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Inhibitory Effects of Water Extracts of Longan Leaves on Mutation and Tyrosinase

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Abstract The antityrosinase and antimutation effects of longan leaves and its bioactive compounds was investigated. The water extracts of longan leaves (WLL) inhibited the mutagenicity of 2-aminoanthracene, an indirect mutagen, and 4-nitroquinoline-*N*-oxide, a direct oxidative mutagen toward *Salmonella* typhimurium TA 98 and TA 100. WLL at 0-0.6 mg/ml displayed free radical scavenging activity, reducing power, chelating ability and protection against lipid oxidative damage. In addition, the inhibitory actions of WLL on tyrosinase activity and nitric oxide (NO) production in lipopolysaccharide (LPS) stimulated macrophages RAW 264.7 cell increased in concentration-dependent manner. According to HPLC-DAD analysis showed that epicatechin, ellagic acid and gallic acid, the major phenolic compounds, were present in WLL. The phenolic components may in part account for contributing the protective effects of WLL. On the basis of the results obtained, WLL can display biological functions and effectively protective against oxidation, mutation, tyrosinase, and inflammation.

Keywords: *longan leaves, mutation, tyrosinase, oxidative stress*

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1. Introduction

Food lipid is the component of food that is high susceptible to oxidation, which is a major cause of deterioration in the quality of food. Lipid oxidations induced by reactive oxygen precursor and free radicals lead to cellular oxidative damage and cause sever health problem such as heart disease and carcinogenesis [1]. To avoid the oxidation of lipids, many phytochemicals have been explored to protect the lipid components in foods against oxidation. In addition, the enzymatic brown reaction in food is another unpleasant effect which changes food color, flavor, nutritive value and shelf life. Many researches have reported that tyrosinase plays a crucial role in the browning reaction in food processing by catalyzing the oxidation of phenol and regulating the initial steps of melanogenesis [2]. Consequently, many reporters have focused on natural additives in order to decrease the browning progression in food production [3]. In addition to food browning, tyrosinase activation and further melanogenesis would reduce the glutathione levels and decrease the antioxidant capacity in food, as well as promoting the progression of melanin hyperpigmentation in different skin diseases [4]. Apart from lipid oxidation and food browning, various environmental mutagens present in contaminated foods cause oxidative damage and induce DNA mutation production. For example, 2-aminoanthracene (2-AA), an indirect-acting mutagen-carcinogen polycyclic aromatic amine, can induce tumors primarily in the liver [5]. However, 2-AA must submit to metabolism activation to display its carcinogenicity. Further, 4-nitroquinoline-N-oxide, a direct oxidative mutagen, is a quinoline derivative carcinogen that generate superoxide radicals and reactive oxygen species by redox recycling [6]. A consequence is that whenever DNA are damaged, DNA mutation may increase, eventually increasing the risk of tumor progression. Aside from the formation of direct adducts with DNA, these mutagens may increase oxidative stress in cells, which also induces cell damage and causes mutations.

Foods containing health beneficial components are defined as those which prevent the occurrence and development of oxidative damage by inhibiting the activation of a mutagen, inhibiting its interaction with cellular macromolecules, or by inducing deactivation and clearance of the mutagen. Further, these health beneficial components exhibiting protective capacity in vivo are considered to be safe and free of any harmful side effects [7]. Longan (*Dimocarpus longan* Lour.), a genus of the Sapindaceae family, is a commercially attractive fruit and is widely distributed in Asian regions including Taiwan, China, and Thailand. Many studies on the biological activities from different tissues of longan, including fruits, pericarp, flower and seed. Longan is widely used as health

beneficial foods. However, this is the first study to determine the biological effects of longan leaves, an agriculture waste material, on mutation, oxidation, inflammation and tyrosinase activity. Consequently, the aim of present study is to explore the effects of water extracts of longan leaves on anti-mutagenicity, anti-tyrosinase, anti-inflammation.

2. Materials and Methods

2.1. Materials

1, 1-diphenyl-2-picyl-hydrazyl (DPPH), kojic acid, 4-nitroquinoline-N-oxide, 2-aminoanthracene, lipo-polysaccharide (*Escherichia coli* O127:B8), Folin-Ciocalteu reagent, thiobarbituric acid (TBA), 3-[4,5-dimethyl-thiazol-2-yl]-2,5- diphenyl tetrazolium bromide (MTT), epicatechin, ellagic acid, and gallic acid and mushroom tyrosinase were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). L-3, 4- dihydroxyphenylalanin (L-DOPA) was obtained from Acros Organic (Geel, Belgium). Medium was added 1.6 g of nutrient broth in 200 ml of water. The minimal agar plates were composed of 20g glucose, 0.35 g $NaHNH_4PO_4{\cdot}4H_2O$, 10 g K_2HPO_4, 2 g citric acid monohydrate, 0.2 g $MgSO_4{\cdot}7H_2O$ and 15 g agar in 1000 ml of sterile water. Top agar was added 2.5 g NaCl and 3 g agar in 500 ml of sterile water. The leaves of longan (*Dimocarpus longan* Lour.) were harvested in Tainan, Taiwan.

2.2. Sample Preparation

The leaves of longan were cut into small pieces, frozen at −20 °C and then lyophilized for 48 h. These freeze-dried leaves were then ground to a fine powder. The leave powder (100 g) was extracted with boiling water (1000 ml) and stirred for 10 min. The extract was filtered and the residue was re-extracted under the same conditions. The combined filtrate was freeze-dried. The final dehydrated powder (4.63 g) was then dissolved in phosphate buffer saline (PBS). The sample was called as the water extract of longan leaves (WLL).

2.3. Total Polyphenolics

Total polyphenolics content were determined as gallic acid equivalents (GAE). The different concentrations of WLL were added to a 10 ml volumetric flask, to which 2 ml sodium carbonate (20% (w/v)) was added. After 5 min, 0.1 ml Folin-Ciocalteu reagent 50% (v/v) was added and the volume was made up to 10 ml with H_2O. After 1 h incubation at 30 °C, the absorbance was measured at 750 nm and compared to a gallic acid calibration curve [8].

2.4. Total Flavonoid Content

A 1ml aliquot of WLL was incubated with 0.1 ml (2-aminoethyl) diphenyl borate (0.2% in ethanol). After 20 min of incubation, the absorbance was measured at 405 nm. The absorbance of rutin solutions was detected under the same conditions. The total flavonoid content in WLL (rutin equivalents, RE) was calculated [8].

2.5. Determination of DPPH Radical Scavenging Activity

The effect of WLL on the DPPH radical scavenging activity was estimated as described previously [9]. The samples were added to a methanolic solution (1 ml) of DPPH radical (final concentration of DPPH was 0.2 mM). The mixture was shaken vigorously and keep for 30 min at room temperature; the absorbance of the resulting solution was then measured at 517 nm. Trolox was used as a reference standard.

2.6. Determination of ABTS Radical Inhibition

The $ABTS^{\cdot+}$ cation free radical scavenging activity was measured as previously described [10]. The $ABTS^{\cdot+}$ was generated by reacting 1 mM ABTS with 0.5 mM hydrogen peroxide and 10 Units/ml horseradish peroxidase in the dark at 30 °C for 2 h. After 1 ml $ABTS^{\cdot+}$ was added to WLL, the absorbance at 734 nm was recorded after 10 min. A lower level of absorbance indicated a stronger inhibitory activity of samples.

2.7. Determination of Reducing Activity

The WLL were added to potassium ferricyanide (2.5 ml, 10 mg/ml), and the solution was incubated at 50 °C for 20 min. TCA (2.5 ml, 100 mg/ml) was added to the mixture, which was then centrifuged at 650g for 10 min. The supernatant (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml, 1.0 mg/ml), and then the absorbance was read at 700 nm. The reducing activity was plotted as a function of concentration and the ascorbic acid equivalent reducing activity was calculated against an ascorbic acid calibration curve [11].

2.8. Determination of Chelating Activity

The chelating activity of WLL on Fe^{2+} was measured as previously described [11]. The samples thus prepared (0.6 ml) were mixed with $FeCl_2$ (2 mM, 0.2 ml) and added ferrozine (5 mM, 0.2 ml) incubated for 10 min, and the absorbance at 562 nm was assayed. A lower level of absorbance indicated a higher chelating activity of samples.

2.9. Determination of Liposome Protection

The mixture containing the lecithin (580 mg) and phosphate buffer (58 ml, 10 mM, pH 7.4) was sonicated by an ultrasonic cleaner (Branson 8210, Branson ultrasonic Corporation, Danbury, CT, USA) in an ice-cold water bath for 2 h. The sonicated solution, $FeCl_3$, ascorbic acid and samples (0.2 ml) were mixed to produce a final mixture with a concentration of 3.12μM $FeCl_3$ and 125μM ascorbic acid and incubated at 37 °C for 1 h. The levels of liposome protection were determined by the thiobarbituric acid (TBA) method [11]. The absorbance of the sample group was read at 532 nm against a blank, which contained all reagents except lecithin. A lower level of absorbance indicated a stronger lipid peroxidation inhibitory activity of sample.

2.10. Tyrosinase Activity Determination

The mushroom tyrosinase was used for the assay. Diphenolase inhibitory activity was determined by measuring the dopachrome accumulation at 475 nm using spectrophotometer [12]. All the works were performed in sodium phosphate buffer (pH 6.8). The reaction mixture consisting of 0.1 ml of samples, 0.1 ml of mushroom tyrosinase (1000 Unit/ml) and L-DOPA (3.8 mM) was added in this order to read the absorbance at 475 nm for 5 min at 25°C. The value in the absence of samples was represented as the control. The inhibitory activity of mushroom tyrosinase was calculated with the following formula: Inhibition (%) = (1-(OD_{475} in sample /OD_{475} in control)) × 100%.

2.11. Determination of NO Production in RAW 264.7 Cells

Murine macrophage cells, RAW 264.7 cells (ATCC number: TIB-71), were purchased from Bioresources Collection and Research Center (Shin-chu, Taiwan) and cultured in RPMI-1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 1 mM pyruvate and maintained in humidified 5% CO_2/95% air, at 37 °C. The nitrite (NO_2^-) levels in the cultured media, which reflect the intracellular nitric oxide synthase (NOS) activity, were assayed by the Griess reaction. RAW 264.7 cells were cultured with WLL and treatment with or without LPS (0.2 μg/ml) for 24 h. Then, the growth medium was mixed with the same volume of Griess reagent; then the absorbance of the mixture at 550 nm was measured. After the cells were cultured as mentioned above, MTT assay was applied to determine the cytotoxicity of samples.. Briefly, the original medium was removed, then, MTT (final 0.5 mg/ml) was added to each well. After 1 h incubation, the reaction was terminated and the plates were incubated for 30 min, the formazan dye was dissolved by the dimethyl sulfoxide. The absorbance of each well was measured at 570 nm [13].

2.12. Mutagenicity Assay

The mutagenic activity of WLL was evaluated by the Ames test with a 20 min first incubation at 37 °C [13]. The histidine-requiring strains of *Salmonella typhimurium* TA 98 and TA 100 were obtained from Taiwan Agricultural Chemicals and Toxic Substances Research Institute (Taichung, Taiwan). The external metabolic activation system, S9 mix (Organ Teknika Co., Switzerland) was prepared from liver of Sprague-Dawley male rats treated with Aroclor 1254. WLL (0.1 ml, 50-100 mg/ml corresponding to 5-10 mg/plate, respectively) were added to the overnight cultured S. typhimurium TA98 or TA 100 (0.1 ml) and S9 mix (0.5 ml) or 0.1 M phosphate buffer (0.5 ml, pH 7.4) in place of the S9 mix. The entire mixture was incubated at 37 °C for 20 min. Next, 2.0 ml of top agar was added and then spread out in a Petri dish containing 20 ml of minimum agar. The plates were incubated at 37 °C for 48 h and the mixture were counted manually. To examine the toxic effects of WLL on S. typhimurium TA 98 and TA 100, the mixtures after incubation were diluted with 0.1 M phosphate buffer (0.5 ml, pH 7.4), and the diluted mixtures were poured into minimal agar plates containing MgCl2, KCl, glucose-6-phosphate, and NADP. The plates were incubated at 37 °C for 2 days, and the numbers of colonies were counted.

2.13. Antimutagenic Activity Assay

The antimutagenic activity of WLL was conducted by the Ames method except for the addition of mutagen before incubation. The concentrations of mutagens were tested as in a previous study [13]. The mutagens used were 4-NQO (0.5 μg/plate), a direct mutagen; 2-AA (2.5μg/plate), which required S9 mix for metabolic activation. Mutagen (0.1 ml) was added to the mixture of a strain (TA 98 or TA 100), and samples were added with the S9 mix for 2-AA or with phosphate buffer (0.1 M, pH 7.4) for 4-NQO. The mutagenicity of each mutagen in the absence of samples is defined as 100%. The number of spontaneous revertants in the absence of mutagens and samples was used as reference. The inhibition (%) of mutagenicity of the WLL was calculated as following:

% of inhibition={1-[(No. of his^+ revertants with mutagen and WLL-No. of spontaneous revertant)/ (No. of his^+ revertants with mutagen-No. of spontaneous revertant)]} × 100.

2.14. RP-HPLC Analysis

Sample (50 mg/ml) was filtered through of 0.45 μm filter before injected HPLC (Hitachi Ltd., Tokyo, Japan), consisting of two model L-7100 pumps, and one model L-7455 photodiode array detector. The injection volume was 0.02 ml and the flow rate was 0.8 ml/min. The column temperature was set at 25°C. The column was a Mightysil RP-18 GP (5μm, 250 x 4.6 mm I.D). The method involved the use of a binary gradient with mobile phases containing: (A) phosphoric acid in water (0.1%, v/v) and (B) H_2O/CH_3CN (3:7, v/v). The solvent gradient elution program was as follows: 0-10 min, 100-80% A, 0-20% B; 10-20 min, 80-70% A, 20-30% B; 20-30 min, 70-55% A, 30-45% B; 30-40 min, 55-45% A, 45-55% B; 40-45 min, 45-20% A, 55-80% B; and finally 45-60 min, 20-0% A, 80-100% B.

2.15. Statistical Analysis

Data are expressed as mean±SD, and ANOVA was conducted by using the SPSS software. When a significant F ratio was obtained ($p < 0.05$) a post hoc analysis was conducted between groups by using a Duncan's multiple range tests or a Dunnett's test. p-Values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. RP-HPLC Analysis of Marker Components in WLL

The radical scavenging activities of longan from various tissues are contributed to various natural phytochemicals including epicatechin, ellagic acid, and

gallic acid, [14,15]. Thus epicatechin, ellagic acid, and gallic acid were selected as marker constituents for chromatographic finger-print analysis of WLL. Butyl p-hydroxybenzoate is an internal standard (IS). According to the plot of the peak-area ratio (y) vs. concentration (x, mg/ml), the regression equations of three constituents and their correlation coefficients (r) were as follows: gallic acid, y = 0.0588x + 0.2488 (r = 0.996); epicatechin, y = 0.036x + 0.0597 (r = 0.997) ; ellagic acid, y = 0.0249x + 0.1539 (r = 0.999). Table 1 show the HPLC-PAD chromatography for WLL, and displays a comparison of the amounts of the three marker constituents present in the WLL, ellagic acid (24.64 mg/g of WLL) > epicatechin (17.31 mg/g of WLL) > gallic acid (1.42 mg/g of WLL). Meanwhile, the levels of total polyphenols and total flavonoid in WLL determined as gallic acid and rutin equivalents were 268.0±8.7 and 48.3 ±2.5 mg/g of WLL, respectively.

Table 1. HPLC analysis of three marker components in water extracts of longan leaves (WLL)

Compound	Retention time (min)	λmax (nm)	Contents (mg/g of WLL)
gallic acid	22.67	258.6	1.42±0.02
epicatechin	31.92	266.6	17.31±0.04
ellagic acid	37.28	253.1	24.64±0.02

Values are express as the means ± SD for n=3.

3.2. Effects of WLL on Antioxidative Activity

Many reports have noted that the excessive levels of ROS decreased the intracellular antioxidant capacity and produced oxidative stress. Therefore, ROS reduction can contribute to the inhibition of oxidative damage. The effects of WLL and three maker components on radical scavenging and reducing activities are summarized in Table 2. Reducing activity of natural products is regarded as a hydrogen donating capacity, which can terminate radicals' chain reactions. The reducing ability of WLL and its marker constituents were determined in comparison with ascorbic acid. At 0.05-0.1 mg/ml, WLL showed reducing ability that increased in concentration-dependent manner. The reducing ability of WLL at 0.1 mg/ml was equivalent to 36.5 μg/ml of ascorbic acid. Further, as for the reducing activities of the three polyphenols in WLL, at the concentration of 0.01 mg/ml, gallic and ellagic acid exhibited reducing power superior to that of epicatechin. The relatively stable DPPH and ABTS radical has been widely used to the ability of natural products. From 0.05-0.1 mg/ml, the radicals scavenging activities on DPPH and ABTS of WLL were 43.3-91.0% and 27.4-94.2%, respectively, indicating that WLL is a potential radical scavenger as well as a strong radical terminator. As shown in Table 2, also displayed that the inhibitory effects of epicatechin, ellagic acid, and gallic acid on DPPH were 61.3%, 87.1%, 98.1%, while the inhibitory effects on ABTS were 99.4%, 98.1% and 99.1%. The data indicate that the radical scavenging activity of the three marker polyphenols might contribute to the antioxidant and reducing activity of WLL.

Table 2. Effects of water extracts of longan leaves (WLL) on radical scavenging and reducing activity

Sample	(mg/ml)	DPPH inhibition (%)	ABTS inhibition (%)	Reducing activity (μg Vit C/ml)
WLL	0.050	43.3±0.4	27.4±3.2	14.8±0.8
	0.075	65.2±4.1	66.2±4.1	26.5±0.5
	0.100	91.0±4.6	94.2±4.6	36.5±1.1
epicatechin	0.010	61.3±4.2	99.4±0.6	8.7±0.5
ellagic acid	0.010	87.1±1.8	98.1±0.8	18.9±1.0
gallic acid	0.010	95.9±1.3	99.1±0.4	23.9±1.0

Values are express as the means ± SD for n=3.

Table 3. Effects of water extracts of longan leaves (WLL) on chelating activity and liposome protection.

Sample	(mg/ml)	Chelating activity (%)	Liposome protection (%)
WLL	0.2	18.3±2.6	61.4±8.5
	0.4	48.6±0.8	97.8±5.1
	0.6	85.8±1.2	99.0±1.6
epicatechin	0.1	45.6±1.4	47.5±2.2
ellagic acid	0.1	79.1±1.6	26.2±2.4
gallic acid	0.1	33.7±1.4	42.8±2.9

Values are express as the means ± SD for n=3.

The chelating activity could serve as a potential inhibitory factor in oxidation processes; thus, the metal ions chelating capacity of WLL was determined. The results are summarized in Table 3, WLL in the range of 0.2-0.6 mg/ml exhibited 18.3-85.8% chelating activity on ferrous ions. The chelating capacities of epicatechin, ellagic acid, and gallic acid were 45.6%, 79.1%, and 33.7%. Lipid peroxidation plays a key role in the pathogenesis of diseases, producing toxic aldehyde and promoting cellular pathological metabolism. In this research, liposome protection was used as an index to assay the protective action of WLL on lipid oxidation. At 0.2-0.6 mg/ml, WLL showed a dose-dependent inhibitory effect, 61.4-99.0%, on the liposome oxidation induced by the Fe^{3+}/H_2O_2 system. The liposome protection of epicatechin, ellagic acid, and gallic acid was 47.5%, 26.2%, and 42.8%. These data indicated WLL could be an effective protector by chelating metal ions and reducing lipid oxidation in vitro.

Table 4. Effects of water extracts of longan leaves (WLL) on mushroom tyrosinase activity in acellular system and nitric oxide (NO) production in lipopolysaccharide (LPS) stimulated macrophages

Sample	(mg/ml)	Inhibition of tyrosinase activity (% of control)
WLL	0.5	38.9±3.2
	1.0	58.6±4.1
	1.5	87.2±4.6
epicatechin	0.05	27.2±1.9
ellagic acid	0.05	58.6±2.4
gallic acid	0.05	64.5±2.1
		Inhibition of NO production (% of LPS group)
WLL	0.05	15.6±2.9
	0.10	36.0±1.1
	0.15	53.9±2.4
	0.20	76.6±3.7

Values are express as the means ± SD for n=3. The macrophages cells were exposed to 200 ng/ml lipopolysaccharide for determination of NO production.

3.3. Effects of WLL on Tyrosinase Activity and LPS-Induced NO

Tyrosinase is a copper-containing and a key enzyme for melanogenesis. In this study, WLL exhibited a significant inhibitory activity on mushroom tyrosinase activity with an IC_{50} value of 0.83 mg/ml (Table 4). At 0.5-1.5 mg/ml, WLL exhibited 38.9-87.2% inhibitory activity on tyrosinase. In this test, the tyrosinase inhibitory activity of WLL increased with increasing sample concentration. Epicatechin, ellagic acid, and gallic acid (at 0.05 mg/ml) showed 27.2%, 58.6%, and 64.5% inhibitory actions on tyrosinase activity, respectively. In the cellular test, the protective effect of WLL was tested on the nitric oxide (NO) scavenging activity in LPS induced macrophages. NO plays a vital role in the pathogenesis of inflammation, the production of NO by activated macrophages was measured as nitrite in medium through the Griess reaction. As shown in Table 4, WLL at 0.05-0.2 mg/ml decreased NO production to 15.6-76.6% of that observed in the LPS group. No cell toxicity was observed in the presence of WLL, as measured by the MTT assay. The results suggested that WLL could reduce the RNS production in LPS stimulated macrophages.

3.4. The Mutagenicity and Antimutagenicity of WLL

The Ames test is a widely employed method to measure the mutagenicity of natural products. In this mutation study, if a lethal toxicity occurred in a test treated with samples, the results of the mutagenicity could be affected and confuse the numbers of revertants of TA 98 and TA 100. Therefore, the toxicities of WLL against *S. typhimurium* TA 98 and TA 100 were determined first. The WLL in the range of 2-10 mg/plate did not show any toxicity against TA 98 and TA 100, respectively, indicating that the WLL did not display toxicity in TA 98 and TA 100 (data not shown). Further, the mutagenicity of WLL was determined by comparing the ratio of induced revertants and spontaneous revertants in plates. As shown in Table 5, at 2-10 mg/plate, WLL did not significantly ($p > 0.05$) increase the numbers of colonies in *S. typhimurium* TA 98 and TA 100 with or without S9 activation. Therefore, the dose below 10 mg/plate was selected for the antimutagenic assay. For testing the antimutagenicity of WLL, 4-NQO and 2-AA were used as direct and indirect acting mutagens, respectively, to induce the *S. typhimurium* TA 98 and TA 100 mutation. As displayed in Table 6, WLL exhibited dose-dependent protective effects against 4-NQO induced mutagenicity in *S. typhimurium* TA 98 and TA 100 without S9 activation. WLL at levels of 2-10 mg/plate showed 10-40% inhibitory effects against 4-NQO induced mutagenicity in TA 98; meanwhile, they showed 4-44% inhibitory effects in TA 100. Table 6 also showed the antimutagenicity of WLL on 2-AA induced mutation in *S. typhimurium* TA 98 and TA 100 with S9 activation. WLL at levels of 2-10 mg/plate showed 13-72%, inhibitory effects against 2-AA induced mutagenicity in TA 98; meanwhile, they showed 27-92% protective effects in TA 100.

Table 5. The mutagenicity of water extracts of longan leaves (WLL) toward *S. typhimurium* TA98 and TA100 with and without S9 mix

Sample (mg/plate)	His^+ revertants/plate (% of spontaneous)	
	TA98	TA100
Spontaneous group	19±1 (100)a	126±2 (100)a
2	19±3 (100)a	123±3 (98)a
5	19±3 (100)a	123±6 (98)a
10	17±1 (89)a	120±4 (95)a
	TA98 + S9	TA100 + S9
Spontaneous group	25±4 (100)a	138±4 (100)a
2	26±2 (104)a	136±3 (99)a
5	26±3 (104)a	137±2 (99)a
10	24±3 (96)a	134±2 (97)a

Values are express as the means ± SD for n=3. Values with different superscripts in a column are significantly different ($p < 0.05$). % of spontaneous = [(no. of his^+ revertants in the presence of sample)/ (no. of spontaneous revertants)] ×100. The number of spontaneous revertants was determined without samples and mutagens.

The oxidative damage derived from RNS and ROS induces oxidation in macromolecules including lipids, DNA, and proteins, and then leads to mutation and cell death [13]. On the other hand, tyrosinase not only catalyzes undesirable reactions which change the color and flavor, and decrease in nutritional quality and economic loss of food product, but also increases the oxidative risk in different physiological systems [16]. Phytochemicals are rich natural sources with a complex array of antioxidants. They exhibit different protective effects against harmful stress in various models [17]. From the obtained data of this study, WLL showed a significant capacity in radical scavenging, lipid peroxidation and NO production prevention, as well as in tyrosinase and mutation inhibition. Moreover, the data indicated that the protective effects of WLL might be attributed mainly to its bioactive compounds.

Table 6. The antimutagenicity of water extracts of longan leaves (WLL) toward S. typhimurium TA98 and TA10

Sample (mg/plate)	His+ revertants/plate (% of inhibition)	
	TA98+4-NQO	TA100+4-NQO
0	382±14 (0) d	1204±10 (0) d
2	345±14 (10) c	1156±16 (4) c
5	306±11(21) b	835±20 (34) b
10	235±8 (40) a	732±12 (44) a
	His+ revertants/plate (% of inhibition)	
	TA98+2-AA +S9	TA100+2-AA +S9
0	405±16 (0) d	1298±33 (0) d
2	356±13 (13) c	986±25 (27) c
5	205±6 (53) b	319±14 (84) b
10	131±3 (72) a	233±13 (92) a

Values are express as the means ± SD for n=3. 4-NQO, 4-nitroquinoline N-oxide. 2-AA, 2-anthramine.Values with different superscripts in a column are significantly different ($p < 0.05$). % of inhibition = {1-[(no. of revertants with mutagen and sample - no. of spontaneous revertants)/ (no. of revertants with mutagen -no. of spontaneous revertants)]} ×100. The number of spontaneous revertants was determined without samples and mutagen.

Free radicals are unstable and highly-reactive species that have been reported to induce pathological events such as inflammation, aging and carcinogenesis [18]. In this study, WLL displayed significant protective activities. Three polyphenols were identified as epicatechin, ellagic

acid, and gallic acid in WLL. These compounds have been reported to possess strong antioxidant activities. Beside these, epicatechin, ellagic acid, and gallic acid are widely recognized to decrease oxidation of LDL and increase the antioxidant capacity of cells. As shown in Table 2, WLL exhibited a significant reducing ability. In particular, gallic acid showed greater reducing effects than the other marker constituents. Accumulating studies has revealed that the reducing effects of phenolic compounds could play a critical role, not only in free radical scavenging, but also in the prevention of cell damage. Thus, WLL and its phenolic component might protect biomolecules from oxidative damage. Additionally, reducing agents (e.g. ascorbic acid) could be used as tyrosinase inhibitors by reducing oxidized substrate, indicating that WLL and its maker constituents with reducing activity are potential inhibitors of tyrosinase [19].

Lipid peroxidation is recognized as the main mechanism involved in long term damage to cell and the toxicity process that lead to cell death [20]. As shown in Table 3, in the liposome model system, WLL showed protective activity against the damage caused by lipid oxidation. Moreover, epicatechin, in the liposome model system, showed the greatest protective action among the marker phenolic compounds. Obviously, WLL and its phenolic components could show the potential ability in the inhibition of lipid oxidative damage and thereby protect biomolecules against oxidative damage in organs. Additionally, lipid peroxidation yield exocyclic propane, etheno and malondialdehyde adducts in DNA to induce mutation [21]. The obtained data indicated that WLL could be an effective protector in preventing lipid peroxidation.

Tyrosinase, a copper-containing enzyme, plays an important role in catalyzing the melanogenic pathway and promotes the reactive metabolites produced in the process of melanin generation [19]. In the process of polyphenol oxidation, quinonic compounds were formed, which then reacted with the free nucleophilic group of proteins, denatured the protein structure and irreversibly destroyed the enzyme function [22]. Meanwhile, the synthesis of melanin causes glutathione depletion and the formation of hydrogen peroxide. As shown in Table 4, WLL clearly decreased tyrosinase activity in vitro. This finding shows that WLL can decrease the danger posed by a reduction in glutathione and quinone induced cell damage. Recent studies have supported the role of metal ion chelation in the processes of tyrosinase inhibition [19]. These data imply that polyphenols have different biological activities due to their redox properties, allowing them to act as reducing agents, hydrogen donators, and tyrosinase inhibitors. Consequently, it is possible that the polyphenolic constituents in the WLL were able to contribute to their physiological activity.

Accumulating studies have revealed that NO can act as a mutagen through deamination of methylcytosine in DNA [23]. Excess NO also plays a critical role in production more toxic peroxynitrite anions and further induces the formation of 8-nitroguanine DNA adducts [24]. In this study, WLL inhibited NO production to less than 25% by 0.2 mg/ml in LPS stimulated macrophages. The obtained results indicated that WLL decreases the nitrosative stress formation activated phagocytes and may further decrease the nitration of biological molecules, mutation of DNA, and progression of aging diseases. In contrast, polyphenolic compounds display biological function due to their redox properties, allowing them to act as reducing agents, hydrogen donators, and reactive oxygen quenchers [17]. Thus, it is possible that the polyphenols in WLL could contribute to their RNS reduction ability. Note that WLL could inhibit NO overproduction, and consequently decrease NO-induced DNA damage as well as cytotoxicity. Polyphenolic compounds such as gallic acid of WLL showed inhibitory activity against NO production [25]. These findings imply that WLL containing protective components may potential protect organ from injury during inflammation by suppressing the NO associated metabolism. However, the mechanism underlying the action could also be attributed to WLL inhibition NO production in activated macrophages.

Foods may contain mutagenic and carcinogenic substances as very minor components: nitrosamine, polycyclic aromatic hydrocarbons, and heterocyclic amines. These mutagenic compounds have planar structure that can be inserted between the base pairs of double-stranded DNA. The multiple genetic alterations could be produced. In the present study, WLL exhibited multiple health beneficial functions including antimutation, antioxidant activities, and decreased the nitrosative stress. Neither toxicity nor mutagenicity was found in WLL in *S. typhimurium* TA 98 and TA 100 with or without S9 activation. The obtained results displayed that none of the WLL or their constituents showed frame shift, base pair mutagenicity or cytotoxicity in this study. In fact, recent researches have reported that natural products may play an important role in preventing mutagen activation in the *S. typhimurium* bacterial model. For example, molasses has displayed antimutagenic activity against different mutagens induced mutations of *S. typhimurium* TA 98 and TA 100 [13]. In this study, 4-hydroxyaminoquinoline 1-oxide (4-NQO) produced the ultimate mutagenic compound, which binds to DNA generating stable quinoline-purine mono adducts [26]. On the other hand, 2-AA undergoes the biotransformation pathway by a cytochrome P450-dependent monooxygenase to produce a nitrene moiety, which can bind to DNA [13]. According to the data reported in Table 6, WLL displayed inhibitory activity on the mutagenicity of 4-NQO and 2-AA in *S. typhimurium*. The obtained results indicated that WLL could play an antimutagenic role by scavenging the active metabolic electrophile of 4-NQO and 2-AA. The conjugated reaction between WLL and the toxic electrophile molecules might be an important detoxification pathway. Beside these, the antimutagenic activities of the WLL could be attributed to the decreased metabolic activation and the levels of toxic reactive intermediates, which would further prevent cell from oxidative damage.

4. Conclusions

WLL, a natural product of agriculture waste, decreased mutation, oxidation and tyrosinase activity, and also inhibited RNS generation in LPS stimulated macrophages. The biological functions might be closely attributed to

their phenolic compounds, epicatechin, ellagic acid, and gallic acid, which showed protective effects in the various models of this research. The data suggests that WLL can exhibit biological functions in antimutation, antityrosinase, antioxidant and anti-inflammation in vitro. The results also could be useful in effectively utilizing discarded longan leaves as functional products.

Conflicts of Interest

The authors declare no conflict of interest.

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