



DNA Damage Effect of Botanical Insecticides Using Chinese Hamster Lung Cells

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Abstract

BACKGROUND: Botanical insecticides, especially *Azadirachta Indica* extract (AIE) and *Sophorae radix* extract (SRE) are widely used in Agriculture field. In our previous studies on genotoxicity test of AIE and SRE samples, a suspicious clastogenic properties was shown. Herein, we investigated the DNA damage effect of these botanical insecticide samples through the *in vitro* comet assay.

METHODS AND RESULTS: Chinese hamster lung (CHL) fibroblast cell line was used, and methyl methanesulphonate was as positive control. Respective two samples of AIE and SRE were evaluated using Single Cell Gel Electrophoresis (Comet) assay and measured as the Olive tail moment (OTM). Results from this study indicated that all tested AIE and SRE samples did not show DNA damage in comet assay using CHL cells, compared with control.

CONCLUSION: AIE and SRE samples used in this study were not cause genetic toxicity and are suitable for use as organic materials.

Key words: Botanical insecticide, Comet assay, DNA damage

Introduction

Unlike synthetic chemical pesticides, which leave harmful residues in the aquatic environment, botanical insecticides are believed to be more environmentally friendlier because they are easily biodegraded and leave no residues in the environment and their use is growing in agriculture field (Bhat *et al.*, 2012). As of August 2013, there are approximately 1,140 environmental-friendly organic materials that has been registered in South Korea. Especially, botanical insecticides such as *Azadirachta Indica* extract (AIE) and *Sophorae Radix* extract (SRE) account for 20% of the registered environmental-friendly organic materials.

Azadirachtin (Aza), as active ingredient of AIE, belongs to the organic compounds group known as tetranortriterpenoid and has known to act as an ecdysone blocker and an insect anti-feedant (Muangphra and Gooneratne, 2011). Matrine, one of the major active ingredient extracted from the traditional medicinal herb *Sophora flavescens*, has been known to be a very effective for botanical insecticide.

Despite their frequent use, studies on their

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toxicities and side effects are still sparse. Aza and matrine have generally been regarded as a relatively nontoxic substance for agricultural use and, for instance, there are only few reports on its genotoxic potential in the literature (Vinod *et al.*, 2011; Cho *et al.*, 2013; Yoon *et al.*, 2014).

We previously investigated the genotoxic effects of AIE and SRE through chromosomal aberration (CA) (Yoon *et al.*, 2014) and *in vitro* micronucleus (MN) assay (Cho *et al.*, 2013) using Chinese hamster lung (CHL) cells. Based on those results, all SRE samples had no genotoxic effect in both tests, but in the chromosomal aberration test, one of the SRE samples had potential clastogen properties, showing a suspicious positive result at 250 µg/ml in the presence of S-9 mix. However, genotoxicity tests that are designed to detect changes that occur in a particular index are not possible to detect any genetic toxicity.

Among many genotoxicity tests, comet assay, or single cell gel electrophoresis assay (SCGE) has been reported to evaluate DNA damage in single cells under alkaline conditions (Singh *et al.*, 1988). Recently, the popularity of the comet assay has increased because of its relatively simple and rapid procedures and high sensitivity (ability to detect carcinogens as positive). The reason why we focused on the comet assay in this study is that the comet assay was reported to be equally effective at detecting carcinogens that are gene mutagens or clastogens, and only declines slightly in sensitivity with compounds positive for MN *in vitro*. For instance, some compounds positive for MN *in vitro* that were negative in the comet assay (Kirkland and Speit, 2008).

In view of the above, we investigated the DNA damage effect of botanical insecticides having a suspicious clastogen property in our previous CA test through the comet assay in order to confirm the genotoxic evaluation of those samples using CHL cells. Respective two samples of SRE and ARE were tested for their possible genotoxic potential according to the alternative *in vitro* comet assay recently suggested internationally.

Materials and Methods

Cell culture and Materials

Cell culture. Chinese hamster lung (CHL) fibroblast cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells were

maintained in Eagle's minimum essential medium (EMEM, Gibco, Carlsbad, CA) supplemented with 1% penicillin-streptomycin and 10% heat-inactivated fetal bovine serum at 37°C in a 5% CO₂ atmosphere. The doubling time was about 13 h and cells were subcultured every 2-3 days.

Materials. Respective two samples of AIE and SRE were purchased from commercial products that are circulating in Korea. All AIE samples were from India, and their active ingredient azadirachtin were respectively 0.03% (AIE sample A) and 0.35% (AIE sample B). All SRE samples were from China, and their active ingredient matrine were respectively 0.3% (SRE sample A) and 0.26% (SRE samples B).

Reagents. Methyl methanesulphonate (MMS, CAS 66-27-3), sodium hydroxide (NaOH, CAS 1310-73-2), sodium chloride (NaCl, CAS 7647-14-5), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA-Na₂, CAS 6381-92-6), triton X-100 for molecular biology (CAS 9002-93-1), trizma base (CAS 77-86-1), ethidium bromide (EtBr, CAS 1239-45-8) and dimethyl sulfoxide (DMSO, CAS 67-68-5), normal melting agarose (NMA, CAS 9012-36-6) and low melting agarose (LMA, CAS 39346-81-1) were purchased from Sigma Aldrich (St Louis, MO, USA). NMA and LMA were diluted to 1% and 0.5% in Phosphate buffer saline (PBS, Gibco, Carlsbad, CA). MMS and test substances were dissolved in DMSO.

Experimental methods

Treatment. Prior to treatment, cells were seeded in 6 well plates at 1×10⁵ cell/mL and incubated for 24 h at 37°C. Three hours after the treatment of SRE, cells were collected and then gently resuspended with PBS. The cells were treated with 5 µM MMS, as a positive control.

Slide preparation. First layer on each clean slide was precoated with 1% NMA (200 µL). Second layer containing mixture of cell suspension (500 µL) and 1% LMA (500 µL) were spread onto each first layer and covered using coverslip. Slides were dried at 4°C for 10 min. Third layer on slide was coated with 0.5% LMA (200 µL). After hardening third layer, coverslips were removed and then slides were dried 4°C for 10 min.

Lysis, unwinding and electrophoresis. All steps were carried out in dark room. Slides were put in a stain jar that contained a lysis solution (pH 10.0) for 1 h at 4°C. The lysis solution consisted of 2.5 M NaCl,

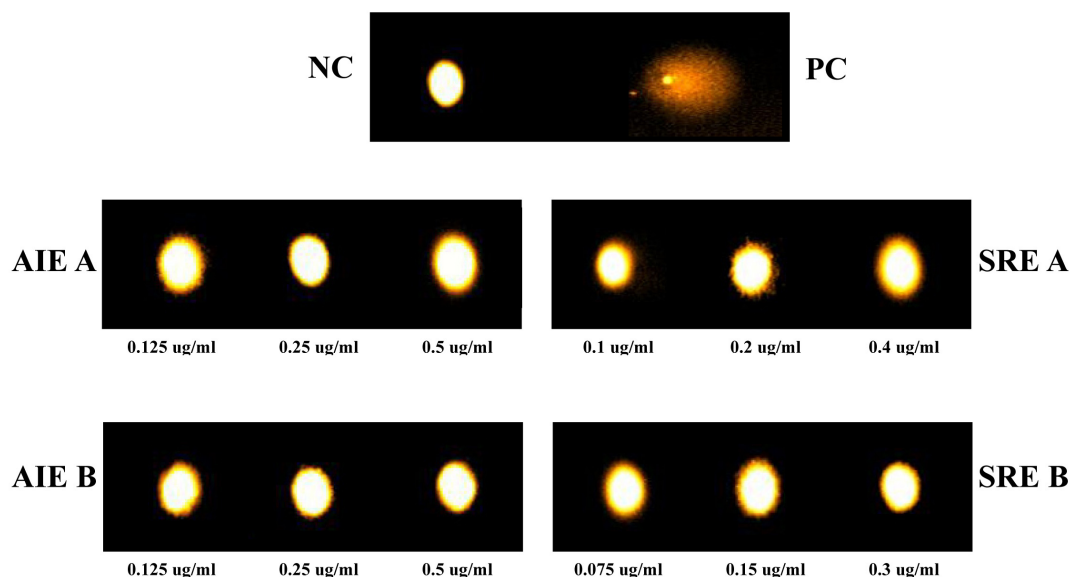


Fig. 1. Representative images of DNA damage of negative control, positive control, and the tested samples from comet assay.

NC; negative control(DMSO), PC; positive control(5 μ M MMS), AIE; *Azadirachta Indica* extract, SRE; *Sophorae radix* extract.

500 mM Na₂EDTA, 1 M Trizma base% and 10% DMSO. Prior to unwinding of DNA, the slides were washed three times with distilled water and kept in stain jar with unwinding buffer (pH>13) that consists of 10 N NaOH and 200 mM Na₂EDTA for 30 min at 4°C. Electrophoresis was carried out at 25 V and 300 mA for 20 min, using the alkaline buffer solution used for unwinding of DNA. After electrophoresis, slides were washed three times for 5 min with 0.4 M Tris buffer (pH 7.5), soaked in 70% ethanol and 100% ethanol for each 5 min, and then dried at room temperature for overnight.

Image analysis. EtBr was used for staining. The cell images were examined at 200 X magnification using a fluorescence microscope (Nikon TE2000-U, Japan). Captured images of 50 cell per slide were analyzed using image analysis software (Andor Komet 7.0, UK) to obtain olive tail moment. Olive tail moment, the parameter of DNA damage, was referred as distance between the center position of the head and the center of % DNA in tail.

Statistical analysis

The data was tested by one way analysis of variance (ANOVA) followed by Duncan's test using SPSS (Statistical Package for the Social Sciences, version 18.0). A probability of less than 0.05 was considered as statistically significant.

Results and Discussion

Azadirachta Indica extract (AIE)

Comet assay has been known as a simple method for measuring DNA damage in eukaryotic cells. The principle of the assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field, whereas undamaged DNA migrates slower and remains within the confines of the nucleoid when a current is applied (OECD, 2014).

Singh *et al.* (1988) developed the alkaline version of the comet Assay in which they used the length of DNA migration (tail length) to quantify the extent of damage. However, with time, most of them were not of frequent or wide use. After then, Olive *et al.* (1990) reported the concept of the tail moment to describe DNA migration. The tail moment came to be known as the Olive tail moment (OTM). This parameter is considered to be particularly useful in describing heterogeneity within a cell population, as OTM can pick up variations in DNA distribution within the tail. Therefore, we measured the OTM to describe DNA damage of CHL cells after treatment of AIE and SRE samples (Fig. 1).

Table 1 shows the effects of the AIE sample A and B on the DNA damage of CHL cells. OTM value of DMSO-treated negative control was 11.29 \pm 1.96, and

Table 1. Effects of the *Azadirachta Indica* extract sample A and B on the DNA damage in Chinese hamster lung cells

Treatment	Conc. ($\mu\text{g/ml}$)	OTM ^{a)}
Negative control (3% DMSO)		1.29 \pm 1.96
Positive control (5 μM MMS)		31.23 \pm 15.66*
AIE ^{b)} sample A	0.125	2.24 \pm 1.90
	0.25	1.83 \pm 1.83
	0.5	2.52 \pm 2.52
AIE sample B	0.125	2.56 \pm 2.19
	0.25	2.83 \pm 2.54
	0.5	4.00 \pm 3.60

DNA damage was measured as the OTM (olive tail moment); tail length \times % DNA in the tail. ^{a)} Value of olive tail moment \pm SD of 50 cells per slide of three experiments. *indicates significant at $p < 0.05$. ^{b)} AIE; *Azadirachta Indica* extract

that of positive control treated with 5 μM MMS was 31.23 \pm 15.66. OTM value of positive control showed significant increase of compared with that of negative control, indicating 5 μM MMS-treated group induced DNA damage of CHL cells.

The effects of AIE sample A and B were examined at three dose levels: 0.5 0.25 and 0.125 $\mu\text{g/ml}$. OTM values after treatment of AIE sample A of CHL cells were from 1.83 \pm 1.83 to 2.52 \pm 2.52, and those values after treatment of AIE sample B were 2.56 \pm 2.19 to 4.00 \pm 3.60 for three treated dose levels.

Although active ingredient content of AIE sample B was 10 times higher than that of the sample A, OTM values were similar between AIE sample A and B. All AIE samples treated groups did not show significant increase of OTM compared with negative control groups. Therefore, we evaluated that two AIE samples tested in this study were no genotoxic effect of DNA damage of CHL cells.

Sophorae radix extract (SRE)

Table 2 shows the effects of the SRE sample A and B on the DNA damage of CHL cells. The effects of SRE sample A was examined at three dose levels: 0.1, 0.2 and 0.4 $\mu\text{g/ml}$ and the OTM values after treatment of SRE sample A of CHL cells were from 4.91 \pm 0.40 to 6.86 \pm 0.95. The effects of SRE sample B was examined at three dose levels: 0.075, 0.15 and 0.3 $\mu\text{g/ml}$ and the OTM values after treatment of SRE

Table 2. Effects of the *Sophorae radix* extract sample A and B on the DNA damage in Chinese hamster lung cells

Treatment	Conc. ($\mu\text{g/ml}$)	OTM ^{a)}
Negative control (3% DMSO)		1.29 \pm 1.96
Positive control (5 μM MMS)		31.23 \pm 15.66*
SRE ^{b)} sample A	0.1	5.18 \pm 0.53
	0.2	4.91 \pm 0.40
	0.4	6.86 \pm 0.95
SRE sample B	0.075	5.18 \pm 0.25
	0.15	3.25 \pm 0.36
	0.3	3.44 \pm 0.43

DNA damage was measured as the OTM(olive tail moment); tail length \times % DNA in the tail. ^{a)} Value of olive tail moment \pm SD of 50 cells per slide of three experiments. *indicates significant at $p < 0.05$. ^{b)} SRE; *Sophorae radix* extract

sample B of CHL cells were from 3.25 \pm 0.36 to 5.18 \pm 0.25. Although active ingredient content of the sample B was less than that of the sample A, OTM values were similar between SRE sample A and B. All SRE samples treated groups did not show significant increase of OTM compared with negative control groups. Therefore, we evaluated that two SRE samples tested in this study were no genotoxic effect of DNA damage of CHL cells.

Our previous studies for respective two samples of AIE and SRE used in this study showed that all samples had no genotoxic effect in the reverse mutation test and the MN test, but in the chromosomal aberration test, one of SRE samples had potential clastogenic properties (Yoon *et al.*, 2014; Cho *et al.*, 2013). According to the genotoxicity battery system (ICH, 2012), SRE samples showing potential clastogenic properties in our previous study was need to be followed by sequential the *in vivo* genotoxic test such as *in vivo* MN test or *in vivo* comet assay. Herein, we tested the *in vitro* comet assay instead of the *in vivo* test to finally confirm the genotoxic effect of the SRE sample, because of recent growing preference on animal alternative tests. The SRE sample that showed potential clastogenic properties of CHL cells was no genotoxic effect of DNA damage of same cell line.

The substance used as positive control in our previous CA study was MMC, which is a bi-functional alkylating agent used in the treatment of

human cancers and one of the most potent direct-acting clastogens (Vinod *et al.*, 2011). Based on our previous CA study, AIE samples did not have a clastogenic properties of CHL cell. Vinod *et al.* also reported that neem oil sample probably exerts the anticlastogenic effect by influencing the enzymes required for repair or probably by the inhibition of the phase I enzymes required for the metabolic activation *in vivo* MN test (Vinod *et al.*, 2011). As described above, neem-based products seemed to be used more functional-friendly agents such as cosmetics, not focusing on the side effects when it was used.

Although some studies reported the clastogenic potential of azadirachtin for use of AIE or SRE samples, other influencing factor such the extraction method, extraction solvents, the extracted parts of plant, or active ingredient contents of the extracts, etc were suggested to induce directly clastogenic properties (EFSA, 2011, Cho *et al.*, 2013). In addition, botanical insecticides such as AIE or SRE samples are a complex mixture containing several chemicals such as fatty acids, aliphatic compounds, polyphenolics (flavonoids and their glycosides), and an active ingredient such as terpenoids is difficult to speculate regarding the nature of chemicals that are responsible for the clastogenic or mutagenic action (Vinod *et al.*, 2011).

Overall, results from this study indicate that AIE and SRE samples are not mutagenic and clastogenic effects which is any adverse genotoxic effects which may arise during long-term application. Thus, these samples can be useful as botanical insecticides in cultivating the environmental-friendly organic agricultural crops.

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