

***In Vitro* FREE RADICAL SCAVENGING AND ANTI-GENOTOXIC ACTIVITIES OF *THUNBERGIA LAURIFOLIA* AQUEOUS LEAF EXTRACT**

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ABSTRACT:

Background: Lead (Pb) is a xenobiotic, which causes numerous health defects like neurological, metabolic, kidney disorders, as well as mutation including DNA damage. *Thunbergia laurifolia* (TL) is a renowned medicinal plant for its antidote properties in Thailand. These properties caused us to investigate its anti-genotoxic effects against Pb in human peripheral blood leukocytes.

Methods: The TL aqueous leaf extract was prepared and the 50% inhibition concentration (IC₅₀) on DPPH, superoxide, nitric oxide and hydroxyl radical scavenging activity determined. An *in vitro*, genotoxicity test was used to determine the magnitude of DNA damage of human peripheral blood leukocytes classified by a comet assay. TL extract at doses of 25, 50 and 100 µg/mL were used in combination with Pb at doses of 40 and 80 µg/mL, respectively, on *in vitro* human peripheral blood leukocytes and incubated at 37°C for 1 hrs. H₂O₂ (40 mM) was used as a DNA damage positive control.

Results: Genotoxicity was induced by Pb and H₂O₂ exposure in human peripheral blood leukocytes leading to DNA damage. TL was found to be a potent free radical scavenger of superoxide, hydroxyl radical, DPPH, hydrogen peroxide and nitric oxide with IC₅₀ value of 0.298±0.004, 0.402±0.001, 0.869±0.030, 1.600±0.089 and 1.624±0.140 µg/mL, respectively. TL treatment ameliorated Pb induced genotoxicity in human peripheral blood leukocytes. Moreover, TL provided a protective effect on DNA damage against H₂O₂ in human peripheral blood leukocytes.

Conclusion: TL aqueous leaf extract possesses an anti-genotoxic effect, possibly due to its antioxidant capacity, and could prove useful as an antidote to lead toxicity.

Keywords: *Thunbergia laurifolia*; Genotoxicity; Lead; Lead poisoning; Antioxidant

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INTRODUCTION

Lead poisoning worldwide is a major public health risk, but is particularly high in developing countries. Burning Pb containing fossil fuels, paints, unrestricted mining and disposal of industrial waste are the prime source of Pb mediated environmental pollution. Human beings can be intoxicated by inhaling contaminated air, drinking contaminated water and consuming food such a fish, shrimp and shellfish. Exposure to Pb produces various deleterious effects on the hematopoietic, renal, reproductive and central nervous system, mainly

through increased oxidative stress [1-3]. Different pathological conditions have been recorded in experimental studies of human beings including renal and liver dysfunction, damage to the central nervous system [4, 5]. Cardiovascular mortality, mutagenicity and cancer are associated adverse effects of Pb induced oxidative cellular damage [6-8]. Oxidative stress is a well-recognized mechanism of Pb toxicity, resulting in lipid peroxidation, DNA damage and the oxidation of protein sulfhydryl groups [9, 10]. Prevention of Pb toxicity is thus a major international public health priority.

Thunbergia laurifolia (Linn.) or Rang Jued, a Thai medicinal plant, is well known for its medicinal properties against food and chemical toxins. Fresh

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TL and dried leaves, bark and roots are used as antidotes for insecticide, drug, arsenic, strychnine, and alcohol toxicity [11-14]. TL is also recognized to have antioxidant, anti-inflammatory, hepatoprotective and anti-diabetic properties [12, 15-18]. Recently, we have been reported that TL plays a functional role against Pb-induced oxidative stress and neurotoxicity [5, 19]. Free radical generation starts a chain reaction that results in lipid peroxidation, disruption of the cell membrane, protein oxidation and oxidation of nucleic acids like DNA and RNA leading to cancer. Reactive oxygen species (ROS) also play a role in Pb induced DNA strand breaks in the lymphocytes of battery plant workers [1, 7]. Based on the potent antioxidant property of TL aqueous extract and the prophylaxis against the majority of Pb mediated oxidative damage which may be used to treat Pb induced genotoxicity. This study aimed to evaluate genotoxicity of Pb and anti-genotoxicity of TL against Pb induced DNA damage in human peripheral blood leukocytes alongside *in vitro* antioxidant properties.

MATERIALS AND METHODS

Plant materials and preparation of aqueous crude extract

The TL leaves were obtained from The Organic Herbal Center, Nakhon Si Thammarat which was identified by the Thai Traditional Center of Nakhon Si Thammarat and was confirmed following Oonsivilai [12]. Leaves were oven dried at 60°C and grounded in a blender to a fine powder. The fine powder (10 g) was boiled extracted with distilled water (100 mL) using an autoclave at 120°C, 15 pounds for 15 min. The TL aqueous leaf extract was filtered and then freeze dried under vacuum at -80°C for 18 hrs using an evaporator (EYELA, Tokyo, Japan). The yield of the dry powder of the TL extract was 9.65% which was stored at -30°C.

Free radical scavenging activity of TL in cell free system

DPPH scavenging assay

Free radical scavenging capacity was evaluated according to Chen et al. [20]. Briefly, 200 µL of methanolic solution of DPPH (100 µM) was added with 50 µL of extract in a 96 well microplate, thoroughly mixed, incubated in the dark at room temperature for 20 min. The final reaction of decolorizing was measured at 517 nm using a microplate reader (MULTISKAN GO, Thermo scientific) against a blank.

$$\text{Scavenging activity (\%)} = \left[\frac{(A_0 - A_1)}{A_0} \right] \times 100$$

Where A₀; absorbance of all reagents except TL extract, A₁; absorbance in presence of TL extract samples.

Nitric oxide (NO) scavenging assay

NO scavenging activity was measured as described by Marcocci et al., [21]. Briefly, a reaction mixture of 1 mL with sodium nitroprusside in PBS, pH 7.4 (SNP, 5 mM final concentration) and a different concentration of sample was made, incubated at 25°C for 2.5 hours. Then the solution was thoroughly mixed with Griess' reagent (1% sulfanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 5% H₃PO₄) and the absorbance of the chromophore formed by diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylenediamine measured at 570 nm. Scavenging activity of TL was calculated compared to the absorbance of standard solutions of sodium nitrite salt treated in the same way with Griess' reagent.

Superoxide scavenging assay

Based on autoxidation of pyrogallol, the superoxide anion assay was performed according to Phyu and Tangpong [22]. Briefly, a reaction mixture of 100 mL of 3 Mm pyrogallol, 3 mL Tris buffer (pH 8.2) and 0.5 mL TL extract in different concentrations were prepared. Following autoxidation of pyrogallol, superoxide is formed and the rate of autoxidation was determined at 325 nm by UV-Vis spectrophotometer. The TL scavenging activities on the superoxide anion were calculated by comparing ΔA₁/min (without TL) and ΔA₂/min (with TL), according to the following equation:

$$\left[\frac{\Delta A_1 - \Delta A_2}{\Delta A_1} \right] \times 100$$

IC₅₀ value was defined as the concentration required to give 50% scavenging activity.

Hydroxyl radical scavenging assay

The assay followed the method described by Halliwell et al. with a slight modification [23]. As this method based on the Fenton reaction, FeCl₃ (10 mM, 0.01 mL) was added with H₂O₂ (10 mM, 0.1 mL), EDTA (1 mM, 0.1 mL) and, Deoxyribose (10 mM, 0.36 mL). 1 mL of different concentrations of TL extract prepared in PBS and 0.33 mL of phosphate buffer (50 mM, pH 7.9) were added to the

mixture. The reaction was initiated by adding 0.1 mL of ascorbic acid, followed by incubation at 37°C for 1 hour. After incubation, 1.0 mL of 10 % TCA and 1.0 mL of 0.5 % TBA (in 0.025 M NaOH containing 0.025 % BHA), was added to 1 mL of the incubated mixture, which was boiled for 20 min to develop the pink chromogen. This mixture was immediately kept in ice to avoid over oxidation, centrifuged at $1000 \times g$ for 3 min. The pink chromogen was then measured at 532 nm against a blank solution. The hydroxyl radical scavenging activity of the extract is reported as % inhibition of deoxyribose degradation calculated using the following equation;

$$\left[\frac{(A_0 - A_1)}{A_0} \right] \times 100$$

Where A0 is the absorbance of the H₂O₂ solution without TL and A1 with TL.

Hydrogen peroxide scavenging assay

The measurement of the ability of TL to inhibit hydrogen peroxide (H₂O₂) was performed as described previously [24]. A solution of H₂O₂ (40 mM) was prepared in a phosphate buffer (pH 7.4). 0.6 mL of TL was added to the H₂O₂ solution. The absorbance of the reaction mixture was measured at 230 nm by UV-Vis spectrophotometer. A blank solution containing the phosphate buffer without H₂O₂ was prepared. The inhibition of TL on H₂O₂ was calculated according to the following equation:

$$\left[\frac{(A_0 - A_1)}{A_0} \right] \times 100$$

Where A0 is the absorbance of the H₂O₂ solution without TL and A1 with TL.

Blood samples collection and determination of DNA damage by comet assay

Blood samples were obtained by venipuncture using heparinized tubes from three healthy male non-smoking donors. The donors had no previous known contact with high concentrations of Pb. These blood samples were kept at 4°C to omit any extra DNA damage. Then the human peripheral blood leukocytes were treated with TL (25, 50 and 100 µg/mL) and Pb (40 and 80 µg/dL), respectively. Treatment with 40 mM H₂O₂ and normal saline were used as positive and negative control.

The comet assay was done according to

Tice et al. [25] with a slight modification done as reported Azqueta et al. [26]. Briefly, microscopic slides were percolated with 1% normal agarose (NA) and kept for overnight at 4°C to make the slides more adherent to a subsequent layer of agarose. Then, mixture of whole blood (15 µL) and 140 µL low melting point agarose heated at 37°C was placed on the slides and coverslip added, immediately to spread the mixture. The slides were then placed in refrigerator for 10-15 min to solidify the gel. The coverslip was removed and immersed in a coupling jar containing cold lysis solution (89 mL of lysis stock solution - NaCl 2.5 M, EDTA 100 mM, Tris 10 mM and ~8.0 g of NaOH, 10% DMSO and 1% Triton X-100, pH 10.0), kept at 4°C for 1 hours and placed in the dark to reduce unwanted DNA damage. Thereafter, these slides were transferred into an electrophoresis vat and covered with an alkaline buffer (NaOH 300 mM + EDTA 1 mM, pH >13.0) for 30 min to unwind the DNA and electrophoresis for 20 min at 35 V (1.0 V/cm) and 300 mA. Following electrophoresis, the excess alkaline buffer was removed by dipping into a jar containing a neutralization buffer (0.4 M Trizma Hydrochloride, pH=7.5) for 15 min (3x5 min). Prior to analysis, slides were kept at 4°C. Finally, slides were stained with 50 µL ethidium bromide (20 µg/mL) for 15 min and analyzed under a fluorescence microscope at 400× magnification (ECHLIPS E600, NIKHON). 200 nucleoids were counted and visually defined according to Roberto et al. [27]. Comets were classified into four classes: class 0 (no damage/no tail), class 1 (little damage/small tail smaller than the head diameter), class 2 (medium damage/medium tail with size equal to or larger than the head diameter), and class 3 (extensive damage/large tail with size two times larger than the head diameter) and scored using the following formula:

$$\text{Total score} = (0 \times n_1) + (1 \times n_2) + (2 \times n_2) + (3 \times n_3)$$

Where, n is the number of nucleoids in each class analyzed.

Statistical analysis

DNA damages was expressed as the mean ± standard error of mean (SEM) of three independent experiments. The significance value ($p < 0.05$) was evaluated with one way-ANOVA followed by Bonferroni post-test using GraphPad Prism 5.0 software.

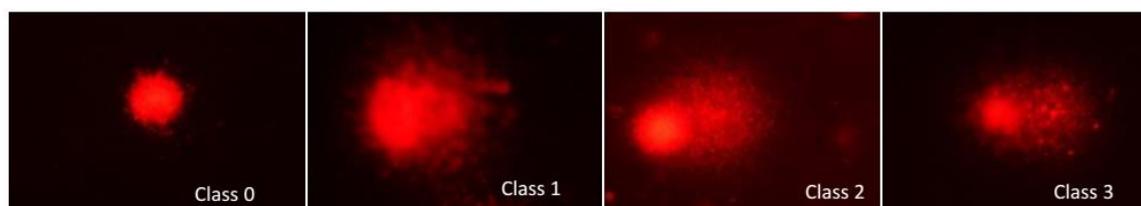


Figure 1: DNA-damage evaluations by comet assay in human peripheral leukocytes after treatment with Pb for 1 hour. Representative images of different classes of comets are shown. Class 0; no damage, Class 1; minimal damage, Class 2; medium damage, and Class 3; high damage, respectively. The total comet score was calculated and presented as mean \pm SEM from three independent experiments

Table 1 Free radical scavenging activity of TL

DPPH scavenging (IC 50, mg/mL)	Super oxide scavenging (IC 50, mg/mL)	Hydroxyl radical scavenging (IC 50, mg/mL)	Hydrogen peroxide Scavenging (IC 50, mg/mL)	Nitric oxide scavenging (IC 50, mg/mL)
0.869 \pm 0.030	0.298 \pm 0.004	0.402 \pm 0.001	1.600 \pm 0.089	1.624 \pm 0.140

Data were presented as mean \pm SEM, n=3

Table 2 Genotoxicity evaluation of TL and Pb in human peripheral blood leukocytes by comet assay

Treatment	Class				Comet score
	0	1	2	3	
Negative control	95.00 \pm 0.57	5.00 \pm 0.57	0.00 \pm 0.00	0.00 \pm 0.00	5.00 \pm 0.57
H ₂ O ₂ (40 mM)	62.66 \pm 0.88 ^a	18.66 \pm 0.66 ^a	8.66 \pm 1.85 ^a	10.00 \pm 1.52 ^a	66.00 \pm 0.57 ^a
Lead (40 μ g/dL)	83.66 \pm 2.40 ^{a,b}	14.33 \pm 0.66 ^{a,b}	0.66 \pm 0.66	1.33 \pm 1.33	19.66 \pm 5.69 ^{a,b}
Lead (80 μ g/dL)	80.33 \pm 2.72 ^{a,b}	14.66 \pm 2.02 ^{a,b}	3.00 \pm 0.00 ^{a,b}	2.66 \pm 0.66 ^{a,b}	28.66 \pm 3.84 ^{a,b}
TL (25 μ g/mL)	93.00 \pm 1.15 ^{c,d}	7.00 \pm 1.15 ^{c,d}	0.00 \pm 0.00 ^{c,d}	0.00 \pm 0.00 ^{c,d}	7.00 \pm 1.15 ^{c,d}
TL (50 μ g/mL)	92.00 \pm 1.00 ^{c,d}	6.66 \pm 0.33 ^{c,d}	1.33 \pm 0.66 ^{c,d}	0.00 \pm 0.00 ^{c,d}	9.33 \pm 1.66 ^{c,d}
TL (100 μ g/mL)	91.00 \pm 1.00 ^{c,d}	8.33 \pm 0.66 ^{c,d}	0.66 \pm 0.33 ^{c,d}	0.00 \pm 0.00 ^{c,d}	9.66 \pm 1.33 ^{c,d}

Data were expressed as mean \pm SEM of three independent experiments. ^a P < 0.05 within the column compared between negative control and treatments groups, ^b P < 0.05 Pb compared with H₂O₂ treatment. ^{c,d} P < 0.05 TL compared with H₂O₂ and Pb treatment

RESULTS

Free radical scavenging activity of TL leaf aqueous extract in cell free system

TL was able to neutralize the DPPH radical to colorless or yellowish solution. This is because of the proton donating capacity of the TL extract which decrease the purple color DPPH radical, reflecting its free radical scavenging activity. Table 1 shows that the TL can scavenge 50% of DPPH at 0.869 \pm 0.03 mg/mL.

Excess production of superoxide is also responsible for initiating oxidative stress as well as oxidative damage. In our study, we found that TL is highly active (IC₅₀= 0.298 \pm 0.004 μ g/mL) in scavenging superoxide compared to other radicals. TL can quench superoxide to form further series of radical formation and protect against oxidative damage. Hydroxyl radical and hydrogen peroxide scavenging capacity of TL showed an IC₅₀ of

0.402 \pm 0.001 and 1.600 \pm 0.089 mg/mL, respectively, showing the ability of TL to quench hydroxyl radicals and hydrogen peroxide and decrease the rate of the propagation step in lipid peroxidation. TL also possesses NO scavenging activity, and its IC 50 value is 1.624 \pm 0.140 mg/mL (Table 1).

Genotoxicity and anti-genotoxicity of TL and Pb in human peripheral blood leukocytes

In vitro experiments showed that Pb treated in human peripheral blood leukocytes at concentrations 40 and 80 μ g/dL showed a clear DNA-damage comet score and thus genotoxicity. On the other hand, TL treated leukocytes showed no genotoxic affect at any dose of treatment differing from H₂O₂ which show the most extensive DNA damage as classified in Figure 1 and Table 2. These results indicated that Pb was less genotoxicant in comparison to H₂O₂ and DNA damage was mainly found in class I. However, a combination treatment

Table 3 Anti-genotoxicity evaluation of TL co-treatment with Pb in human peripheral leukocytes by comet assay

Treatment	Class				Comet score	% amelioration
	0	1	2	3		
Negative control	95.00±0.57	5.00±0.57	0.00±0.00	0.00±0.00	5.00±0.57	-
H ₂ O ₂ (40 mM)	62.66±0.88 ^a	18.66±0.66 ^a	8.66±1.85 ^a	10.00±1.52 ^a	66.00±0.57 ^a	-
Lead (40 µg/dL)	83.66±2.40 ^a	14.33±0.66 ^a	0.66±0.66 ^a	1.33±1.33 ^a	19.66±5.69 ^a	-
Lead (80 µg/dL)	80.33±2.72 ^a	14.66±2.02 ^a	3.00±0.00 ^a	2.66±0.66 ^a	28.66±3.84 ^a	-
Lead (40 µg/dL)+TL (25 µg/mL)	94.00±1.00 ^b	3.66±0.88 ^b	0.66±0.33	1.66±1.66	10.00±4.50 ^b	49.15
Lead (40 µg/dL)+ TL (50 µg/mL)	91.66±2.33 ^b	6.33±2.33 ^b	1.33±0.33	0.66±0.33	11.00±2.00 ^b	44.06
Lead (40 µg/dL)+ TL (100 µg/mL)	91.66±0.33 ^b	5.66±0.33 ^b	1.66±0.33	1.00±1.00	12.00±2.00 ^b	38.98
Lead (80 µg/dL)+ TL (25 µg/mL)	92.00±1.00 ^b	7.66±1.33 ^b	0.00±0.00 ^b	0.33±0.33 ^b	8.66±0.33 ^b	69.76
Lead (80 µg/dL)+ TL (50 µg/mL)	89.33±0.33 ^b	8.00±2.51 ^b	1.66±1.66	1.00±1.00	14.33±3.84 ^b	50.00
Lead (80 µg/dL)+TL (100 µg/mL)	86.33±0.88 ^b	10±0.57 ^b	2.00±0.00 ^b	1.66±0.66	19.00±2.08 ^b	33.72
H ₂ O ₂ (40 mM)+TL (25 µg/mL)	76.66±0.88 ^c	20.00±1.73	2.66±1.20 ^c	0.66±0.33 ^c	27.33±1.45 ^c	58.56
H ₂ O ₂ (40 mM)+TL (50 µg/mL)	84.33±1.45 ^c	12.33±1.45 ^c	1.66±0.88 ^c	1.00±0.00 ^c	18.66±1.20 ^c	71.74
H ₂ O ₂ (40 mM)+TL (100 µg/mL)	89.33±0.94 ^c	5.33±0.94 ^c	2.66±0.47 ^c	2.00±0.81 ^c	16.66±1.69 ^c	74.69

Data represent the mean ± SEM of three independent experiments. ^a*P*< 0.05 means significant difference between negative control, ^b*P*< 0.05 combination treatment of TL and Pb compared to Pb treated only.

with TL ameliorated both Pb and H₂O₂ caused DNA-damage according to the comet classification and score as shown in Figure 1 and Table 3.

DISCUSSION

This study was designed to evaluate the anti-genotoxicity and antioxidant activity of TL against Pb induced human peripheral blood leukocytes. It has been reported that the cytotoxicity of TL on different cell lines such as L929, BHK-21 [C13], HepG2 and Caco-2 cell lines demonstrated that over than 100 µg/mL are toxic to cells [28]. Whereas, cytogenetic studies in humans exposed to Pb, blood Pb levels >40 µg/dL, have been demonstrated to significantly increased the level of chromosome aberration and single-strand DNA breaks [29].

In the present results, we found that Pb treatment at the doses of 40 and 80 µg/dL exhibited significant genotoxicity of DNA breakage as found in direct exposure to H₂O₂ (40 mM) (*P*<0.05). The Comet assay of Pb and H₂O₂ were mainly in Class I which indicates little damage and a tail smaller than the diameter of the head of the human peripheral blood leukocytes. However, chronic exposure to either Pb or H₂O₂ may increase the number of DNA damage and cell death. As previously reported, Pb

can generate reactive oxygen species (ROS) such as hydroperoxide (-OOH), singlet oxygen and H₂O₂ which may be a major mechanism of Pb induced oxidative stress resulting in DNA damage [30]. Evidence of genetic damage following exposure to Pb, shows an increased ROS imbalance subsequently facilitating DNA damage and elevated caspase 3 activity [30-33]. In addition, apoptosis leukocyte cells are also correlated with an increase in the level of Pb in the blood [32]. Moreover, oxidative damage with genotoxicity indicated by depletion of the antioxidant enzymes, Cu, Zn-SOD and CAT, as well as depleted thiols and increased protein degradation products carbonyl groups and nitrites has been reported [34]. Our data suggests Pb oxidized DNA damage at high Pb concentration in agreement with results reported by Wozniak and Blasaik [30]. In this situation, TL may play a protection role, in part, in scavenging free radicals in human peripheral blood leukocytes against Pb and H₂O₂ induced DNA damage.

Previously, we have reported the free radical scavenging of TL aqueous extract on ABTS + scavenging radical compared with Trolox, the vitamin E derivative, as a known positive control and showed that the total expression 346.60±20.18

mmol TEAC/g of extract. 1 mg of TL aqueous extract, the dose of *in vitro* treatment used in this study, is equal $3.46.60 \pm 20.18$ mmol Trolox equivalent indicating that TL potent scavenging free radical (Table 1). TL aqueous extract also showed the highest phenolic content and antioxidant activity in the presence of different compounds including glucoside and polyphenols [28]. TL leaves extract contains apigenin, apigenin glucosides, iridoid glucosides, grandifloric acid, glucopyranosides as well as phenolic acids such as caffeic, gallic and protocatechuic acid [12, 28, 35]. Studies *in vitro* and *in vivo*, show that phenolic compounds like gallic acid, ellagic acid rhamnoside and glucoside derivatives exhibited protective effects on DNA oxidative damage [28, 36]. Moreover, the toxic effects of perinatal Pb exposure on the brain of rats which involves of oxidative stress and the beneficial role of antioxidants as mentioned by Antonio-Garcia and Masso-Gonzalez [37], and combined administration of antioxidants (vitamins) and thiol chelators significantly ameliorated chronic Pb intoxication [38]. Taken together, our results suggest that TL possesses high phenolic content, and high free radical scavenging activity evidenced with the amelioration and reduced severe classification of Pb and H₂O₂ induced DNA damage, Table 1 and 3 ($P < 0.05$). The results from this experiment suggest that the mechanism of TL on anti-genotoxicity against Pb and H₂O₂ induced DNA damage is, in part, in scavenging free radicals in human peripheral blood leukocytes.

CONCLUSION

An increased level of DNA damage was present in *in vitro* in human peripheral blood leukocytes exposure to Pb and H₂O₂. TL played a role in protection against both Pb and H₂O₂ induced DNA damage. The anti-genotoxic effect of TL could be, at least, due to its antioxidant properties. TL also showed no genotoxicity to human peripheral blood leukocytes.

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