reduction of Cu_2^+ and were assayed by the oxidation of cytochrome *c* (10). In this experiment, the hydroxyl radicals were generated in 3 mL of 1.5 mM sodium phosphate buffer (pH 7.4), containing 100 mM L-ascorbic acid, 100 mM $CuSO_4$, 12 mM cytochrome *c*, and 25, 50, and 100 μg samples, respectively. The mixture was incubated at 25°C for 90 min. The change in transmittance caused by the color change of cytochrome *c* was measured at 550 nm.

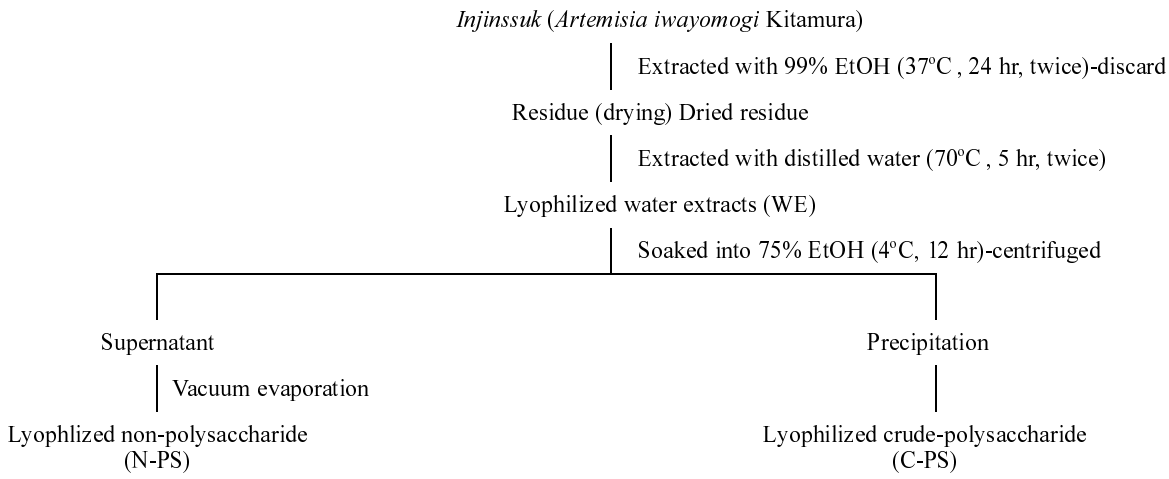

Fig. 1 Schematic diagram for the preparation of water extract and fractionation of crude polysaccharide and non-polysaccharide from *injinssuk*.

The hydroxyl radical quenching ability was calculated by determining the inhibition of the color changes.

Superoxide radical quenching activity Superoxide quenching activities of sample, hydroquinone, L-ascorbic acid, and glutathione were determined photochemicaly, using the assay system consisting of methionine, riboflavin, and NBT (11). The reaction mixture was composed of 1.3 mM riboflavin, 13 mM methionine, 0.63 M NBT, 0.05 M sodium carbonate (pH 10.2), and test sample or antioxidants. Three mL of the mixture was transferred, in triplicate, into a transparent glass serum bottle. The sample tubes were randomly placed in a light storage box and replaced randomly every 5 min for 10 min as previously described in detail (12,13). The temperature within the light storage box was 20°C during light illumination. The light intensity at the sample level was 5,500 lx. During the light illumination, NBT was reduced to blue formazane by the superoxide anion formed. The blue formazane formation was determined by measuring the absorbance at 560 nm. The inhibition of blue formazane formation was expressed as superoxide quenching activity.

Determination of carbohydrate content Total carbohydrate contents were determined in the diluted sugar solution according to the method Dubois *et al*. (14). Maltose used as a standard.

Determination of crude polysaccharide content The crude polysaccharide content (%) was calculated the weight of C-PS/weight of WE, as shown in Fig. 1 according to general method (15).

Statistical analysis The Statistical Analysis System (SAS) software ver. 6.11 was used to perform data analysis. All analysis were determined by Duncan's multiple range test at $p<0.05$.

Results and Discussion

Antimutagenic activity It was found that the water extract of *injinssuk* (WE) leaves exhibited significantly stronger inhibition activity than that of stems against *t*-BOOH-induced mutagenecity. Figure 2 shows the inhibitory activity of the water extracts of leaves and stems from *injinssuk* on the mutagenecity of *t*-BOOH in *Escherichia coli* PQ37 and the carbohydrate contents of both sample. The inhibition rates of WE of leaves and stems at concentration of 50 μg/assay were 20.33 and 12.0% against on the *t*-BOOH-induced mutagenicity, respectively. The WE of leaves showed inhibitory effects with dose response at a concentration of 10 to 250 μg/assay, and IC_{50} was 250 μg/assay (Fig. 3). This result suggested that carbohydrate content in *injinssuk* may be correlated with its' antimutagenic activity. It has been previously reported that some polysaccharides among carbohydrate components in other plants may be responsible for antitumor and antimutagenic activities and immunological property (13-15). We studied the antimutagenicity, carbohydrate, and crude polysaccharide contents of WE of leaves collected at 2 different harvest season. The results showed that there was a positive correlation of antimutagenic activity with crude

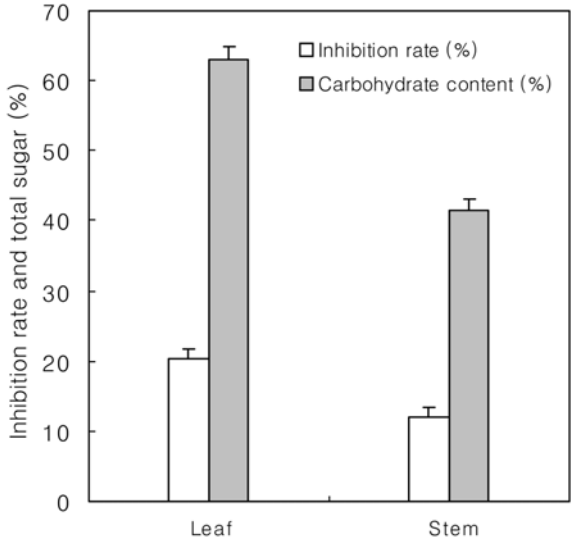

Fig. 2. Antimutagenic activities of water extracts (WE) of leaves and stems of *injinssuk* on the *tert*-buthyl hydroperoxide-induced mutagenicity in *E. coli* PQ37. The concentration of test samples was 50 μg/assay. Each value is the mean±SD (n=3).

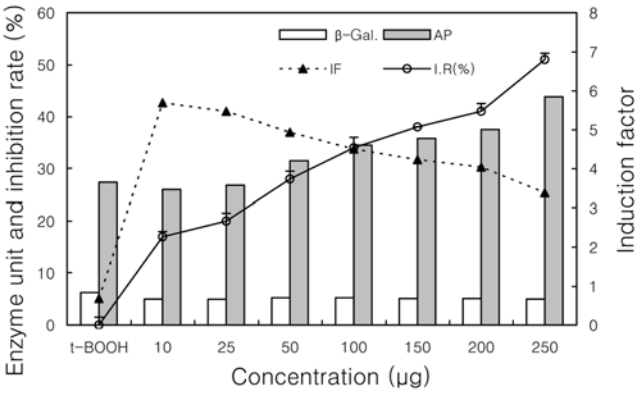

Fig. 3. The dose-dependent inhibitory effects of water extract (WE) obtained from the leaves of *injinssuk* on the *tert*-buthyl hydroperoxide-induced mutagenicity in *E. coli* PQ37. Each value is the mean±SD (n=3).

polysaccharide content of WE, but not with carbohydrate content of WE obtained from leaves. As shown in Fig. 4, the antimutagenic activity and polysaccharide content of WE harvested in May and September was 22.0, 11.0 and 40.0, 31.1%, but carbohydrate content showed 21.5, 29.0%, respectively. These results suggested that antimutagenic substance may be a polysaccharide component, and the general sugars may not be active compounds. Ahn *et al*. (7) reported recently that the ethanol insoluble part among the water extracts of *injinssuk* had the strong inhibitory effect against the mutagenicity of benzo[a]pyrene conversed by a mixed-function oxidase system to an ultimate metabolite (7). However, benzo[a]pyrene, the authors used in the study, was not a direct oxidative stress induced mutagenic compound. Antimutagenic activity by direct oxidative stress inducing mutagen and reactive oxygen scavenging activities of the polysaccharide extract of Artemisia species have never been previously reported.

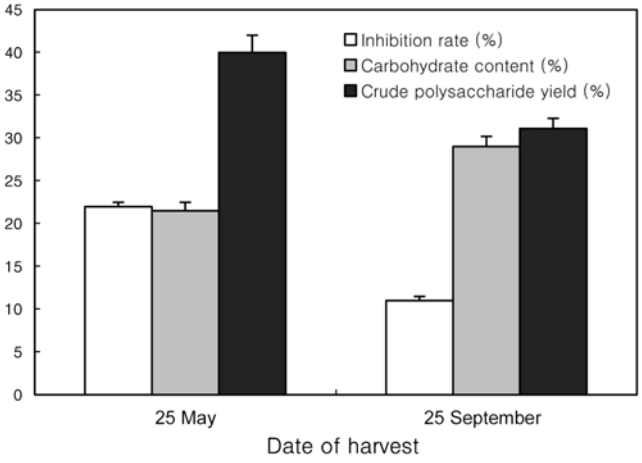

Fig. 4. Comparison of antimutagenic effects, carbohydrate and crude polysaccharide contents of water extracts (WE) of leaves from *injinssuk* collected at different seasons. *tert*-Buthyl hydroperoxide was used as a mutagen. The concentration of test sample was 50 μg/assay. Each value is the mean±SD (n=3).

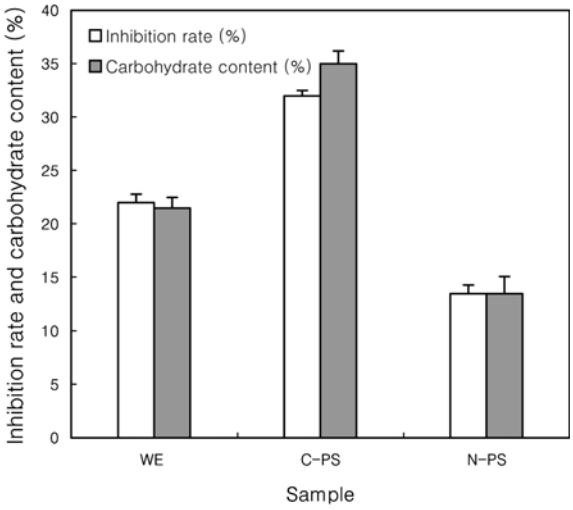

Fig. 5. Comparison of antimutagenic effects and carbohydrate contents of the water extracts (WE), crude polysaccharide (C-PS) and nonpolysaccharide (N-PS) fractions of leaves from *injinssuk* after extracted with ethanol. *tert*-Buthyl hydroperoxide was used as a mutagen. The concentration of test sample was 50 μg/assay. Each value is the mean±SD (n=3).

Antimutagenic activities of polysaccharide and non-polysaccharide fractions To confirm the idea that polysaccharides might be active components for the antimutagenecity of *injinssuk* on the oxidative stress induced mutagenecity, the WE was fractionated into 75% ethanol insoluble part (crude polysaccharide fraction, C-PS) and 75% ethanol soluble parts (non-polysaccharide fraction, N-PS) and the antimutagenecities of these C-PS and N-PS against *t*-BOOH inducing mutagenecity on *E. coli* PQ37 were investigated. Figure 5 showed that the inhibition rates of C-PS and N-PS at concentration of 50 μg/assay were 32.0 and 13.5% against *t*-BOOH-induced mutagenecity, respectively. C-PS showed the markedly higher inhibitory activity against mutagenecity than N-PS, further confirming that polysaccharides were active components for antimutagenic activity. As the C-PS concentration increased in the system, its' inhibitory activity against *t*-BOOH-induced mutagenecity increased in a dose-dependent manner. The treatments with 25, 50, 100, and 125 μg/assay showed 29.0, 37.0, 45.0, and 50.0% inhibition (Table 1). As Table 1 shown, alkaline phosphatase units tend to increased according to increasing concentration of C-PS. Both the decreased induction factor and increased alkaline phosphatase seemed to result from a remarkable increase in survival of *t*-BOOH-treated cells. These results suggested that a high concentration of C-PS was associated with a higher antimutagenic activity, but C-PS had no toxicity. The inhibitory activities of well known radical scavengers (ascorbic acid and glutathione) against the mutagenecity were tested along with C-PS. The 50% inhibition concentration (IC_{50}) of C-PS was obtained with an approximately 3-7 fold higher concentration than that of ascorbic acid and glutathione against on *t*-BOOH-induced mutagenicity (Fig. 5). C-PS showed distinct antimutagencity (IC_{50} 125 μg/assay), although its' activity was weaker than that of ascorbic acid (IC_{50} 25 μg/assay) and glutathione (45 μg/assay). A number of esculetin 6-methylether, esculetin 7-methylether (scopoletin), scopolin, β-sitosterol, chlorogenic acid, essential oil, fatty acid, sesquiterpene lactones, eudesmanolides, and flavonoids have been identified as the constituents of *injinssuk* (4), but the polysaccharide of *injinssuk* has never been studied to posses protective on oxidative DNA damage. These results suggested, for the first time, that C-PS of *injinssuk* had the strong antimutagenicity against direct oxidative stress-induced mutagenecity.

Table 1. Inhibitory effects of crude polysaccharide fraction from *injinssuk* on the mutagenicity of *tert*-buthyl hydroperoxide in *E. coli* PQ37

Concentration[1)] (μg/assay)	β-Gal (unit)	AP (unit)	β-Gal/AP	IF	Inhibition rate (%)
Negative control	2.76	60.73	0.50	1.00	
Positive control	6.96	32.66	0.21	4.68	
25	5.76	38.33	0.15	3.30	29.0±0.50[a]
50	5.44	40.00	0.13	2.96	37.0±0.50[b]
100	4.96	42.13	0.12	2.59	45.0±1.13[c]
125	4.60	42.80	0.10	2.36	50.0±0.50[d]

[1)]β-Gal, β-galactosidase activity; AP, alkaline phosphtase activity; IF, induction factor. Means with different superscript are significantly different at p<0.05.

Table 2. Hydroxyl radical scavenging activities of fractions from the water extraction of *injinssuk* leaves after ethanol washing

Samples	Conc. (μg/assay)	OD[1)] (550 nm)	Inhibition (%)
Control	$CuSO_4$	59.4±0.40	
OH-	Buffer	78.6±0.45	
Caffeic acid	100	75.1±0.05	18.23[a]
	500	60.1±0.02	96.35
Glutathione	25	78.8±0.20	-1.04
	50	75.6±0.62	15.62
	100	74.5±0.70	21.35[b]
WE	25	76.8±0.20	9.38
	50	74.0±0.52	23.95
	100	67.9±0.69	55.72[d]
C-PS	25	75.4±0.41	15.63
	50	69.5±0.30	47.40
	100	62.1±0.52	85.94[e]
N-PS	25	73.3±0.48	27.60
	50	72.5±0.25	31.77
	100	68.7±0.39	51.56[c]

[1)]Optical density are mean±SD of triplicate independent experiments. Means with different superscript at the 100 μg/assay are significantly different at $p<0.05$.

Table 3. Superoxide radical scavenging activities of fractions from the water extraction of *injinssuk* leaves after ethanol washing

Samples	Conc. (μg/assay)	OD[1)] (560 nm)	Inhibition (%)
O_2-	Buffer	0.181±0.002	
Blank	- NADP	0.004±0.00	
Hydroquinone	100	0.001±0.00	99.49[f]
Ascorbic acid	25	0.051±0.001	73.97
	50	0.050±0.002	74.49
	100	0.0034±0.005	82.65[e]
Glutathione	25	0.248±0.002	-26.53
	50	0.277±0.004	-41.32
	100	0.231±0.002	-17.86[a]
WE	25	0.150±0.002	24.74
	50	0.106±0.003	45.92
	100	0.044±0.005	77.55[c]
C-PS	25	0.106±0.005	45.92
	50	0.094±0.004	52.04
	100	0.054±0.006	72.45[b]
N-PS	25	0.083±0.002	57.63
	50	0.045±0.007	77.04
	100	0.040±0.005	79.59[d]

[1)]Optical density are mean±SD of triplicate independent experiments. Means with different superscript at the 100 μg/assay are significantly different at $p<0.05$.

Hydroxyl radicals scavenging activity and superoxide anion quenching ability Different free radicals are not equally reactive, which have certain kinetic and mechanistic properties, i.e., peroxy (ROO·), alkoxy (RO·), hydroxyl (·OH), and superoxide ($O_2^{\cdot -}$) radicals (18). We determined the 2 most representative reactive oxygen species (hydroxyl and superoxide radical) scavenging activities of WE, N-PS, and C-PS along with caffeic acid (a well-known effective hydroxyl radical scavenger) and hydroquinone acid (a well known effective superoxide radical quencher). C-PS showed strongest hydroxyl radical scavenging activity, followed by N-CP and caffeic acid at the same concentration of 100 μg/assay. C-PS, N-PS, and caffeic acid scavenged 85.94, 51.56, and 18.23% of hydroxyl radical at a concentration of 100 μg/assay, respectively (Table 2). It has been well known that caffeic acid is an antioxidant having strong hydroxyl radical scavenging activity. Our results clearly indicated that C-PS possessed exceptionally strong hydroxyl radical scavenging activity, even significantly higher than caffeic acid at the same concentration. It was also interesting to note that C-PS had the greatly stronger hydroxyl radical scavenging activity than N-PS, indicating strong hydroxyl radical scavenging activity of polysaccharides obtained from *injinssuk* possessed. As shown in Table 3, the superoxide anion quenching abilities of C-PS, N-PS, ascorbic acid, glutathione, and hydroquinone at concentration of 100 μg/3 mL were 72.45, 79.59, 82.65, –17.86, and 99.49% in the system, respectively. The results also shows that C-PS showed strong superoxide anion radical scavenging activity, its activity was close to those of ascorbic acid at the same treated concentration. It was interesting to note that glutathione did not show any scavenging activity on superoxide anion radical. The result also showed that the superoxide anion radical scavenging activity of C-PS was slightly lower than that of N-PS. It is also interesting to note that even though the superoxide quenching ability of C-PS was lower than that of N-PS, but antimutagenic and hydroxyl radical scavenging activities of C-PS was greater than N-PS. In brief summary, WE obtained from the leaves of *injinssuk* collected on May showed much higher protective activity on the oxidative stress induced antimutagenecity than that collected on September. From the study on the antimutagenicity, carbohydrate and crude polysaccharide contents of WE of leaves collected at 2 different harvest season, it was found that there was a positive correlation of antimutagenic activity with crude polysaccharide content of WE, but not with carbohydrate content of WE obtained from leaves.

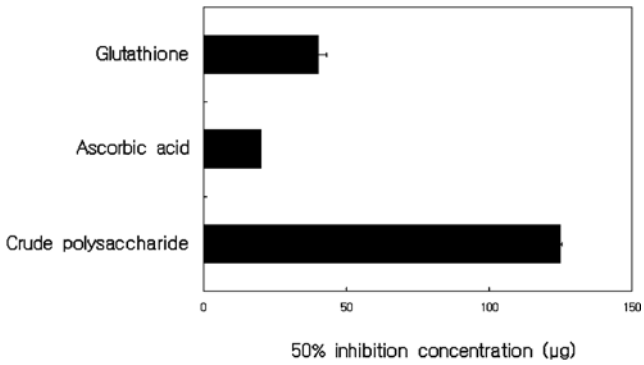

Fig. 6. IC50 values of C-PS, ascorbic acid and glutathione for the inhibition of *tert*-buthyl hydroperoxide-induced mutagenicity in *E. coli* PQ37. Each value is the mean±SD (n=3).

Crude polysaccharide fraction possessed exceptionally strong scavenging activity on the hydroxyl radical. It was also found that crude polyssacharide fraction had relatively strong scavenging activity on superoxide anion radical. Our result clearly shows for the first time that crude polysaccharide was an active component for the protective activity of *injinssuk* on the oxidative stress induced mutagenecity, and that the strong antimutagenetic activity might be due to its strong radical scavenging activity.

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