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Effect of Acanthopanax extract on the DNA and erythrocyte damage induced by herbicides

Yoo-Na Seo[1], Jum-Ji Kim[1], Kwang-Soo Sung[2] and Mi-Young Lee[1*]
[1]**Department of Medical Biotechnology, Soonchunhyang University**
[2]**Sushin Ogapy Co., Ltd**

제초제로 인한 DNA와 적혈구 손상에 미치는 오가피 추출물의 효과

서유나[1], 김점지[1], 성광수[2], 이미영[1*]
[1]순천향대학교 의료생명공학과
[2](주) 수신오가피

Abstract In order to investigate whether the ethanol extract of *Acanthopanax sp.* might inhibit herbicide-induced DNA damage and erythrocyte damage, the suppression of the oxidative DNA damage of lymphocyte and erythrocyte damage in the presence of the extract were evaluated by comet assay and hemolysis assay, respectively. Phenoxy herbicides, named 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) and bipyridyl herbicide paraquat induced oxidative DNA damages of lymphocytes. However, the oxidative DNA damage by 2,4-D, 2,4,5-T or paraquat was inhibited *in vitro* upon treating *Acanthopanax* extract. Moreover, the erythrocyte damage was also suppressed *in vitro* by *Acanthopanax* extract treatment.

요 약 오가피 에탄올 추출물이 제초제로 인한 DNA와 적혈구 손상을 억제할 수 있는지 알아보기 위해 코멧 어세이와 용혈반응을 사용하여 오가피 추출물 존재하에서 DNA 산화손상 억제와 적혈구 손상 억제 정도를 측정하였다. 페녹시계 제초제인 2,4-D (2,4-dichlorophenoxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) 그리고 바이피리딜계 제초제인 paraquat은 임파구 DNA에 산화적 손상을 유발하였다. 그러나 2,4-D, 2,4,5-T, 혹은 paraquat으로 인한 DNA 산화손상은 오가피 추출물 처리에 의해 시험관에서 억제되었다. 또한 적혈구 손상도 오가피 추출물 처리에 의해 시험관에서 억제되었다.

Key Words : Paraquat, 2,4-D, 2,4,5-T, Comet assay, Hemolysis, Acanthopanax

1. Introduction

2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) are widely used phenoxy herbicide family [1]. They are the chemicals related to the growth hormone indole acetic acid, thus they induce rapid and uncontrolled growth of plant and eventually kill them when sprayed on broad-leaf plants [2]. There have been many studies in the literature reporting adverse effects of phenoxy herbicides on environmental and human health [3]. Especially, 2,4,5-T is known to contain the contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) which is regarded as one of the most toxic compounds known to human [4]. Apart from agricultural uses, 2,4,5-T was a major ingredient of 'Agent Orange', a herbicide blend used by the U.S. military in Vietnam between January 1965 and April 1970 as a defoliant. Bipyridyl herbicide paraquat (1,1-dimethyl-4,4- bipyridinium dichloride, PQ), also known as a methyl viologen, has been commonly used as a weed controller and defoliant that induces oxidative stress in mammals by participating in redox cycling [5].

*Corresponding Author : Lee Mi-Young (miyoung@sch.ac.kr)
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Paraquat has been reported to be highly toxic, largely due to serious and irreversible effects, and paraquat-induced oxidative DNA damage was also known [6]. Paraquat is a suspected etiologic factor in the development of Parkinson's disease. Paraquat increased p53 protein level and its target genes, Bax [6]. The toxicity mechanism of paraquat was reported to involve: the generation of the superoxide anion, which can lead to the formation of more toxic reactive oxygen species, named hydrogen peroxide and hydroxyl radical; and the oxidation of the cellular NADPH, the major source of reducing equivalents for the intracellular reduction of paraquat, which results in the disruption of NADPH-requiring biochemical processes [7, 8]. The major cause of death in paraquat poisoning is respiratory failure due to an oxidative insult to the alveolar epithelium with subsequent obliterating fibrosis [8]. However, there has been little report concerning the natural substances existed in herbs that ameliorate the toxic effects produced by phenoxy herbicides and bipyridyl herbicides.

Acanthopanax is a typical oriental medicinal herb that enhances the strength, energy and general well-being for humans. The major active constituents of *Acanthopanax sp.* were eleutheroside, acanthoside, daucosterine, β-sitosterol, sesamine, and savinine [9]. It has been used clinically to treat cirrhosis, chronic bronchitis, hypertension, ischemic heart disease, gastric ulcer, rheumatism, and diabetes [10]. We also reported that *Acanthopanax sp.* has a suppressive effect on the allergic inflammation [11]. It remains unclear whether the *Acanthopanax* could suppress the DNA and erythrocyte damage induced by herbicides. Therefore, we investigated the inhibitory effect of *Acanthopanax* on the oxidative DNA damage and erythrocyte damage induced by herbicides.

2. Material and Methods

2.1 Sample preparation

The powdered ethanol extracts of *Acanthopanax sp.* were provided by Sushin Ogapy Co., Ltd (Cheonan-City, Chungnam, Korea), and used in this investigation.

A 400 μL of fresh whole blood from rats was added to 600 μL of phosphate-buffered saline (PBS) and layered onto 400 μL of Histopaque 1077 (Sigma-Aldrich, St. Louis, MO). After centrifugation at 1,450 rpm for 5 min at room temperature, the lymphocytes were collected from the layer just above the Histopaque 1077 boundary and washed in 1 mL PBS [12].

2.2 Determination of DNA damage by comet assay

To investigate the ability of *Acanthopanax* extract to inhibit oxidative DNA damage, lymphocytes were pre-incubated with various concentrations of *Acanthopanax* extract for 30 min at 37°C in the dark, and then treated with 50 μM paraquat, 2,4-D or 2,4,5-T for 5 min on ice. PQ was dissolved in PBS, and 2,4-D and 2,4,5-T were dissolved in DMSO. PBS or DMSO-treated sample was used as a control.

The alkaline comet assay was performed according to Singh *et al*. [13] with slight modifications, as reported previously [14]. The lymphocytes were mixed with 75 μL of 0.7% low-melting-point agarose and added to slides precoated with 1.0% normal-melting-point agarose. After the agarose solidified, the slides were covered with 100 μL of 0.7% low-melting-point agarose and immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl (pH 7.5), 1% sodium lauryl sarcosine, 1% Triton X-100, and 10% DMSO) for 1 h at 4°C. The slides were placed in an electrophoresis tank containing 300 mM NaOH and 10 mM Na_2-EDTA (pH 13.0) for 20 min to allow the DNA to unwind. Electrophoresis was performed at 25V/300 mA for 20 min at 4°C. The slides were washed with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) three times for 5 min at 4°C, and then treated with ethanol for 5 min.

2.3 Image analysis

The slides were stained with ethidium bromide (20 μg/mL) and covered with coverslips. The image was analyzed using Komet 5.5 software (Kinetic Imaging, Liverpool, UK) and fluorescence microscope (Leica, Wetzlar, Germany). To quantify DNA damage in the comet assay, the olive tail moment was calculated as: (Tail.mean-Head.mean) × Tail% DNA/100 [15]. A total of 150 randomly captured comets were examined from each slide.

2.4 Hemolysis assay

100 μL of erythrocyte suspension (2×10^8 cells/mL) was incubated with varying concentration of *Acanthopanax* extract, and then 7 mM 2,4-D, 2,4,5-T or paraquat was added to the mixtures for 1 h at 37°C. The degree of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm, as previously reported [16]. The absorbance of the control group was used as the blank.

2.5 DPPH assay

The DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical-scavenging assay was carried out as described earlier [17]. In brief, an aliquot of 100 μL sample at different concentrations was mixed with 100 μL of freshly prepared 500 μM DPPH. After incubation at 37°C for 30 min, absorbance at 520 nm was measured, and the percent of the activity was calculated.

2.6 Statistical analysis

The comet assay data were the means of three determinations and were analyzed using the SPSS package for Windows version 13 (SPSS Inc., Chicago, IL). The mean values of DNA damage for each treatment were compared using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Erythrocyte damage was measured using Duncan's multiple range test. $P < 0.05$ was considered significant.

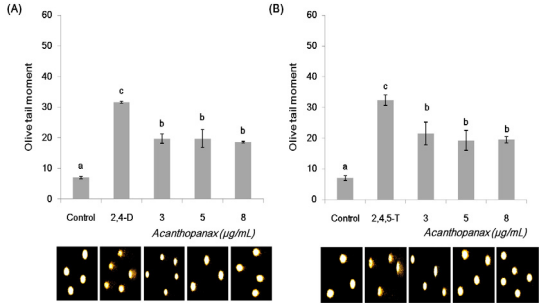
3. Results

3.1 Suppressive effects of *Acanthopanax* extract on the herbicide-induced DNA damage

The comet assay (single-cell gel electrophoresis assay) is a well-established genotoxicity test for estimating oxidative DNA damage at the individual cell level, both in blood and in cells [18].

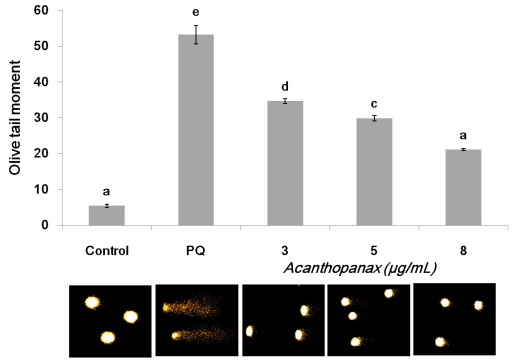
As shown in Figure 1 (A), lymphocytes treated with 2,4-D showed notable DNA damages, evaluated by the olive tail moment in a comet assay. The olive tail moment at 50 μM 2,4-D was about 31.60±1.51, compared with 7.10±0.38 in the DMSO-treated control, indicating a severe DNA damage with 50 μM 2,4-D. The addition of *Acanthopanax* extract inhibited the oxidative DNA damage caused by 2,4-D as demonstrated by the reduction of the olive tail moment. The olive tail moment at 3 μg/mL *Acanthopanax* was reduced up to 19.75±2.91, and similar levels of suppressive effects were also seen at 5 and 8 μg/mL of *Acanthopanax*-treated group.

The olive tail moment at 50 μM 2,4,5-T was about 32.35±1.66, compared with 7.03±0.76 in the DMSO-treated control, indicating a great DNA damage as shown in Figure 1 (B). Upon treating *Acanthopanax* extract the oxidative DNA damage by 2,4,5-T was suppressed to some extent, as demonstrated by the reduction of the olive tail moment. The olive tail moment at 3 μg/mL *Acanthopanax* was approximately 21.51±3.72, and the similar suppressive effects were also seen at 5 and 8 μg/mL of *Acanthopanax*.

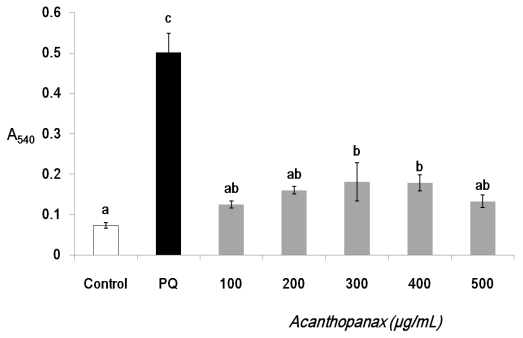


[Fig. 1] Effect of *Acanthopanax* extract on the phenoxy herbicide (A) 2,4-D and (B) 2,4,5-T-induced oxidative DNA damage in lymphocyte. Values not sharing the same letter are significantly different from one another ($P < 0.05$).

Figure 2 shows that *Acanthopanax* could inhibit paraquat-induced oxidative DNA damage, evaluated with comet assay. The DNA damage was induced by 50 μM paraquat, showing 53.20±2.52 of olive tail moment. The olive tail moment at 3 μg/mL *Acanthopanax*-treated group was approximately 34.66±0.70, and those at 5 and 8 μg/mL of *Acanthopanax*-treated group were approximately 29.91±0.75 and 21.17±0.29, respectively. The result suggests that *Acanthopanax* extract could suppress the oxidative DNA damage induced by paraquat *in vitro*.



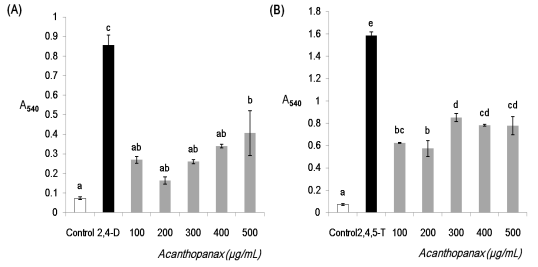
[Fig. 2] Effect of *Acanthopanax* extract on the bipyridyl herbicide PQ (paraquat)-induced oxidative DNA damage in lymphocyte. Values not sharing the same letter are significantly different from one another (P < 0.05).



[Fig. 4] Effect of *Acanthopanax* extract on the bipyridyl herbicide PQ (paraquat)-induced erythrocyte damage. Values not sharing the same letter are significantly different from one another (P < 0.05).

3.2 Suppressive effect of *Acanthopanax* extract on the herbicide-induced hemolysis

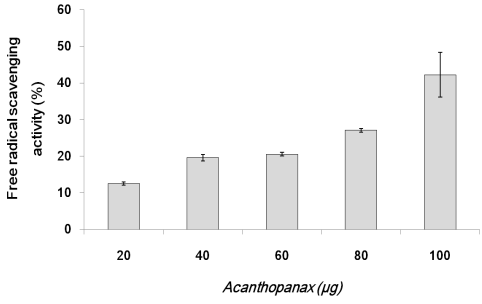
Figure 3 shows the suppressive effect of *Acanthopanax* extract on erythrocytes damage by 2,4-D and 2,4,5-T treatment, evaluated with hemolysis assay. The erythrocyte was damaged at 7 mM 2,4-D and 2,4,5-T with release of content hemoglobin into surrounding fluid [19]. However, 2,4-D and 2,4,5-T-induced hemolysis were prominently reduced by *Acanthopanax* treatment, demonstrated by the decrease in the absorbance at 540 nm (Fig. 3). The result suggests the hemoprotective effect of *Acanthopanax* extract against 2,4-D and 2,4,5-T *in vitro*. Moreover, *Acanthopanax* extract could suppress the hemolysis induced by paraquat as shown in Fig. 4, showing notable reduction in the absorbance of *Acanthopanax* extract-treated group.



[Fig. 3] Effect of *Acanthopanax* extract on the phenoxy herbicide (A) 2,4-D and (B) 2,4,5-T-induced erythrocyte damage. Values not sharing the same letter are significantly different from one another (P < 0.05).

3.3 Free radical scavenging ability of *Acanthopanax* extract

In order to examine whether the suppressive effects of *Acanthopanax* extract against DNA and erythrocyte damage induced by phenoxy herbicides and bipyridyl herbicide might be associated with the antioxidant activity of *Acanthopanax,* the free radical scavenging ability of *Acanthopanax* was determined by DPPH assay (Fig 5). DPPH assay evaluates the ability of *Acanthopanax* to scavenge free radicals of DPPH. The present DPPH assay suggest that *Acanthopanax* extract might possess free radical scavenging activity as shown in Fig 5.



[Fig. 5] Free radical scavenging activity of *Acanthopanax* extract by DPPH assay.

4. Discussion

The herbicides, named 2,4-D, 2,4,5-T and paraquat,

have been demonstrated to be a highly toxic compound for humans and animals and many cases of acute poisoning, diseases and death have been reported over the past few decades. The high mortality rate of herbicide exposure has been attributed to the lack of an antidote or effective treatment to reduce the toxic effects of the poison. However, the use of antioxidants as a therapeutic treatment for paraquat toxicity have been emphasized since the recognition that paraquat induces its toxic effects mainly via oxidative stress-induced mechanisms [20]. In this investigation, oxidatively damaged DNA and erythrocyte by 2,4-D, 2,4,5-T and paraquat were suppressed to some extent by *Acanthopanax* extract treatment *in vitro*. The results raise the important possibility that the suppressive effect of *Acanthopanax* might be due to the components with antioxidant activity, probably acting as radical scavengers. Most of the antioxidants used in treating herbicide-exposed humans and animals have failed to modify the toxicity of the herbicide largely due to their inability to cross cell membrane barriers [8, 20]. Therefore, their ability to cross cell membrane barrier might be important to detoxify the poison of the herbicide *in vivo*. Multiple strategy will be necessary to reduce the herbicide toxicity. Further studies will be required to understand the action mechanism and exact components responsible for the ability of *Acanthopanax* extract to suppress the herbicide-induced DNA damage and erythrocyte hemolysis in this investigation.

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Mi-Young Lee [Regular member]

- Feb. 1987 : Yonsei Univ., Biochem, MS
- Aug. 1991 : Yonsei Univ., Biochem, PhD
- Mar. 1992 ~ current : Soonchunhyang Univ., Dept. of Medical Biotehcnology, Professor

<Research Interests>
Biotechnology

Kwang-Soo Sung [Regular member]

- Jan. 1984 ~ current : Sushin Ogapy Farm
- Jan. 2001 ~ current : Sushin Ogapy Co. Ltd, President

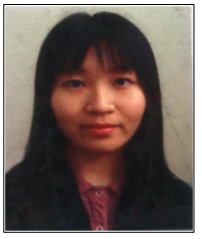
<Research Interests>
Functional Foods

Yoo-Na Seo [Associate member]

- Mar. 2009 ~ current : Soonchunhyang Univ., Dept. of Medical Biotechnology, Graduate school student

<Research Interests>
Biotechnology

Jum-Ji Kim [Regular member]



- Mar. 2007 ~ current : Soonchunhyang Univ., Dept. of Medical Biotechnology, Graduate school student

<Research Interests>
Biotechnology