



## Research Article

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### **MORINGA OLEIFERA LAM. LEAVES EXTRACT REDUCES HUMAN T-CELL HYPORESPONSIVENESS AND DNA DAMAGE INDUCED BY OXIDATIVE STRESS**

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#### ABSTRACT

Chronic inflammatory conditions lead to the generation of excessive reactive oxygen species which can give rise to T cell hyporesponsiveness. Thus there has been an interest to target these mediators with nutrients which have anti-oxidant properties. Accordingly *Moringa oleifera* extract has been reported to have anti-inflammatory and anti-oxidant properties. We have now examined the effects of *Moringa oleifera* Lam. on the depressed human T-cell responses, (IL-2 and IL-2 mRNA production) and DNA damage (quantifying the oxidized derivative of deoxyguanosine, 8-OHdG) induced oxidative stress. T-cells treated with oxidative substances, uric acid, aluminum chloride, hydrogen peroxide, and ultraviolet irradiation showed marked reduction in IL-2 production, which was prevented by pre-treating the cells with Moringa extract. These effects correlated with its prevention of the down-regulation of *IL2* gene expression and a reduction in the DNA damage caused by oxidative stress. Thus the ability of Moringa extract to reduce inflammation is likely to relate to its ability to prevent T-cell hyporesponsiveness experienced in inflammatory diseases, through an action at the pre-transcriptional level.

**Keywords:** *Moringa oleifera* extract; oxidative stress; T-cell responses; Oxidative DNA damage

#### INTRODUCTION

In tissues of patients with chronic inflammatory disease excessive reactive oxygen species are generated. At least one of the consequences of prolonged exposure to these is the inhibition of T-cell responses, differential regulation of T<sub>H1</sub> versus T<sub>H2</sub> development and inhibition of T regulatory cell responses.<sup>1</sup> The ability of cells to regulate their levels of reactive oxygen species (ROS) through intracellular anti-oxidants such as Glutathione is critical in the deployment of physiological responses. Unfortunately in chronic inflammatory conditions both the intracellular environment and inflammatory fluids are skewed towards excess ROS.<sup>2</sup> Several studies have demonstrated that exposure of T-cells to either ROS or reactive nitrogen species (RNS) leads to decreased T-cell proliferation and migration.<sup>1,3,4</sup>

Supplementation with nutrient anti-oxidants has become of wide interest in chronic inflammatory diseases where patients experience increased oxidative stress. *Moringa oleifera* extracts have been considered as supplements in the treatment of chronic inflammatory diseases. Previous studies have reported that *Moringa oleifera* Lam. protects in experimental inflammatory models.<sup>5</sup> This includes collagen-induced paw inflammation and colitis.<sup>6</sup> However, despite these results little is known on the mechanisms of action of the extract. We have examined the effects of *Moringa oleifera* Lam. on human T-cell responses and DNA damage under an oxidative stress environment.

For the first time we present evidence that human T-cell responses, production of IL-2, impaired by an oxidative stress environment, is normalized by the addition of *Moringa oleifera* Lam. extract, seen at the gene transcription level. Furthermore the extract was found to prevent DNA damage precipitated under these oxidative stress conditions.

#### MATERIALS AND METHODS

##### *Moringa oleifera* Lam. leaves extract

Fresh leaves of *Moringa oleifera* Lam. were collected, washed, and dried using paper towels. The extract was prepared by homogenizing the Moringa leaves followed by acidified aqueous-methanol (1% acetic acid, 50% methanol) (RCI Lab scan Limited, Bangkok, Thailand) treatment for 7 days. The aqueous crude extract was prepared under evaporation, and the preparation was partitioned with diethyl ether (RCI Lab scan Limited, Bangkok, Thailand) to fractionate the non-polar substances. The pH of the extract was adjusted to 8.5 using sodium bicarbonate (Thermo Fisher Scientific, Pittsburgh, PA, USA) and then partitioned with chloroform (RCI Lab scan Limited, Bangkok, Thailand) to separate the non-phenolic fraction. The pH of the aqueous fraction of the extract was then adjusted to 3.5 with acetic acid and partitioned with ethyl acetate (RCI Lab scan Limited, Bangkok, Thailand) to obtain the phenolic-enriched fraction of the *Moringa oleifera* Lam. leaves extract that was used in the present study.<sup>7</sup>

### Isolation of human T-cell lymphocytes

Human blood "buffy coat" was kindly provided by Naresuan University Hospital, Phitsanulok, Thailand. The use of buffy coat for this project received ethical approval from Naresuan University Ethics Committee. Mononuclear cells (PBMCs) were isolated by density gradient centrifugation using 1.077 g/ml Lymphoprep solution (Axis-Shield PoC AS, Rodeløkka, Oslo, Norway) at  $600 \times g$ . Lymphocytes were then isolated from the PBMC by size sedimentation centrifugation using 1.068 g/ml Percoll solution (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) at  $400 \times g$ .<sup>7</sup> The lymphocytes were then passed through nylon wool column, allowing B-cells to bind to the nylon wool and collecting the T-cell rich eluate.<sup>8</sup> Cells were washed twice with Hank's balanced salt solution (HBSS) by centrifuged at  $400 \times g$ . The purity of isolated T-cells was determined by immunostaining with APC-conjugated anti-CD3 antibody (BD Biosciences, San Jose, CA, USA), specific to the T3 complex of the T-cell receptor, for 20 minutes at 4°C. Cells were analyzed by fluorescence-activated cell sorting using FACSCalibur Cell Analyzer (BD Biosciences, San Jose, CA, USA).

### Cytotoxicity bioassay

The cytotoxicity concentrations of vitamin-E (United States Biological, Salem, MA, USA), uric acid (Bio Basic Inc., Markham, ON, Canada), hydrogen peroxide (Bio Basic Inc., Markham, ON, Canada), aluminum chloride (QR&C, Auckland, New Zealand), ultraviolet irradiation and Moringa extract was determined by the neutral red toxicity bioassay.<sup>7,9</sup> T-cells were plated at a density of  $5 \times 10^5$  cells/well in 24-well tissue culture plates and then exposed to varying concentrations of test substances for 24 h. Then neutral red dye at a concentration of 50 µg/ml was added and cells incubated for 2 h. After washing the cells twice, the cell pellet was dissolved in acid alcohol solution. Absorbance at 540 nm was measured using microplate reader (PerkinElmer Inc., Waltham, MA, USA). Cell viability of each sample was calculated and cellular cytotoxicity of test substances were analyzed using dose response relationships/sigmoidal curve fitting analysis (Origin Lab Corporation, Northampton, MA, USA).

### T-cell responses

Cells were cultured in RPMI-1640, supplemented with 10% FBS, 1% penicillin/streptomycin mixture (Thermo Fisher Scientific, Inc., New York, NY, USA), 1 µg/ml phytohemagglutinin (PHA) (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) and 1000 ng/ml phorbol-12-myristate-13-acetate (PMA) (United States Biological, Salem, MA, USA) to stimulate IL-2 production.<sup>7,9</sup> Cells were collected in microfuge tubes and centrifuged at  $400 \times g$  for 5 min. The culture fluid was collected for the quantification of IL-2 by ELISA. The cell pellet was used to extract RNA and DNA for analyzing IL2 gene expression and oxidative DNA damage, respectively.

### Enzyme-linked immunosorbent assay (ELISA)

The ELISA was used to measure the secreted IL-2 (BioLegend, Inc., San Diego, CA, USA). In this assay, 100 µl of cell culture fluid was added to anti-human IL-2 antibody pre-coated plates and plates incubated for 2 h at room temperature. These were washed 4 times with 300 µl of washing buffer and blocked with 100 µl of BSA assay buffer for 1 h with shaking at room temperature. After washing the plates the reaction was detected by adding biotin-conjugated anti-human IL-2 antibody and

incubating for 1 h with shaking. After washing, 100 µl avidin-HRP conjugate was added to each well and plates incubated for 30 min with shaking. Plates were then washed 5 times and 50 µl of HRP substrate added to each well and incubate for 15 min in the dark at room temperature. Finally, 100 µl of 1M sulfuric acid was added to stop the reaction and the absorbance of each reaction was measure at 450 nm using microplate reader (PerkinElmer Inc., Waltham, MA, USA).

### RNA and DNA extraction

RNA and DNA were extracted by the guanidinium thiocyanate phenol-chloroform method using TRIzol reagent (Thermo Fisher Scientific Inc., New York, NY, USA). The cell pellet was lysed by adding 1 ml of TRIzol reagent for 5 min at room temperature. For the isolation of RNA, 200 µl of chloroform was added to the homogenized sample, mixed, incubated for 3 min and centrifuged at  $12000 \times g$  for 15 min at 4°C. The aqueous phase layer containing RNA was collected and mixed with 500 µl of isopropanol for 10 min to precipitate the RNA. The RNA sample was centrifuged at  $12000 \times g$  for 15 min at 4°C, and then the RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at  $7500 \times g$  for 5 min at 4°C. The isolated RNA was re-suspended with nuclease-free water and store at -20°C. For the isolation of DNA, the phenol-chloroform layer from the previous step was collected, mixed with 300 µl of ethanol for 3 min and centrifuged at  $2000 \times g$  for 5 min at 4°C. The DNA pellet was then washed with 1 ml of sodium citrate/ethanol solution and centrifuged at  $2000 \times g$  for 5 min at 4°C. The isolated DNA was then re-suspended with Tris-EDTA buffer and stored at -20°C. The concentrations of RNA and DNA of each sample were measure by spectrophotometry using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., New York, NY)

### Gene expression analysis

Measurement of IL-2 mRNA levels was performed by real-time quantitative reverse transcription PCR (RT-qPCR). Each PCR reaction contains 1 µl of 1 ng/ml RNA template, 0.8 µl of 10 µM forward primer, 0.8 µl of 10 µM reverse primer, 10 µl of SCRIPT One-Step RT-qPCR GreenMaster (Jena Bioscience GmbH, Jena, Germany), and 7.4 µl of nuclease-free water. Reverse transcription and PCR reaction were performed using CFX96 real-time PCR detection system (Bio-Rad Laboratories Inc., Hercules, CA, USA). The reaction was started with reverse transcription at 50°C for 15 min, initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C and annealing/elongation at 60°C for 15 sec and 1 min, respectively. Forward and reverse primers specific to IL2 gene were TCCTGTCTTGCATTGCACTAAG and CATCCTGGTGAGTTTGGGATTC. Forward and reverse primers specific to beta actin gene were CATGTACGTTGCTATCCAGGC and CTCCTTAATGTCACGCACGAT. Expression of IL2 gene was evaluated by normalized gene expression analysis using  $2^{-\Delta\Delta C_T}$  method.<sup>10</sup>

### T-cell DNA damage

Oxidative DNA damage was measured by the acetyl cholinesterase competitive ELISA (Cayman Chemical Company, Ann Arbor, MI, USA). To goat anti-mouse IgG-coated plate was added 50 µl of a DNA sample, 50 µl of acetylcholine tracer, and 50 µl of mouse anti-oxidative DNA damage (anti-8-OHdG) to the wells. The plates were incubated, with shaking, for 2 h, and then washed 5 times with 300 µl of washing buffer. Color development was precipitated by adding

200  $\mu$ l of Ellman's reagent and further incubation for 2 h. Absorbance of reactions was measured at 412 nm using microplate reader (PerkinElmer Inc., Waltham, MA, USA).

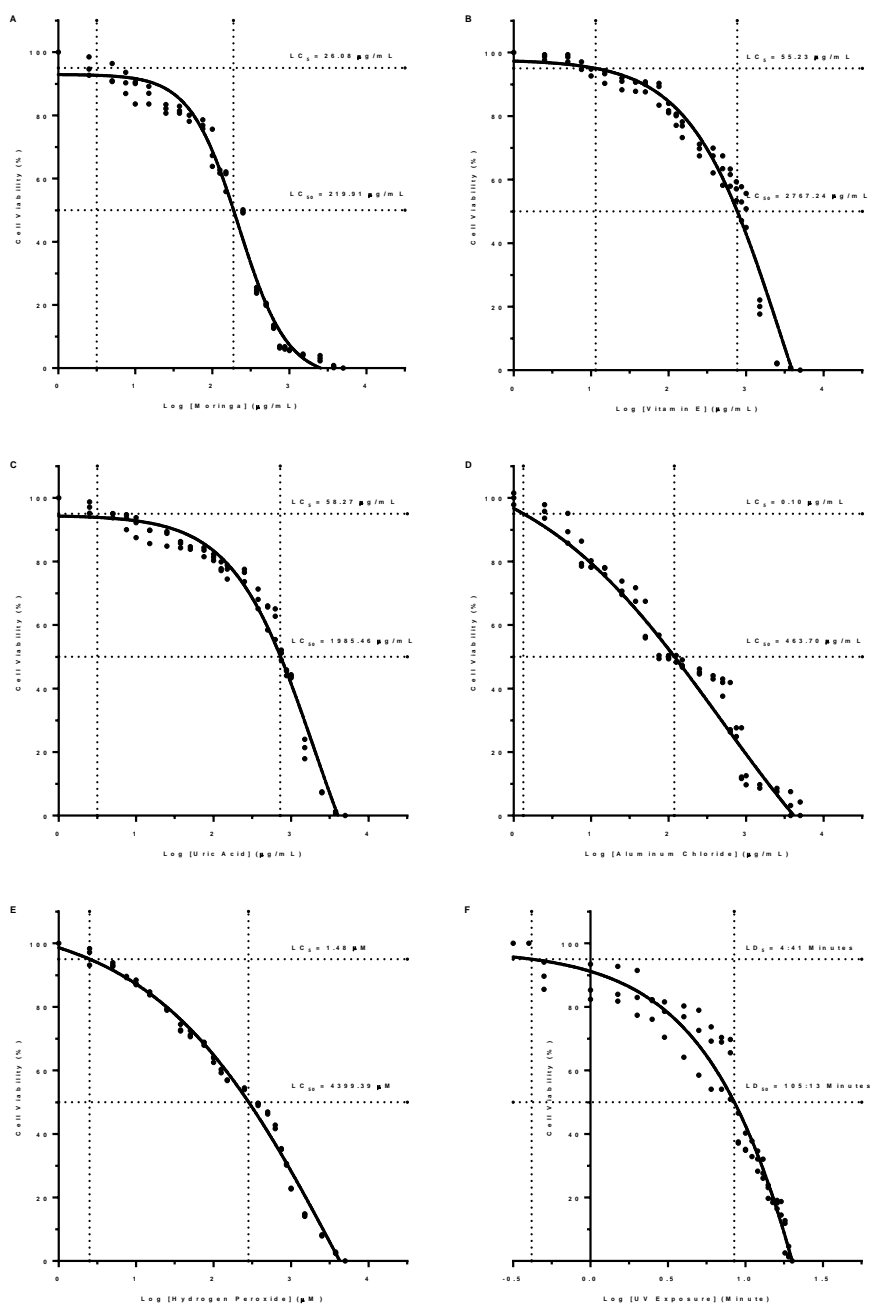
### Experimental design and statistical analysis

Results were analyzed by one-way ANOVA with Bonferroni multiple comparisons test and plotted as bar graph using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). Confidential interval of 99% ( $p = 0.01$ ) was used in all statistical analysis.

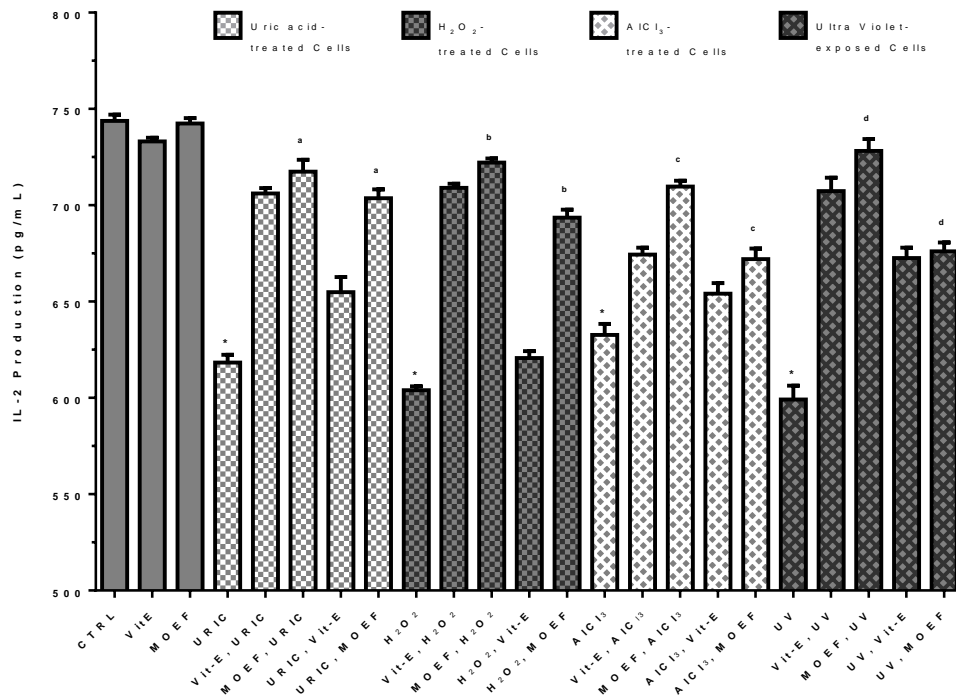
## RESULTS

### Cytotoxicity of test substances

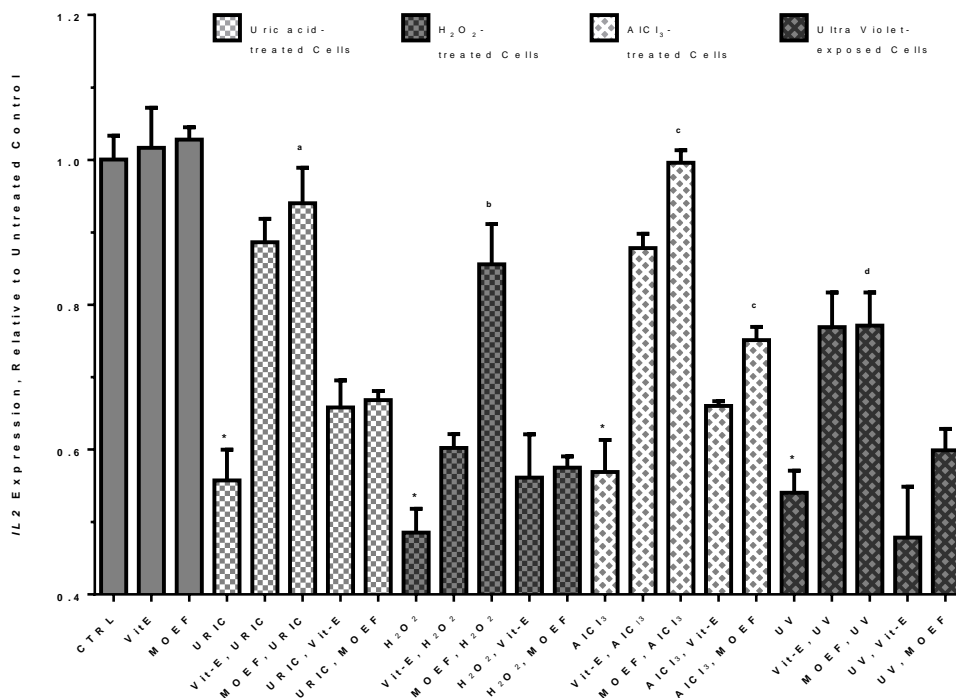
Prior to initiating experiments to test the effects of Moringa extract on oxidative stress induced cell hyporesponsiveness, all agents were tested for cytotoxicity. The cytotoxicity of test substances was calculated according to the sigmoidal curve fitting analysis (Figure 1). The 5% lethal concentration of Moringa extract, vitamin-E, uric acid, aluminum chloride, and hydrogen peroxide were 26.08, 55.23, 58.27, 0.10  $\mu$ g/ml and 1.48  $\mu$ M, respectively. The 5% lethal dose of ultraviolet irradiation was 4:41 min. These concentrations/doses were used in T-cell studies.



**Figure 1.** Dose-response curve of test substances. showing lethal dose or lethal concentration of 50% and 5% of Moringa extract (A); vitamin-E (B); uric acid (C); aluminum chloride (D); hydrogen peroxide (E); and ultraviolet irradiation (F).

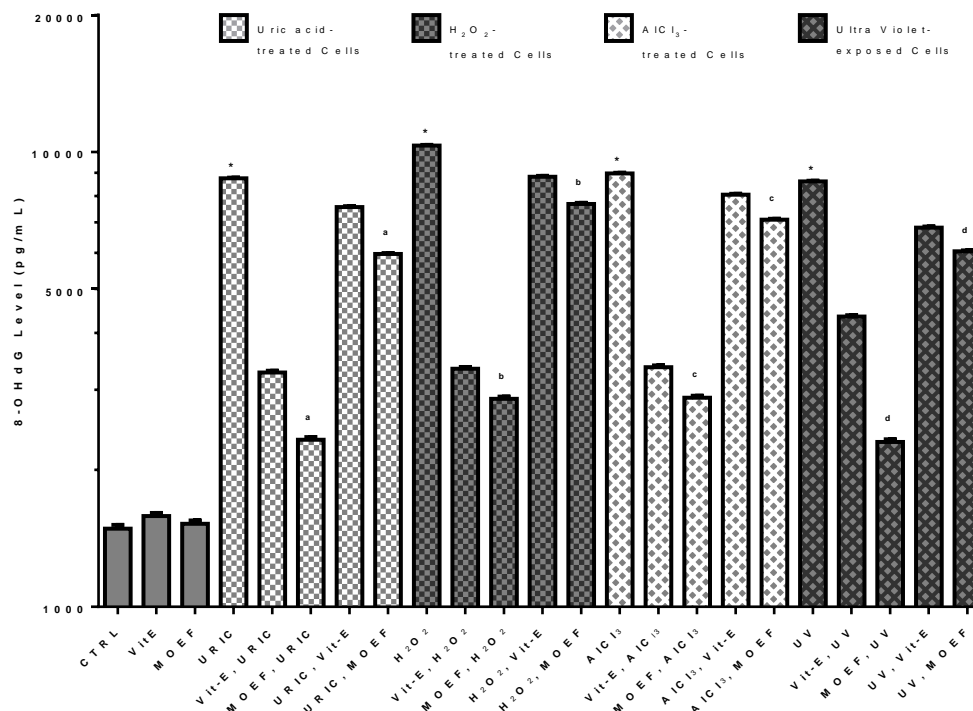


**Figure 2. Moringa extract protects T-cells against oxidative stress-induced depression of IL-2 production.** T-cells were subjected to oxidative stress using, uric acid, hydrogen peroxide, aluminum chloride and ultraviolet irradiation as indicated the bars. The T-cells were treated with Moringa extract either 6 h prior or 6 h after expose to stress. The cells were stimulated with PHA-PMA and IL-2 production was measured in the T-cell culture fluids by ELISA. The data are presented as mean  $\pm$ SD of 3 independent experiments. Statistical analyses: \*compared to untreated control cells;  $p < 0.01$ , a compared to uric acid-treated cells;  $p < 0.01$ , b compared to hydrogen peroxide-treated cells;  $p < 0.01$ , c compared to aluminium chloride-treated cells;  $p < 0.01$ , d compared to ultraviolet-exposed cells,  $p < 0.01$



**Figure 3. Moringa extract protects T-cells against oxidative stress-induced depression of IL2 expression.** T-cells were subjected to oxidative stress using, uric acid, hydrogen peroxide, aluminum chloride and ultraviolet irradiation as indicated under the bars. The T-cells were treated with Moringa extract either 6 h prior or 6 h after expose to stress. The cells were stimulated with PHA-PMA and IL2 expression was measured from RNA by RT-qPCR. The data is presented as mean  $\pm$  SD of 3 independent experiments. Statistical analyses: \*compared to untreated control cells;  $p < 0.01$ ,

a compared to uric acid-treated cells;  $p < 0.01$ , b compared to hydrogen peroxide-treated cells;  $p < 0.01$ , c compared to aluminium chloride-treated cells;  $p < 0.01$ , d compared to ultraviolet-exposed cells,  $p < 0.01$



**Figure 4. Moringa extract protects T-cells against oxidative stress-induced DNA damage (8-OHdG).** T-cells were subjected to oxidative stress using, uric acid, hydrogen peroxide, aluminum chloride and ultraviolet irradiation as indicated under the bars. The T-cells were treated with Moringa extract either 6 h prior or 6 h after expose to stress. The cells were stimulated with PHA-PMA and oxidative DNA damage was measured from DNA of T-cells by 8-OHdG competitive ELISA. The data are presented as mean  $\pm$  SD of 3 independent experiments. Statistical analyses:

\*compared to untreated control cells;  $p < 0.01$ , a compared to uric acid-treated cells;  $p < 0.01$ , b compared to hydrogen peroxide-treated cells;  $p < 0.01$ , c compared to aluminium chloride-treated cells;  $p < 0.01$ , d compared to ultraviolet-exposed cells,  $p < 0.01$

#### Effects of Moringa extract on the production of IL-2 by T-cell in the presence of oxidative substances

T-cells were plated at a density of  $5 \times 10^5$  cells/well in 24-well plates. Cells were treated with Moringa extract 6 h prior or 6 h after treatment with the oxidative substances (uric acid, aluminum chloride and ultraviolet irradiation). The concentration of substances used was that which gave  $< 5\%$  toxicity. The data in Figure 2 show that untreated control T-cells produced 743.74 pg/ml of IL-2. Exposure of T-cell to oxidative substances significantly decreased the production of IL-2 ( $p < 0.01$ ). Uric acid, hydrogen peroxide, aluminum chloride, and ultraviolet irradiation decreased the level of IL-2 to 618.28, 603.95, 632.73, and 599.17 pg/ml, respectively. Pretreating T-cells with Moringa extract significantly increased the production of IL-2 under the stress substance exposure ( $p < 0.01$ ). Treatment with Moringa extract post stress substance exposure greatly reduced the effectiveness of the extract, although some significant effect was still seen. The effects of Moringa extract was comparable to that of vitamin-E (Figure 2).

#### Effect of Moringa extract on the expression of IL2 gene in T-cells under oxidative stress

T-cells were treated with stress substances as described above. The oxidative substances, uric acid, hydrogen peroxide, aluminum chloride, and ultraviolet irradiation, significantly down-regulated the expression of IL2 mRNA to 0.56, 0.49, 0.57, and 0.54, respectively when compared with untreated control ( $p < 0.01$ ). Pre-treatment with Moringa extract caused a significant restoration of IL2 mRNA expression reduced by these oxidative

substances ( $p < 0.01$ ) (Figure 3). Post-treatment with Moringa extract had very little effect apart from that reduced by aluminum chloride (Figure 3).

#### Moringa extract protects against oxidative stress-induced DNA damage

T-cells were treated with the oxidative stress agents as described above. All oxidative substances increased the level of 8-OHdG, the oxidized form of deoxyguanosine from the chain of DNA (Figure 4). Level of 8-OHdG increased from 1485.58 pg/ml in untreated control T-cells to 8747.27 pg/ml in uric acid-, 10331.71 pg/ml in hydrogen peroxide-, 8972.40 pg/ml in aluminum chloride-, and 8610.99 pg/ml in ultraviolet-treated/exposed cells. Pre-treatment with Moringa extract significantly decreased the levels of 8-OHdG in oxidative-treated cells ( $p < 0.01$ ), comparable with the effect of vitamin-E. However post-treatment with Moringa extract was much less effective in this action ( $p < 0.01$ ).

#### DISCUSSION

ROS and RNS normally produced after cell metabolism, while managed by anti-oxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutase, become more difficult to balance when excessive production occurs through oxidative stress seen at inflammatory sites in chronic inflammatory diseases, infection and cancer.<sup>11,12</sup> Since T-cells play critical roles in the outcomes of these diseases, it is important to know how T-cells affected by oxidative stress could be protected by nutritional and pharmacological approaches. T-cell

hyporesponsiveness and loss of their regulatory function has been demonstrated under excessive oxidative stress.<sup>13,14</sup> Oxidative damage of T-cells can lead to a declined in T-cell function.<sup>15,16</sup> Moringa extract is one of the medicinal plant with antioxidant activity, anti-inflammatory, anti-proliferative, and anti-cancer activity.<sup>7,17</sup> However little is known how these effects are induced but most likely involve their polyphenolic compounds such as phenolic acid, flavonoid and tannin present in the extract.<sup>18</sup> Here we demonstrate that Moringa extract protects against oxidative stress-induced hyporesponsiveness of T-cell and DNA damage.

The data demonstrate that the Moringa extract protects human T-cell against oxidative stress-induced hyporesponsiveness, by monitoring the ability of T-cell to produce IL-2. Using a range of oxidative substances, uric acid, hydrogen peroxide, aluminum chloride, and ultraviolet irradiation, to mimic the oxidative stress of the cells inside the body, it was evident that in all cases the down regulation of IL-2 production could be prevented by the Moringa extract. Uric acid is a heterocyclic compound produced by the metabolically breakdown of purine nucleotides inside the cells. Production of uric acid can lead to oxidative stress of the cells and other medical conditions such as diabetes and kidney stone.<sup>19-21</sup> Hydrogen peroxide is a reactive oxygen species produced by electron transport chain of oxidative phosphorylation in the mitochondria. Without antioxidant defense enzymes, hydrogen peroxide can be toxic to cells due to its reactive oxidation stage.<sup>22,23</sup> Aluminum chloride can be separated to aluminum ion and chloride ion when dissolved in water. The aluminum ion is reactive and induces ion-initiated lipid peroxidation and also increases the oxidative capacity of superoxide ion.<sup>24,25</sup> Ultraviolet irradiation is known to be the source of oxidative stress due to the interaction of photons and intracellular chromophore, which can result in genetic damage and activation of redox reaction that can cause oxidative stress of cells.<sup>26,27</sup>

To study the antioxidant activity of Moringa extract, we used an ethyl acetate fraction of the extract, which is rich in phenolic acid. Phenolic acid can interact with oxidative oxygen species, donate the hydroxyl ion to the reactive oxygen and lead to the less reactive oxygen form. We used the antioxidant substance, vitamin-E as a positive control of antioxidant activity, for the evaluation of Moringa extract. The results revealed that the action of the Moringa extract was most likely to be at the pre-transcriptional level since we found that the oxidative stress-induced decrease in IL-2 mRNA production by the T-cell could be prevented by the extract. Furthermore this may be related to our finding that the Moringa extract prevented the oxidative DNA damage, assessed by measuring the levels of 8-Oxo-2'-deoxyguanosine (8-OHdG). This view is furthermore, consistent with our data that if the addition of Moringa extract was post to the treatment with oxidative substances the effectiveness in preventing the T-cell was severely reduced. This suggests that its action is prior to the onset of DNA damage and gene transcription.

## CONCLUSION

The data demonstrate that the Moringa extract prevents the development of oxidative stress-induced T-cell hyporesponsiveness, most likely related to its effects on the early events at the transcriptional or pretranscriptional level and by protecting against DNA damage. Since T-cells and their regulation of immune reactivity at inflammatory sites is the key to disease outcomes, the results are consistent with the extract's *in vivo* anti-inflammatory properties.

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