



Original Article

Cytotoxic and genotoxic activities of an aqueous extract from Thai Noni leaves in human lymphocytes *in Vitro*

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Abstract

The aims of this study were to investigate the cytotoxic and genotoxic activities of an aqueous extract from Thai Noni's leaf (ANL) in human lymphocytes *in vitro*. Chromosome aberration and sister chromatid exchange (SCE) assays were performed. Treatment of ANL (0.8-25 mg/ml) for 3 h did not significantly induce chromosomal aberration nor SCEs ($p < 0.05$). Nevertheless, ANL could induce cytotoxic activities as shown by their toxic level of mitotic index in a dose dependent manner. Only toxicity induced at lower doses of ANL (≤ 3.1 mg/ml) could be recovered as shown by their normal level of the proliferation index. ANL at higher dose of 50 mg/ml induced potent cytotoxicity as no mitotic cell was found. Interestingly, metaphase chromosomes undergoing tetraploidization were found from ANL treatment at 12.5 and 25 mg/ml. These indicate that ANL treatment could interrupt cell cycle that progressed after S phase. Therefore, the optimum dose of ANL as food supplement needs to be considered carefully for human safety. Nevertheless, ANL at doses lower than 3.1 mg/ml might be useful for treatment of human hyperproliferative disorder at appropriate dose since ANL could induce temporary cytotoxicity without genotoxicity. Scientific study is also needed to verify the usefulness of an aqueous extract of Noni's leaf.

Keywords: cytotoxicity, genotoxicity, Noni's leaves, chromosome aberration assay, sister chromatid exchange assay

1. Introduction

Noni (*Morinda citrifolia* L.; Rubiaceae) is an evergreen tree commonly found in Southeast Asia, Australia, Africa, Hawaii and the island of French Polynesia. The fruits, leaves, and roots of Noni have been traditionally used to treat various symptoms and diseases such as cancer, malaria,

diabetes, arthritis and topical inflammation (McClatchey, 2002; Olajide *et al.*, 1999; Makinde and Obih, 1985). West *et al.* (2009) reported that the crude ethanol extract of *M. citrifolia* leaves could mitigate UVB-induced erythema and exhibit potential anti-inflammatory activity in a histamine H-1 receptor antagonism assay. The methanol extract of *M. citrifolia* leaves showed antibacterial activities against gram positive *S. aureus* and Methicillin Resistant *S. aureus* (Zaidan *et al.*, 2005). Previous phytochemical study reported that Noni's leaves contain a variety of phytochemical constituents such as terpenoids, phytosterol, fatty acids, flavonoid glyco-

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sides, iridoid glycosides and anthraquinone (Calzuola *et al.*, 2006; Takashima *et al.*, 2007). Noni's leaves are reported to have high antioxidant activity probably belonging to the families of reducing glycosides and polyphenols (Calzuola *et al.*, 2006; Chong *et al.*, 2004). The nitric oxide scavenging activity of these leaves was also reported which could explain a mechanism of anti-inflammation of Noni's leaves (Basu and Hazra, 2006). The use of these leaves to make hot water beverages is increasing in popularity, especially in Japan, Malasia, Thailand and the United States. However, there have been very few studies on cytotoxic and genotoxic activities of Noni's leaf extract. Some mutagenic and genotoxic activities of *Morinda lucida* (root) using *Escherichia coli* (0157:H7) mutagenic test (modified Ames test) and the Allium cepa assay in onion bulb were reported (Akintonwa *et al.*, 2009). In this research, we examined the cytotoxic and genotoxic activities of an aqueous extract from Thai Noni's leaves (ANL) by cytogenetic assays in mammalian cells. Chromosomal aberration and SCE assays were conducted in human lymphocytes *in vitro*.

2. Materials and Methods

2.1 Thai Noni's leaves

Noni's leaves were collected from Noni trees grown in the central part of Thailand. Only leaves that were not too young or too old with the medium size about 6x10 cm were used and cut into small pieces. Then they were dried in an incubator at 50°C. The yield of the dried leaves was about 29 g % (w/w).

2.2 Analysis of the composition profile of Noni's dried leaves

The dried leaves were analyzed for carbohydrate, protein, ash, and moisture using AOAC methods (Official Methods of Analysis, 1995). Total fat was determined by the Caviezel method, based on a gas chromatographic technique (Pendl *et al.*, 1998).

2.3 An aqueous extract from Thai Noni's leaves (ANL)

An aqueous extract from Noni's leaves was prepared by the Research Center, Faculty of Medicine, Thammasat University, Thailand. Briefly, dried leaves were placed in a conical flask containing water and boiled for 30 min. The extract was filtered under slight vacuum through a Buchener funnel lined with filter paper. The solid residue was extracted twice more under the same conditions. All the extracts were pooled and lyophilised. The dried sample was weighed and kept it in the refrigerator at 4°C until used. An aqueous extract of the Noni's leaves (ANL) was obtained by adding distilled water and mixed vigorously achieving as concentrated liquid extract. Finally, the extract was filtered through 0.2 µm syringe filter before used.

2.4 Cytotoxic and genotoxic studies

2.4.1 Cell cultures

Fresh blood samples for all experiments were obtained by venipuncture from 5 healthy volunteers, age 25-35 years with no recent exposure to radiation or drugs. These studies were approved by our institutional ethical committee. Lymphocyte-enriched buffy coat from 1 ml whole blood (~0.8-2 million lymphocytes) was cultured in 10 ml sterile culture tube containing culture medium of RPMI1640 (Hyclone, U.S.A.), fetal bovine serum (Hyclone, U.S.A.), autologous plasma, penicillin-streptomycin (Seromed, Germany), phytohemagglutinin (Seromed, Germany) and L-glutamine (Hyclone, U.S.A.) using standard blood culture conditions as previously described (Chiewchanwit and Au, 1994). At 24 h after initiation of the culture, the lymphocyte cultures were centrifuged for packed cells and the supernatant medium was removed and saved for reuse after treatment. The remaining lymphocytes were treated with ANL at concentrations of 0.8-50 mg/ml in plain RPMI 1640 culture medium at 37°C for 3 h. For positive control, the lymphocytes were treated with a chemotherapeutic agent, Mitomycin C (MMC) (Sigma-Aldrich, U.S.A.) at a concentration of 3 µg/ml for 3 h. Lymphocytes treated with plain RPMI 1640 for 3 h were used as a negative control. After treatment, all lymphocyte cultures were centrifuged for packed cells. The supernatant medium was discarded and the saved culture medium was reused. Bromodeoxyuridine (BrdU) solution (Sigma-Aldrich, U.S.A.) was added to the culture medium for the final concentration at 5 µM and cells were continued to culture at 37°C in the dark.

2.4.2 Cell harvesting, staining and scoring

1) For chromosomal aberration assay

According to cell cycle delay resulting from our treatment, we extended the harvest time to be at 53 h after initiation instead of at 48 h after initiation for chromosomal aberration study. Prior to the harvest time, colcemid solution (Seromed, Germany) was added to the cells for 1.5 h to block cells at the metaphase stage. Cells were harvested by centrifugation for packed cells and the supernatant was discarded. Cells were continually treated with hypotonic solution and fixed with Carnoy's fixative solution. Slides were prepared and stained with the fluorescent plus Giemsa technique according to our previous protocol (Chiewchanwit and Au, 1994). First, slides were placed in 40 ml of 12.5 µg/ml Bisbenzimide (Hoechst 33258) aqueous solution (Sigma-Aldrich, U.S.A.) for 10 min. Then, they were rinsed through distilled water and mounted with a few drops of McIlvane's buffer. Slides were then exposed to UV light for 30 min on a slide heater. After the UV exposure, slides were washed thoroughly with distilled water and then stained with a 10% aqueous solution of Giemsa (Biotech reagents, Thailand).

Slides were air-dried and mounted. Three independent experiments were performed. From each experiment, 50 cells showing the first metaphase-staining pattern (homogeneously stained chromosomes) were scored from coded slides for the frequencies of chromosomal aberration including chromatid-type aberrations and chromosome-type aberrations. Mitotic Index determined as the total number of mitotic cells/1,000 cells were also evaluated.

2) For sister chromatid exchange (SCE) assay

To harvest cells for the SCE assay, a similar procedure to that for chromosome aberration assay was used except that harvesting was done at 77 h after initiation. Fluorescent plus Giemsa technique was performed for staining. From each experiment, 25 cells showing the second metaphase staining (MII phase) were scored from coded slides for the frequencies of SCEs. Proliferation index determined as (MI+2MII+3MIII)/100 cells were also evaluated.

2.5 Statistical analysis

Raw data obtained from the chromosome aberration and SCE assays were transformed to stabilize the variance by the procedures of Whorton *et al.* (1984):

Transformed chromosome aberration (CA_T):

$$CA_T = 1/2(\sqrt{CA} + \sqrt{(CA+1)})$$

Transformed sister chromatid exchange (SCE_T):

$$SCE_T = \sqrt{SCE}$$

Dunnett's t-test was performed to analyse the difference between the mean of the treated groups and of the control group using the transformed data.

3. Results

Analysis of the composition of dried Thai Noni's leaves was shown in Table 1. The yield of the dried from fresh leaves was about 29 g % (w/w). In comparison with Thai Noni fruit juice (NFJ) we reported earlier (Ratanavalachai, 2008), Thai Noni dried leaves had higher content of carbohydrate, lipid and protein compositions. The yield of an aqueous extract from Noni's dried leaves was about 20 g % (w/w). Moisture content of ANL was about 24 g % (w/w).

and the pH of the ANL dissolved in distilled water was 5.0 which is higher than that of Thai NFJ (pH 3.0).

3.1 *In vitro* genotoxic studies of ANL by chromosome aberration assay using human lymphocyte

As shown in Table 2, no significant induction of structural chromosomal aberration in human lymphocytes *in vitro* was found in ANL treatment at concentrations of 0.8-25 mg/ml for 3 h. However, treatment at doses over than 25 mg/ml of ANL tended to induce more genotoxic activities in a dose dependent manner. ANL treatment at the highest concentration of 50 mg/ml induced strong cytotoxicity as no mitotic cell was observed. Treatment with the positive control (MMC 3 μ g/ml) showed a significant increase in chromosome aberrations as compared to ANL treatment groups and the negative control ($p < 0.05$).

3.2 *In vitro* genotoxic studies of ANL by sister chromatid exchange (SCE) assay using human lymphocytes

As shown in Table 3, no significantly difference in SCE level was observed in human lymphocytes induced by all ANL treatments (0.8-25 mg/ml) for 3 h as compared to the negative control (plain RPMI). In contrast, treatment with MMC (the positive control) at 3 μ g/ml significantly increased the SCE level above the negative control ($p < 0.05$).

3.3 *In vitro* cytotoxic studies of an aqueous extract from Thai Noni's leaves (ANL) as demonstrated by mitotic index (M.I.) and proliferation index (P.I.) value

As shown in Table 4, mitotic index (M.I.) levels of all ANL treatments (0.8-25 mg/ml) measured at 53 h after initiation were significantly lower than that of the negative control ($p < 0.05$). All these M.I. levels are at the same toxic level as that of the positive control (MMC 3 μ g/ml). It should be noted that observation of metaphase chromosomes undergoing tetraploidization as shown in Figure 1b (compared to normal metaphase chromosomes in Figure 1a) were found from ANL treatment groups only at high concentrations of 12.5 and 25 mg/ml. In addition, proliferation index (P.I.) measured at 77 h after initiation was shown that P.I. of ANL treatments at concentrations of 0.8-3.2 mg/ml were signifi-

Table 1. Composition profile of Thai Noni's dried leaves

Composition	Thai Noni's dried leaves	Thai Noni fruit juice (Ratanavalachai <i>et al.</i> , 2008)
Total carbohydrate	61-63 g/100 g	13-15 g/100 g
Total fat	3-5 g/100 g	0.4-0.5 g/100 g
Protein	10-12 g/100 g	0.7-0.9 g/100 g
Moisture	9-11 g/100 g	84-85 g/100 g
Ash	0.1-0.2 g/100 g	0 g/100 g

Table 2. Chromosomal aberration studies in human lymphocytes induced by various concentrations of an aqueous extract from Thai Noni's leaves (ANL) (n=3)

Concentration of Thai ANL (mg/ml)	% Abnormal cell +S.E.M.	Chromatid breaks/ 100 cells+S.E.M.	Chromatid exchanges/ 100 cells+S.E.M.	Total Chromatid- type aberrations/100 cells+S.E.M.
0	0.7±0.7*	0.7±0.7*	0.0±0.0*	0.7±0.7*
0.8	0.7±0.7*	0.7±0.7*	0.0±0.0*	0.7±0.7*
1.6	0.0±0.0*	0.0±0.0*	0.0±0.0*	0.0±0.0*
3.1	0.7±0.7*	0.7±0.7*	0.0±0.0*	0.7±0.7*
6.2	0.0±0.0*	0.0±0.0*	0.0±0.0*	0.0±0.0*
12.5	1.3±1.3*	2.7±1.3*	0.0±0.0*	2.7±1.3*
25	3.4±1.8*	5.4±0.6*	1.4±1.4*	6.8±0.8*
50	toxic	toxic	toxic	toxic
MMC 3 µg/ml	86.7±6.4#	203.7±22.8#	159.7±28.9#	363.3±47.8#

significant different from the negative control (plain RPMI) (p<0.05)

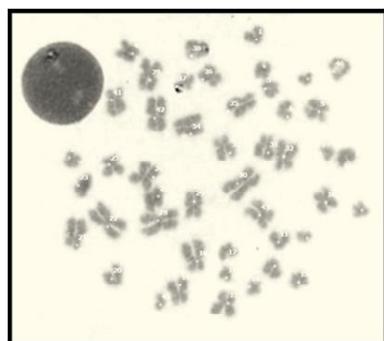
* significant different from the positive control (MMC 3 µg/ml) (p<0.05)

Table 3. Sister chromatid exchange (SCE) studies in human lymphocytes induced by various concentrations of an aqueous extract from Thai Noni's leaves (ANL) (n=3)

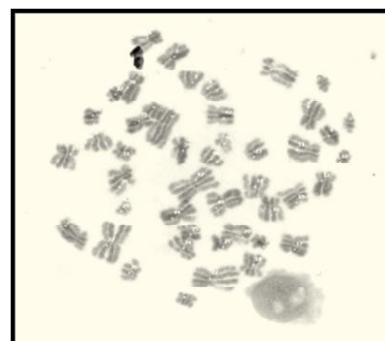
Concentration of Thai ANL (mg/ml)	SCE/cell+S.E.M.	Transformed SCE (SCE _T)/ cell+S.E.M.
0	4.9±0.3	2.1±0.1*
0.8	4.8±0.4	2.1±0.1*
1.6	5.3±0.5	2.2±0.1*
3.1	4.6±0.4	2.0±0.1*
6.2	4.9±0.4	2.1±0.1*
12.5	4.8±0.3	2.1±0.1*
25	5.9±0.6	2.3±0.1*
MMC 3 µg/ml	44.5±2.6	6.5±0.2#

significant different from the negative control (p<0.05)

* significant different from the positive control (MMC 3 µg/ml) (p<0.05)



(a)



(b)

Figure 1. (a) Normal metaphase chromosomes (b) Metaphase chromosomes undergoing tetraploidization

cantly higher than that of the positive control (p<0.05) and were at the same normal level as that of the negative control. Only P.I. of the 12.5 mg/ml ANL treatment were significantly lower than that of the negative control (p<0.05).

4. Discussion and Conclusion

Our data demonstrated that ANL treatment at concentrations of 0.8-25 mg/ml did not induce chromosome aberra-

Table 4. Mitotic index (M.I.) and proliferation index (P.I.) of lymphocyte culture after treatment with various concentrations of an aqueous extract from Thai Noni's leaves (ANL) (n=3)

Concentration of Thai ANL (mg/ml)	M.I. +S.E.M.(53 h)	P.I. +S.E.M.(77 h)
0	47.4 \pm 4.3*	11.2 \pm 1.2*
0.8	17.8 \pm 0.3 [#]	8.3 \pm 2.0*
1.6	15.8 \pm 2.8 [#]	7.6 \pm 2.0*
3.1	20.3 \pm 0.2 [#]	8.6 \pm 1.0*
6.2	16.9 \pm 4.2 [#]	6.4 \pm 1.0
12.5	17.2 \pm 1.5 [#]	5.2 \pm 0.2 [#]
25	16.2 \pm 3.1 [#]	6.3 \pm 0.1
MMC 3 μ g/ml	16.4 \pm 2.8 [#]	2.0 \pm 0.6 [#]

[#] significant different from the negative control (p<0.05)

* significant different from the positive control (MMC 3 μ g/ml) (p<0.05)

Abbreviation: P.I.: proliferation index = (MI+2MII+3MIII)/100 cells;

M.I.: mitotic index = number of mitotic cells/1,000 cells

tions nor SCE in human lymphocytes *in vitro*. Therefore, no genotoxic effect was found in all ANL treatment groups but all concentrations induced cytotoxicity. Mitotic indices of those ANL treatments were nearly at the same toxic level as that of the MMC treatment. In comparison with our previous study using Thai Noni fruit juice (NFJ), NFJ treatments (3.1-100 mg/ml) did not induce any cytotoxic nor genotoxic to human lymphocytes *in vitro* (Ratanavalachai, 2008). However, the cytotoxicity induced by ANL treatments at concentrations of 0.8-3.2 mg/ml could be reversible after cells proliferated further. P.I. of these low doses of ANL treatments were in the same normal level as that of the negative control. These data indicate that ANL treatments at low doses induced just temporary cytotoxicity to human lymphocytes. ANL treatments at higher doses of 12.5 and 25 mg/ml induced tetraploidization of the chromosomes. In general, after cell division, human lymphocytes could generate 2 daughter cells within 24 h of the cell cycle time. All series of events required for the cell division are needed such as centrosome cycle and chromosomal cycle. According to the S phase of the cell cycle, DNA synthesis occurred and sister chromatids could be seen. Our figure 1b had demonstrated that there were sister chromatids pairing together. Therefore, DNA synthesis had occurred, however, they were interrupted to progress further through cell division. A possible mechanism of the interruption could be due to a temporary disruption of the centrosome replication cycle. Supportive evidence has been provided by Meraldi *et al.* (2002) who reported that over-expression of Aurora-A, a protein kinase in centrosome cycle, could lead to tetraploidization as a result of centrosome amplification in Mouse embryo fibroblast (MEF) cells with absence of p53 (p53^{-/-}) cells. On the other hand, Steigemann *et al.* (2009) showed that Aurora B, another protein kinase in centrosome cycle, could protect cells against tetraploidization. Therefore, it is possible that ANL can interfere with

centrosome cycle and lead to tetraploidization at high doses. This possibility needs to be validated by conducting mechanistic studies.

In conclusion, this study demonstrates that ANL treatment at concentrations of 0.8-25 mg/ml were not genotoxic but cytotoxic to human lymphocytes *in vitro*. However, treatment with ANL at low doses (<3.2 mg/ml) were just temporary cytotoxic. Therefore, ANL use as herbal medicine might be possible for treatment of some antiproliferative diseases since they could induce cytotoxicity without genotoxicity. Nevertheless, more scientific data about ANL activities in human *in vivo* are needed to clarify the use of ANL as therapeutic medicine. Drinking tea beverages made from Noni's leaves as food supplement in concentrated form and continuous dosage is a cause for concern. Genetic polymorphism such as extensive or poor metabolizer in each individual is also important in modifying the susceptibility of the ANL toxicity. Good manufacturing practice for the ANL products are needed to be emphasized as well.

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