Hindawi Publishing Corporation
Evidence-Based Complementary and Alternative Medicine
Volume 2013, Article ID 925380, 13 pages
http://dx.doi.org/10.1155/2013/925380

Research Article

Evaluation of the Genotoxic Potential against H_2O_2-Radical-Mediated DNA Damage and Acute Oral Toxicity of Standardized Extract of *Polyalthia longifolia* Leaf

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Received 11 March 2013; Revised 23 May 2013; Accepted 7 June 2013

Academic Editor: Benny Tan Kwong Huat

Medicinal plants have been used in medicoculturally diverse countries around the world, where it is a part of a time-honoured tradition that is respected even today. *Polyalthia longifolia* leaf extract has been previously reported as an efficient antioxidant *in vitro*. Hence, the genotoxic effects of *P. longifolia* leaf were investigated by using plasmid relation, comet, and *Allium cepa* assay. In the presence of $^{\bullet}OH$ radicals, the DNA in supercoil was start nicked into open circular form, which is the product of the single-stranded cleavage of supercoil DNA and quantified as fragmented separate bands on agarose gel in plasmid relation assay. In the plasmid relation and comet assay, the *P. longifolia* leaf extract exhibited strong inhibitory effects against H_2O_2-mediated DNA damage. A dose-dependent increase of chromosome aberrations was also observed in the *Allium cepa* assay. The abnormalities scored were stickiness, c-mitosis, bridges, and vagrant chromosomes. Micronucleated cells were also observed at the interphase. The results of *Allium cepa* assay confirmed that the methanol extracts of *P. longifolia* exerted no significant genotoxic or mitodepressive effects at 100 μg/mL. Thus, this study demonstrated that *P. longifolia* leaf extract has a beneficial effect against oxidative DNA damage. This experiment is the first report for the protective effect of *P. longifolia* on DNA damage-induced by hydroxyl radicals. Additionally in acute oral toxicity study, female rats were treated at 5000 mg/kg body weight of *P. longifolia* leaf extract and observed for signs of toxicity for 14 days. *P. longifolia* leaf extract did not produce any treatment-related toxic effects in rats.

1. Introduction

The consumption of plants, plant extracts, or plant-derived phytochemicals to treat various ailments is a normal therapeutic activity that has been practiced since time immemorial. The World Health Organization estimates that up to 80% of the world's population relies on the traditional medicinal system for some aspects of primary health care [1]. However, it is also essential to note that most of the traditional herbal products have never been the subject of comprehensive toxicological investigations, such as is required for modern pharmaceutical products. Based on their traditional use for long periods of time, they are often assumed to be safe. However, many researchers have exposed that numerous herbal products, which are used as food ingredients or in traditional medicine, have *in vitro* mutagenic or toxic properties [2–4]. *Polyalthia longifolia* var. *angustifolia* Thw. (Annonaceae) is a small medium-sized tree with linear-lanceolate leaves, 1 to 1.5 cm broad, occurring in Sri Lanka and now grown in tropical parts of India along roadsides and in gardens for their beautiful appearance [5]. *P. longifolia* is one of the most important indigenous medicinal plants and is found throughout Malaysia where it is widely used in traditional medicine as a febrifuge and tonic [6].

The diterpenes, alkaloids, steroid, and miscellaneous lactones were isolated from its bark [6]. The stem bark extracts and isolated compounds were studied for various biological activities like antibacterial, cytotoxicity, and antifungal activity [7]. The rareness of evidence from the literature on the possible toxicity, including genotoxicity of the methanolic leaf extract of *P. longifolia*, shows that, although it has not been thoroughly investigated, its pharmacological efficacy does not appear to be in doubt. Therefore, the aim of this study was to determine the possible toxicity of *P. longifolia* methanolic leaf extract using *in vitro* systems.

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between plant extract and cells. This interaction may vary depending on the active ingredient presence in the extract, as it may occur on the cell surface, within the cell body, DNA, or in the tissues beneath as well as at the extracellular matrix. Hence, evaluation of toxic properties of a plant extract is crucial when considering in public health protection because exposure to chemicals can be hazardous and results adverse effects on human beings. In practice, the evaluation typically includes acute, subchronic, chronic, carcinogenic, genotoxic, and reproductive effects [8]. Our previous studies have found that the leaf extract of *P. longifolia* exhibited good antioxidant activity because of the high content of polyphenols and flavonoids (quercetin, rutin, narcissin, isorhamnetin, and kaempferol) in *P. longifolia* [9]. Thus, in this study we predict that antioxidant molecules may also play an important role in the prevention of genotoxic damage. In this study, the antigenotoxic effect of the leaf extract of *P. longifolia* was investigated by using comet assay, plasmid relaxation assay, and *Allium cepa*, which provide sensitive and rapid monitoring of induced genetic damage as primary DNA damage in vero cell, puc18, and meristem root cell against hydroxyl radical. The cellular macromolecules of humans, such as DNA, proteins, and lipids, are continuously at risk to endogenous and environmentally induced damage. Reactive oxygen species (ROS) are an important class of damage agents for cellular macromolecules. ROS, such as O_2^-, OH^-, and H_2O_2, are highly genotoxic/mutagenic and harmful to cellular macromolecules, such as DNA, proteins, and lipids [10]. The adverse effect is represented by the oxidative stress that can arise from a lack of antioxidant defence or by an increase in the oxidative processes in the body [11]. For this reason, it has been proposed that dietary antioxidants in food significantly decrease the adverse effects of ROS, reactive nitrogen species, or both on the normal physiological function in humans [11]. The close relationship between antioxidant activity and antimutagenicity has been consistently reported. Hence, we propose that any possible antigenotoxic effect exhibited by *P. longifolia* leaf extract may have originated from the presence of antioxidative molecules, such as polyphenols and flavonoids in *P. longifolia* leaf extract, which will be investigated in this research. To the extent that antioxidants have attracted much attention with respect to their protective effect against free radical damage, the antigenotoxic potential of *P. longifolia* leaf was invested in the present study. The present study aims to determine the toxicity of *P. longifolia* leaf using an acute oral toxicity test in animal models [12] and genoprotective activity against DNA damage. To the best of our knowledge, this is the first detailed report of the protecting activity of *P. longifolia* leaf against DNA damage.

2. Methods and Materials

2.1. Plant Sample Collection. The leaves of *P. longifolia* were collected from various areas in Universiti Sains Malaysia, Penang, in January 2012, and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample has been deposited (Voucher specimen: USM/HERBARIUM/11306). The leaves were separated and cut into small pieces, which were first washed with tap water and then with distilled water. The leaves were then dried in an oven at 60°C for 7 days, after which the dried leaves were ground into a fine powder using a grinder and stored in clean, labelled airtight bottles.

2.2. Solvent Extraction. The leaf sample was sequentially extracted with methanol by adding approximately 100 g of the dried sample into 400 mL methanol. The extraction was carried out at room temperature by soaking for 7 days with intermittent stirring during the first day. The extracts were filtered through clean muslin cloth and the extraction process was repeated again for a second time by adding another 400 mL of methanol to the sample residue. The filtrate from each extraction was combined and concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at 40°C to 50°C in order to evaporate the excess methanol solvent and until a dark green methanol extract was produced. The concentrated extract was poured into Petri dishes and brought to dryness at 60°C in the oven until a paste-like mass was obtained. Then, a paste form extract was sealed in Petri plates and stored at room temperature (RT). The crude extract was prepared by diluting the paste in methanol and storing in airtight bottles at 4°C for further study. HPLC chromatogram of *P. longifolia* was showed in Figure 1. The chromatographic analyses was performed on a Novapak C18 (4.6 × 250 mm) reversed-phase column and a gradient elution using a mobile phase consisting of methanol/water/formic acid (49 : 59 : 1, v/v) was found to be optimum. To standardize the fingerprint, 10 batches of *P. longifolia* samples from Penang, Malaysia, were analysed with the developed procedure. The peaks that existed in all then ten batches of samples were assigned as "common peaks" for the *P. longifolia* extract (Figure 1). There are nine "common peaks" in the fingerprint.

2.3. Acute Oral Toxicity Study

2.3.1. Target Animal. Female Sprague-Dawley (SD) rats (nulliparous, 8–12-week old) were obtained from the Animal House, University Science Malaysia (USM), Penang. The experimental procedure was approved by Animal Ethical Committee (USM/Animal Ethics Approval/2011/(74) (380)). The animals were housed in well-ventilated animal house with 12 hr light and dark schedule and easy access to water

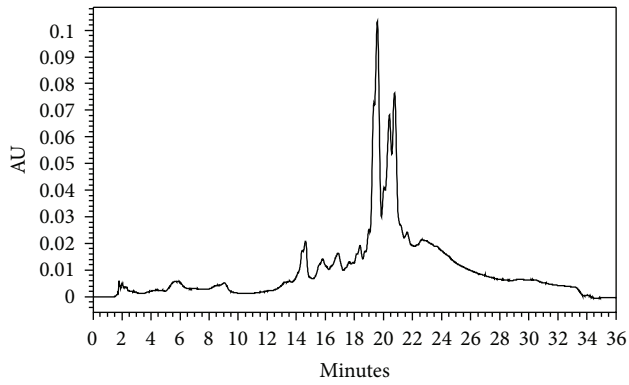

Figure 1: HPLC fingerprint chromatograms of *P. longifolia* leaf.

and standard pellet diet (European Commission, 1986). Animals were randomly selected, marked to permit individual identification, and kept in their cages for at least 5–7 days prior to dosing to allow for acclimatization to the laboratory conditions.

2.3.2. Acute Toxicity Assays. The rats were housed in cages and randomly selected ones were marked on the tail for individual identification. All rats were maintained on a 12 h light/dark cycle and located at room temperature approximately 23°C with constant humidity. Drinking water and food were provided throughout the experiment, except for the short fasting period where the drinking water was still in free access but no food supply was provided 12 h prior to treatment. The acute oral toxicity methanolic leaf extract of *P. longifolia* was evaluated in rats according to the procedures outlined by the Organization for Economic Co-operation and Development (OECD) [13]. A single high dose of 5,000 mg/kg of crude extract was administered to rats in the treatment group by the oral route. The crude extract was suspended in a vehicle (10% Tween 20). The body weight of the rats was determined and the dose was calculated in reference to the body weight as the volume of the extracts' solution given to the rats is 10 mL/kg. Meanwhile, another group of rats were allotted distilled water and were regarded as the control groups. The rats were observed for any indications of toxicity effect within the first seven hours after the treatment period and daily further for a period of 14 days. Surviving animals were weighed and visual observations for mortality, behavioral pattern, changes in physical appearance, injury, pain, and signs of illness were conducted daily during the period.

2.3.3. Organs and Body Weight Analysis. Finishing the 14-day period, all the rats were sacrificed. Vital organs such as heart, kidneys, liver, lung and spleen were isolated and examined for any lesions. All of the individual organs were weighed and their features were compared between both treated and control groups.

2.3.4. Histopathology of Heart, Kidney, Liver, Lung, and Spleen. All the vital organs isolated from each individual were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin wax. Paraffin sections (5 μm) were cut on glass slides and stained with haematoxylin and eosin. The slides were examined under a light microscope and the magnified images of the tissues' structure were captured for further study [14].

2.3.5. Blood Biomarker Assays. After 14 days of treatment with *P. longifolia* leaf extract, the rats blood were further evaluated for biochemical analysis. In the present study, the liver function was evaluated with serum levels of TBIL, ALP, ALT, and AST.

2.3.6. Statistical Analysis. Statistical analysis involved the use of the statistical package for social sciences (SPSS). Data are given as the Mean ± SD; statistics were performed using t-tests, and P values less than 5% were considered statistically significant ($P < 0.05$).

2.4. Plasmid Relaxation Assay. The DNA strand damage was measured by converting circular double-stranded supercoiled DNA into nicked circular and linear forms by *P. longifolia* leaf extract, as described by Kobayashi et al. [15], with some modification. The reaction mixture consisted of supercoiled pUC18 plasmid (20 ng), 20 mM Tris-HCl buffer (pH 7.6), 30 mM hydrogen peroxide, 100 μM ferric chloride, 100 μM ascorbic acid, and leaf extract (1, 0.5, 0.05, 0.005 mg/mL). Quercetin (200 μg/mL) a known phenolic compound was used as a positive control [16]. The mixtures were incubated at 37°C for 30 mins. The DNA were analysed on 0.7% agarose gel electrophoresis followed by ethidium bromide staining.

2.5. Cytotoxicity Screening

2.5.1. Vero Cell Line. The Vero cell line used during the present study was obtained from the Tissue Culture Laboratory of the Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Pulau Pinang, Malaysia. The Vero cell line was initiated from the kidney of a normal adult African green monkey on 27th March 1962, by Yasumura and Kawakita at the Chiba University, Japan (American Public Health Association, 1992). Vero cells were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 raM), penicillin (100 units/mL), and streptomycin (100 μg/mL). The cells were cultured at 37°C in a humidified 5% CO_2 incubator. Vero/HS27 cells were cultured and maintained in RPMI 1640 medium supplemented with 10% FBS. The cells were cultured at 37°C in a humidified 5% CO_2 incubator.

2.5.2. Cytotoxicity Assay. The leaf extracts of *P. longifolia* were tested for *in vitro* cytotoxicity, using Vero cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [16]. Briefly, 100 μL of media (RMPI 1640) was added into each of the 96-well plates (triplicate). Then, the plant extracts were diluted in media by using a micropipette and serial dilution was done. The final concentration of plant extract into each well was between 200 and 3.125 μg/mL.

The cultured Vero cells were harvested by trypsinization, pooled in a 50 mL vial. Then, the cells were plated at a density of 1×10^6 cells/mL cells/well (100 μL) into 96-well microtitre plates. Finally, 200 μL of Vero cells were added as a control. Each sample was replicated three times and the cells incubated at 37°C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 μL of 5 mg/mL) was added into each well and the cells incubated for 2 to 4 h, until a purple precipitate was clearly visible under a microscope. The medium, together with MTT, was aspirated off from the wells, DMSO (100 μL) added, and the plates shaken for 5 min. The absorbance for each well was measured at 540 nm in a microtitre plate reader [17] and percentage cell viability (CV) calculated manually using the formula

$$\text{CV} = \frac{\text{Average abs of duplicate drug wells}}{\text{Average abs of control wells}} \times 100\%. \quad (1)$$

A dose-response curve was plotted to enable the calculation of the concentration that kills 50% of the Vero cells (CC_{50}).

2.6. Comet Assay

2.6.1. Cell Culture and Treatment. The Vero cells were grown in RPMI 1640 culture medium maintained at 37°C in a humidified 5% CO_2 incubator. Cells (0.5×10^5 cell/mL) in a 6-well microplate were preincubated with and without plant extract at the concentration determined previously using MTT assay. The plate was incubated inside the humidified incubator for 24 hr to allow for cellular uptake of the phenolic phytochemicals from the plant extract. Quercetin (50 μM) a known phenolic compound was used as a positive control [18]. The cells were then challenged with 1 mM H_2O_2 10 μM $CuSO_4$ for 30 min. Afterwards, the cells were washed with ice-cold phosphate buffer saline (PBS). Following centrifugation, the cell pellets were resuspended in 1 mL of PBS and the concentration of cells (1×10^5 cells/mL) was determined using a haemocytometer before analysing the extent of DNA damage.

2.6.2. Assessment of Cellular DNA Damage. The alkaline microgel electrophoresis or the "comet assay" was carried out to determine the extent of DNA damage in cells [19]. Briefly, a 7 μL aliquot of cells (1×10^5 cells/mL) was added to 70 μL of molten LM Agarose (0.5% low-melting agarose). After mixing, the sample was pipetted onto an area of the comet slide. The slide was incubated at 4°C for 15 min to accelerate gelling of the agarose disc and then transferred to prechilled lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 1% sodium lauryl sarcosinate, 0.01% triton X-100) for 30 min at 4°C. The denaturation step was performed in an alkali solution (0.3 M NaOH, 1 mM EDTA) at room temperature (RT) for 30 min, shielded from light. The slide was then transferred to 1x TBE (90 mM Tris-borate, 2 mM EDTA) for 10 min to neutralize, then electrophoresis was conducted in a horizontal chamber using fresh 1x TBE at 25 V (0.96 V/cm, approximately 300 mA) for 20 min. Then, the slide was fixed in ice-cold, 100% ethanol for 5 min and air-dried. The slide was then stained with 30 μL of 20 μg/mL ethidium bromide. The photographs of the comet image were taken using an inverted microscope (Fujitsu, Japan), and the length of the whole comet (head and tail) was measured using comet score software.

2.7. Allium cepa Assay

2.7.1. Pretreatment. *A. cepa* bulbs were selected to evaluate chromosomal aberration because it is easy to obtain root meristem and have a small number of chromosomes ($2n = 16$). The *A. cepa* bulbs were grown in tap water at room temperature for 2-3 days. When the roots were 2–4 cm in length, the bulbs were treated with different concentrations of the crude extract (100, 250, 500 μg/mL) for 24 h. Quercetin (10 μg/mL) a known phenolic compound was used as a positive control [20]. Another set of roots was placed in 30% H_2O_2 for 30 min. For the negative control, a set of *A. cepa* was grown in water. Then, the root tips from each bulb were harvested and fixed in Carnoy's fixative (1 : 3 acetic acid : alcohol) for 24 h before proceeding to the preparation of the slides [21].

2.7.2. Preparation of Slides. Preparation of the slides was carried out according to A. K. Sharma and A. Sharma [22]. After pretreatment, the root tips were washed a few times with distilled water. They were hydrolysed with 1 N HCl at 60–70°C for 10 min. After hydrolysis, the roots were washed with distilled water and about 1-2 mm of the root tips were cut and placed on the slide. A small drop of aceto-orcein was dropped on the root tip and left for 2 min. The root tip was squashed with a metal rod and another drop of aceto-orcein was added and left for another 2 min, before the cover slip was carefully lowered to avoid air bubbles and the sides of the slides were sealed with clear nail polish. The slides were observed under the light microscope (Olympus digital camera) at 400x magnification for induction chromosomal aberrations. Nine slides were prepared for each treatment and minimum 100 cells per slide were analysed. The mitotic index, micronucleus in the interphase and chromosome aberrations in mitotic phases, was determined by examination. The experiment was replicated three times. The mitotic index was obtained as follows.

> Mitotic index = Number of cells in mitosis/total number of cells [22].

3. Results and Discussion

3.1. General Signs and Behavioural Observation. The toxic effect of *P. longifolia* leaf extract on the appearance and the general behavioural pattern of rats is shown in Table 1. No toxic symptoms or mortality were observed in any animal, which lived up to 14 days after the administration of the methanolic leaf extract at a single dose level of 5000 mg/kg body weight. The behavioural patterns of the animals were observed for the first 7 h, followed by 14 h after the administration; the animals in both the control and extract treated groups were normal and did not display any significant changes in behaviour, skin effects, breathing, impairment in

Table 1: General appearance and behavioural observations for control and treated groups.

Observation	Control group		Treatment group	
	7 hr	14 hr	7 hr	14 hr
Skin and fur	Normal	Normal	Normal	Normal
Eyes	Normal	Normal	Normal	Normal
Mucous membrane	Normal	Normal	Normal	Normal
Behavioural patterns	Normal	Normal	Rapid heart beat	Normal
Salivation	Normal	Normal	Normal	Normal
Lethargy	Normal	Normal	Normal	Normal
Sleep	Normal	Normal	Normal	Normal
Diarrhea	Normal	Normal	Normal	Normal
Coma	N.O.[a]	N.O.	N.O.	N.O.
Tremors	N.O.	N.O.	N.O.	N.O.

[a] Not observed.

Table 2: Effect of single oral administration of *P. longifolia* leaf extract on organ-to-body weight index in rat.

Organs	Organ body weight index (g)	
	Control	Treated
Heart	0.95 ± 0.02	1.1 ± 0.18*
Kidney	0.35 ± 0.02	0.37 ± 0.05*
Spleen	0.24 ± 0.03	0.26 ± 0.05*
Liver	8.98 ± 0.55	9.16 ± 0.14*
Lung	1.38 ± 0.13	1.66 ± 0.11*

Organ body index = (organ weight × 100)/body weight; crude extract of *P. longifolia* was administered to rat at a dose of 5000 mg/kg; values are mean ± SD ($n = 5$) at 5% level of significance (* $P < 0.05$).

food intake and water consumption, postural abnormalities, and hair loss. In the treated group, in the first 7 h, a rapid heartbeat was observed after the administration; however, it then become normal and may have been due to the stress of receiving the oral administration of the extract.

3.2. Organ and Body Weight Analysis. The body weight and the weights of the vital organs of the animals in both the control and extract treated groups were calculated and are recorded in Table 2. There were no significant changes in body weight. However, the weights of the principal organs showed a significant increment. All animals exhibited a normal increment in body weight without a drastic difference between the two groups. The effect of the extract on the principal organ weights relative to body weight is shown in Table 2. The results revealed that the essential organs, such as kidney, liver, heart, lung, and spleen, were not adversely affected throughout the treatment. Extract treated rats showed increased organ weight. Therefore, the extract was found to be safe at a dose level of 5000 mg/kg and LD_{50} value, which is considered to be higher than 5000 mg/kg. The absolute and relative organ weight of rats between extract treated and control groups showed statistically significant differences ($P < 0.05$).

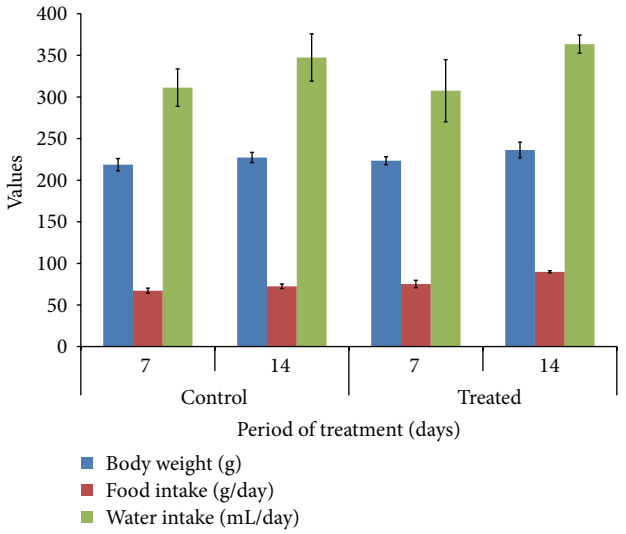

Figure 2: Effect of single dose (5000 mg/kg) administration of the *P. longifolia* leaf extract in rats.

There were no toxicological significant changes in body weight or body weight gain among animals treated with the extract (Figure 2). Figure 2 depicts the body weight profile of the animals tested for acute toxicity of the plant extract. No sign of observable toxicity was detected during the entire experimental course. The food and water consumption levels of animals treated with the plant extract were considered to have been slightly affected by the treatment (Figure 2). In the treated animals, it was observed that the food and water consumption was slightly increased in week 1 and 2 of the treatment phase (Figure 2), which shows a statistically significant difference when compared to the control group ($P < 0.05$).

3.3. Histopathology Analysis. The histopathological analysis revealed no apparent changes in the liver, kidney, lungs, heart, and spleen from both the control and treated rats. The microscopic structures of the organs depicted in Figure 3 show unnoticeable differences between the control and test groups. The microscopic examination revealed that none of the organs from the extract treated rats showed any alteration in cell structure or any unfavourable effects when viewed under the light microscope using multiple magnification power. No pathologies were recorded in the histological sections of the vital organs (heart, liver, spleen, kidney, and lung) of the control group. Figure 3 depicts the histological details of the organs studied. No lesions or pathological changes related to the treatment of the plant extract were seen in the organs of the animals from the treatment groups. However, in the lungs there was evidence of mild inflammation, which could possibly be due to the oral gavage. Nevertheless, the treatment-related results were similar to those of the control group.

3.4. Haematology and Biochemical Analysis. The findings of the histopathological analysis revealed supportive evidence

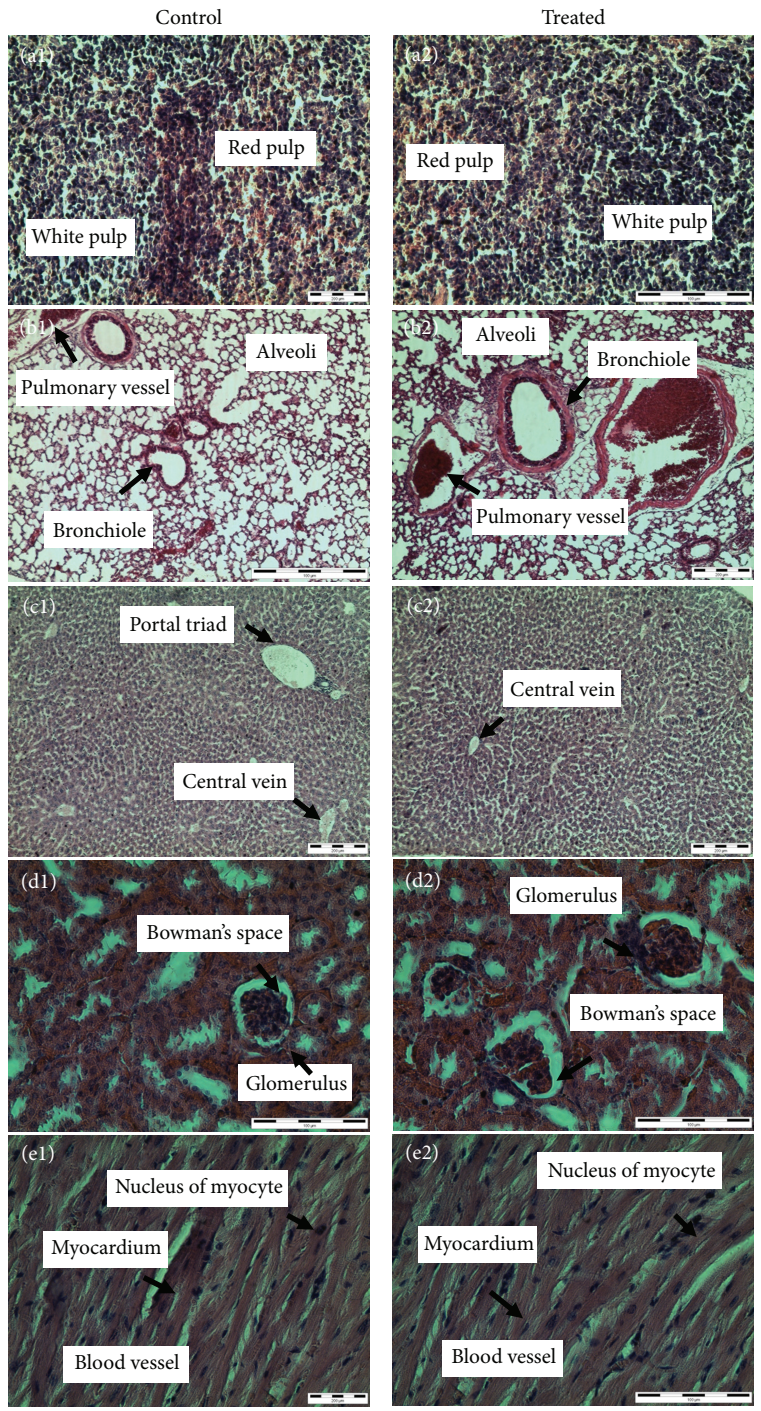

FIGURE 3: Representative histological photomicrographs of (a) kidney, (b) lung, (c) liver, (d) spleen and (e) heartof control and *P. longifolia* leaf extract treated groups (at dose 5000 mg/kg).

for haematological and biochemical analysis. The haematology and biochemicalanalysis of the blood samples taken from animal groups treated with *P. longifolia* leaf extract are presented in Tables 3 and 4. No toxicological significant differences were noted between the animals treated with the *P. longifolia* leaf extract and the control animals. The effects of a single-dose treatment on the haematological parameters are depicted in Table 3. All parameters, except the haemoglobin, red blood cells, and platelet count in rats treated with single dose of plant extract, showed a significantly increased level compared to the untreated group. A slightly increased neutrophil level, as well as a decreased level of monocyte level, was also observed. None of the tested haematological parameters, such as haemoglobin, total

blood count, total white blood cell, neutrophil, lymphocyte, eosinophil, monocyte, basophil, packed cell volume, and platelet count, showed any significant changes.

Meanwhile, the changes in the biochemistry parameters were not statistically significant between the treated and control animals at the end of the experiment. The biochemical parameter profiles of the treated and control groups are shown in Table 4. The oral administration of a single dose of the plant (5000 mg/kg) *P. longifolia* leaf extract did not cause any significant changes in total protein, albumin, globulin, albumin/globulin ratio, total bilirubin, alkaline phosphatase, alanine transaminase aspartate transaminase, gamma glutamyl transferase, urea, potassium, sodium, chloride, creatinine, or uric acid. In addition, the biochemical parameters, such as serum total bilirubin, AST, ALT, GGT, serum total proteins, serum total albumin, and serum total globulin, were within the normal level. In general, no significant biochemical, hematological, or histopathological changes were noted in rats that received single dose level of 5000 mg/kg body weight of *P. longifolia* leaf extract signifying that the extract was not toxic.

In conclusion, the oral dose of 5000 mg/kg of *P. longifolia* leaf extract did not produce any treatment-related signs of toxicity, mortality, or any pathological abnormality in any of the internal organs at necropsy on day 14; thus, its oral lethal dose for female rats is in excess of 5000 mg/kg. The findings of this study also corroborate the findings of Chanda et al. [23] who recently reported the acute oral toxicity of *P. longifolia* leaf extract in rats. They found that the methanol extract of *P. longifolia* leaf up to the dose level 3240 mg/kg body weight did not produce any toxic effect or death; the extract was well tolerated by the rats. Ours is probably the first demonstration on the acute oral toxicity of *P. longifolia* leaf extract at 5000 mg/kg body weight. The present study confirms, in part, the safety of *P. longifolia* leaf extract and is in line with the long history of its safe use in traditional medicine. However, recent investigations have revealed that many plants used as food or in traditional medicine showed mutagenic effects in *in vitro* assays [24–26]. This raises concern about the potential mutagenic hazards resulting from the short-term and long-term use of such plants. Therefore, further study will concentrate on evaluating the genotoxic potential of *P. longifolia* leaf extract by employing *in vitro* models, such as plasmid relaxation assay, comet assay, and *Allium cepa* assay.

3.5. Plasmid Relaxation Assay. In the present study, we have employed the H_2O_2-Fe^{+++}-ascorbate system, which leads to the Fenton reaction to induce oxidative damage of DNA. The plasmid relation assay was used to assess the DNA oxidative damage semiquantitatively using pUC18 plasmid. In the presence of hydroxyl radicals generated by Fenton reaction, the DNA in the supercoil was start nicked into open circular form, which will be the product of single-stranded cleavage of supercoil DNA. Then the open circular DNA can be further cleaved into single-stranded nicked form, which will be the result of double-stranded cleavage. Moreover the extensive oxidative damage would eventually lead to DNA fragmentation and subsequent degradation, depending on the amount of OH- radicals generated [27]. Figure 4 shows H_2O_2, Fe^{+++} or ascorbate alone treated pUC18 plasmid where supercoiled DNA migrated the fastest, nicked to open circular form, which migrated the slowest (lane 2, 3, and 4). However, in the presence of all three reagents together,

Table 3: Effect of single oral administration of the extract on hematological parameters in SD rat.

Hematological parameters	Unit	Acute oral toxicity	
		Control	Plant extract 5000 mg/kg
Haemoglobin	g/L	195 ± 2.3	213 ± 11.82
Total red blood cell	$\times 10^{12}$/L	13.19 ± 0.2	16.24 ± 1.41
Total white blood cell	$\times 10^{9}$/L	10.2 ± 3.5	11.4 ± 0.41
Neutrophil	%	21.7 ± 2.4	27 ± 3.4
Lymphocyte	%	72 ± 8.1	83 ± 2.6
Monocyte	%	8.2 ± 1.1	5.14 ± 0.7
Eosinophil	%	0 ± 0	0 ± 0
Basophil	%	0 ± 0	0.00 ± 0.00
Packed cell volume (PCV)	L/L	0.7 ± 0.02	0.82 ± 0.01
Platelet count	$\times 10^{9}$/L	1083 ± 66.57	1270 ± 112.2

Table 4: Effect of single oral administration of the extract on biochemical parameter in SD rat.

Biochemistry parameters	Unit	Acute oral toxicity	
		Control	Test sample 175 mg/kg
Sodium	mmol/L	157.2 ± 3.4	148.2 ± 6.1
Potassium	mmol/L	23.4 ± 1.5	18.9 ± 1.3
Chloride	mmol/L	164.8 ± 10.3	182.6 ± 1.95
Urea	mmol/L	9.31 ± 2.04	11.07 ± 0.51
Creatinine	umol/L	26.84 ± 2.49	31.5 ± 1.9
Uric acid	mmol/L	0.46 ± 0.03	0.39 ± 0.04
Total Protein	g/L	93.4 ± 4.09	88.8 ± 2.82
Albumin	g/L	37.05 ± 2.36	42.3 ± 3.17
Globulin	g/L	77.1 ± 4.33	69.6 ± 4.71
Albumin/globulin ratio		0.61 ± 0.1	0.58 ± 0.04
Alkaline phosphatase	U/L	347.61 ± 52.14	287.2 ± 24.5
Total bilirubin	umol/L	<1 ± 0	<1 ± 0
(GGT) gamma glutamyl transferase	U/L	<2 ± 0	<2 ± 0
AST aspartate aminotransferase	U/L	1461.3 ± 147.2	1282.1 ± 247.2
ALT Alanine aminotransferase	U/L	320.2 ± 128.52	289.4 ± 118.7
Total cholesterol	mmol/L	7.1 ± 1.4	5.2 ± 0.81
LDL	mmol/L	2.03 ± 0.07	1.22 ± 0.8
HDL	mmol/L	1.18 ± 0.03	1.46 ± 0.08
Triglyceride	mmol/L	5.08 ± 1.4	3.77 ± 0.9

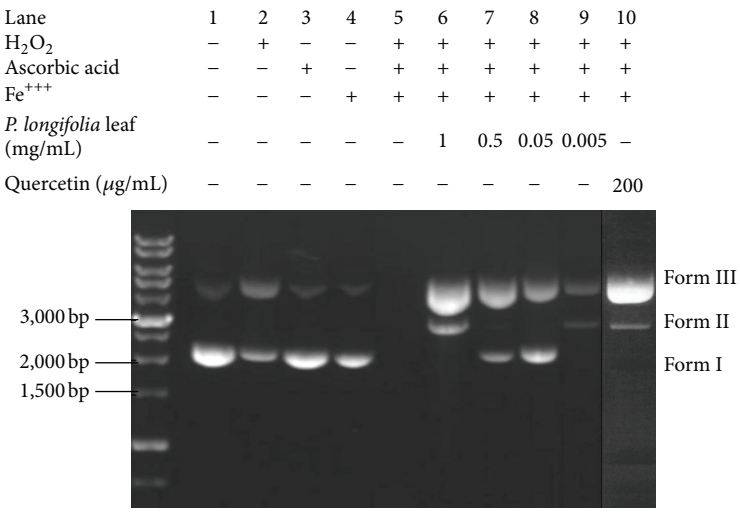

Figure 4: Agarose gel electrophoretic analysis of Fenton-mediated DNA oxidation. Form I = Supercoiled; Form II = Single strand nicked DNA; Form III = Open circular.

the plasmid DNA was fully hydrolysed into linear form and showed that the fragmented DNA migrated between the supercoiled and open circular form (lane 5). The fragmented DNA looks smeared throughout the lane due to the different migration rates of the various fragment sizes. Meanwhile, the plasmid DNA was treated with *P. longifolia* leaf extract, where the DNA damage could be significantly reduced and the protective effects were dose-dependent (lane 6–9). The strongest protective effect, shown as open-circular plasmid DNA, was predominantly produced with 1 mg/mL of *P. longifolia* leaf extract (lane 6). Moreover, when the concentration of the extract decreased from 1 to 0.005 mg/mL the open circular form of DNA was further fragmented to a single-stranded/linear form of DNA mainly due to the strand breaks induced by Fenton reagents (lane 6–9). The effect of Fenton reagent on plasmid DNA was reverted by *P. longifolia* leaf extract. This result suggests that the plasmid DNA exposed to the Fenton reaction is protected from hydroxyl radical-mediated fragmentation when incubated with the leaf extract of *P. longifolia*. Similarly, quercetin effectively protected DNA strand from hydroxyl radical-mediated fragmentation [28].

In the physiological condition, plasmid DNA is composed of mostly supercoiled form (form I) and a small amount of the relaxed form (form II). Plasmid DNA is sensitive to damage caused by a variety of agents. When cleavage of one of the phosphodiester chains of the supercoiled DNA (form I) occurs, it produces a relaxed open-circular form (form II). Further cleavage of the circular strand very close to the site of the initial damage produces linear double-stranded DNA molecule (form III) [29, 30]. The Fenton reagents used in this study are composed of Fe (II) and H_2O_2. They can generate the hydroxyl radical via initiating and catalysing the decomposition of H_2O_2 by Fe (II). Eventually, hydroxyl radicals can react with plasmid DNA to produce a relaxed open circular form. However, in the presence of *P. longifolia* antioxidant, this was repressed since *P. longifolia* leaf extract quenches hydroxyl radicals, as reported in our previous study. This experiment was the first report concerning the protective effect of *P. longifolia* on DNA damage-induced by hydroxyl radical. Furthermore, our previous study showed that the extract exhibited good antioxidant activity with a high content of polyphenol and flavonoid compounds [9]. These findings indicate that the potent antioxidant activity of *P. longifolia* extract partly contributes to the amount of polyphenol and flavonoid compounds.

3.6. Determinations of CC_{50} Concentration. *P. longifolia* leaf extract was evaluated *in vitro* for their cytotoxicity activity against Vero cells by using MTT assay. The results of the cytotoxicity evaluation of extract as CC_{50} (μg/mL) are shown in Figure 5. The CC_{50} value of *P. longifolia* leaf extract against Vero cells was 23.88 μg/mL. The further investigation on the protective effect of *P. longifolia* leaf extract on H_2O_2-induced DNA damage in Vero cells was carried out at CC_{50} value of *P. longifolia* leaf extract in comet assay.

3.7. Comet Assay. Previously, we reported that the methanol extract of *P. longifolia* leaf strongly scavenged various free radicals including hydrogen peroxide *in vitro* [9]. Hence, the extract from *P. longifolia* leaf could be used to protect the cell's DNA. However, to the best of our knowledge, there are no reports on the effective protective effects against DNA damage by methanol extract of *P. longifolia* leaf. Therefore, in this study we used the comet assay, a sensitive biological assay, to measure the DNA damage in Vero cells. In comet assay, DNA strand breaks were represented by the mean tail DNA, that is, the percentage of DNA in the comet tail relative to the total amount of DNA [31]. Untreated control and treated cells with CC_{50} of the *P. longifolia* leaf extract had no detectable or shorter comet tail, whereas cells treated with 1 μmol/L of H_2O_2 showed significant nuclear DNA fragmentation (Figure 6). Compared with the tail DNA of H_2O_2-treated cells, *P. longifolia* leaf extract decreased

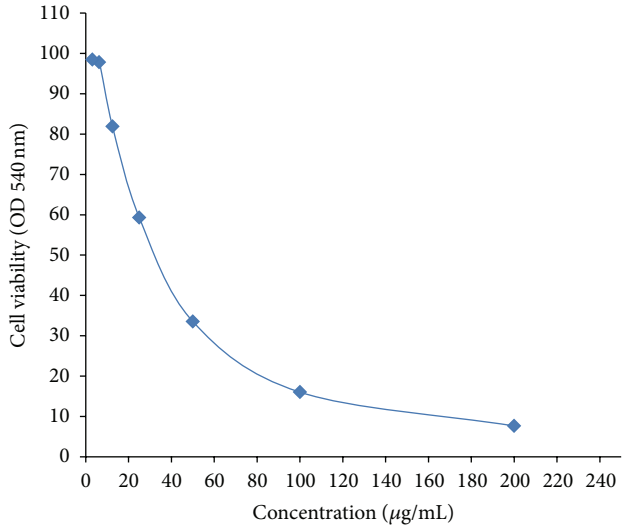

Figure 5: Effect of concentration on cytotoxicity of *P. longifolia* leaf extract on Vero cells.

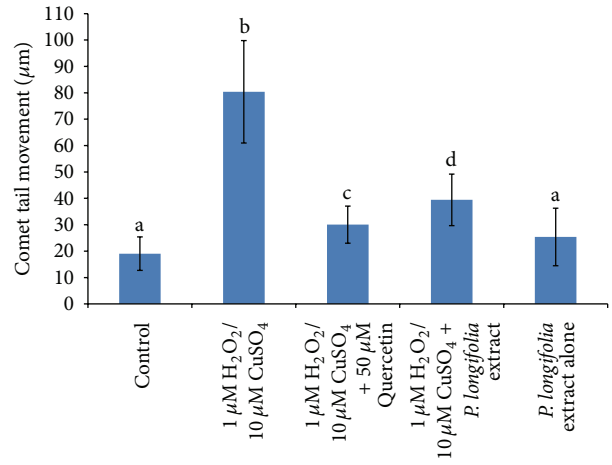

Figure 6: The quantitation of DNA damage and repair in Vero cell line represent the comet tail length (mean ± SD μm) ($P < 0.05$).

the mean tail DNA length by 40.94 μm or 50.94%, at doses of 23.88 μg/mL. In the same way, positive control quercetin decreased the mean tail DNA length by 50.31 μm or 62.60%, at doses of 50 μM compared with the tail DNA of H_2O_2-treated cells [18]. In addition, photomicrographs of different DNA migration profiles were obtained. In the group treated only with hydrogen peroxide, the DNA was completely damaged and the amounts of tail DNA were significantly increased (Figure 7(b)). However, the addition of the *P. longifolia* leaf extract or quercetin with the hydrogen peroxide remarkably reduced the amounts of tail DNA (Figures 7(c) and 7(e)). Hence, the addition of the methanol extracts with the hydrogen peroxide remarkably reduced the amounts of tail DNA.

The exhibited protective effect against H_2O_2-mediated DNA damage might be related to the abilities of bioactive components, especially polyphenols and flavonoids, in the extract [9]. Since polyphenols and flavonoids compounds present in the *P. longifolia* leaf extract are good electron donors, they may accelerate the conversion of H_2O_2 to H_2O [32]. As good electron donors, they show the reducing power. We cannot, however, exclude the possibility that other compounds with genoprotective properties participate in the antigenotoxic effect of *P. longifolia* leaf extract. The results of this study indicate that methanol extracts from *P. longifolia* leaf have efficient antioxidant properties. In addition, our previous studies demonstrated the H_2O_2-scavenging ability of *P. longifolia* leaf in a cell-free system [9]. It has been reported that oxygen free radicals and other reactive oxygen species (ROS) can cause oxidative injury to living organisms and, thus, play an important role in many lifestyle-related diseases, such as cancer [33, 34].

Over the years, many scientific investigations have shown that natural antioxidants purified from plant materials have potential inhibitory effects against H_2O_2-mediated DNA damage and harmful free radicals. Moreover, flavonoids have been reported to prevent DNA damage against H_2O_2 in human lymphocytes assessed by the comet assay [35]. Likewise, numerous studies have demonstrated that polyphenolic compounds reduce DNA damage in the human cell line when assessed with the comet assay [36]. In the present study, we demonstrated the possibility of using *P. longifolia* leaf extract, which is rich in flavonoids and polyphenol, as a potential natural antioxidant resource and inhibitory agent against H_2O_2-mediated DNA damage.

3.8. Allium cepa Assay. The evaluation methods to determine the genotoxicity can be divided into groups based on the biological systems employed and their genetic endpoint detected. Bioassays with prokaryotes enable the detection of agents that induce gene mutation and primary DNA damage. On the other hand, analyses with eukaryotes, such as usage of *A. cepa,* enable the detection of a greater extent of damage, varying from gene mutations to chromosome damage and aneuploidies [37, 38]. Using both the pro- and eukaryotic test systems makes the results both strengthened and correlated to verify if the chemical(s) has/have any really bad effects on the genes. Thus, the *Allium cepa* assay was employed to assess the chromosome aberrations caused by 30% H_2O_2 in this study.

Table 5 shows the cytological effects of the *P. longifolia* leaf extract on root meristem cells of *A. cepa*. The exposure of leaf extract inhibited the mitotic index in a concentration-dependent manner compared with the control groups (distilled water). The mitotic index for the *P. longifolia* leaf extract treated cell was decreased significantly ($P < 0.05$) at 250 μg/mL and 500 μg/mL. The mitotic indexes were 0.303 and 0.209, respectively, compared to the mitotic index at 100 μg/mL, which was 0.424. This indicates that the crude extract may exert a genotoxic effect at 250 and 500 μg/mL. The mitotic index of treated cells was lower compared to the distilled water (negative control), which was 0.503. The Fenton reagent was used as an inducer of gene mutation (30 mM H_2O_2, 100 μM ascorbic acid, and 100 μM $FeCl_3$).

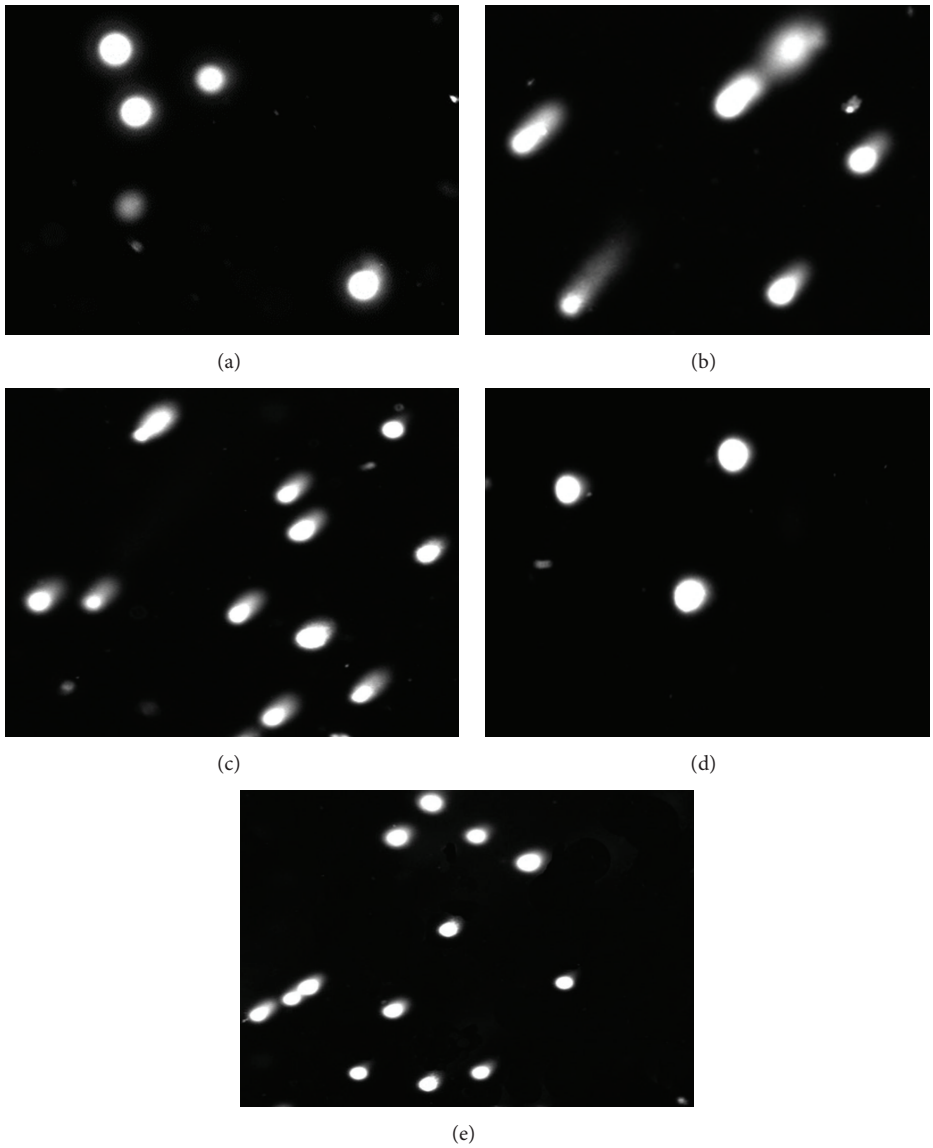

Figure 7: Protective effect of *P. longifolia* leaf extract against H_2O_2-induced DNA damage and migration. (a) Negative control, (b) 1 mM H_2O_2 10 μM $CuSO_4$, (c) 1 mM H_2O_2 10 μM $CuSO_4$ pretreated + 23.88 μg/mL of *P. longifolia* leaf extract, (d) *P. longifolia* leaf extract, and (e) 1 mM H_2O_2 10 μM $CuSO_4$ pretreated + 50 μM quercetin positive control. Magnification 400x.

Table 5: Cytogenetic analysis of *A. cepa* root tips exposed to different concentrations of *P. longifolia* leaf and Fenton reagents.

Treatments			Chromosome aberrations				
Concentration (μg/mL)	No. of cells	Mitotic Index	Stickiness	Bridges	C-mitosis	Vagrant	% of Aberrant cells
P. longifolia leaf							
100	1069	0.424 ± 0.060*	4	3	3	4	1.31
250	1224	0.303 ± 0.045*	5	4	3	8	1.63
500	1046	0.209 ± 0.064*	10	12	7	8	3.54
Fenton reagent (FR)	1105	0.170 ± 0.046*	24	14	20	7	5.88
FR + *P. longifolia* (100 μg/mL)	1225	0.386 ± 0.042*	7	4	3	5	1.55
FR + Quercetin (10 μg/mL)	1239	0.401 ± 0.043*	4	3	2	3	1.07
Control (distilled water)	1194	0.503 ± 0.025	0	2	0	2	0.33

* $P < 0.05$ versus control (distilled water).

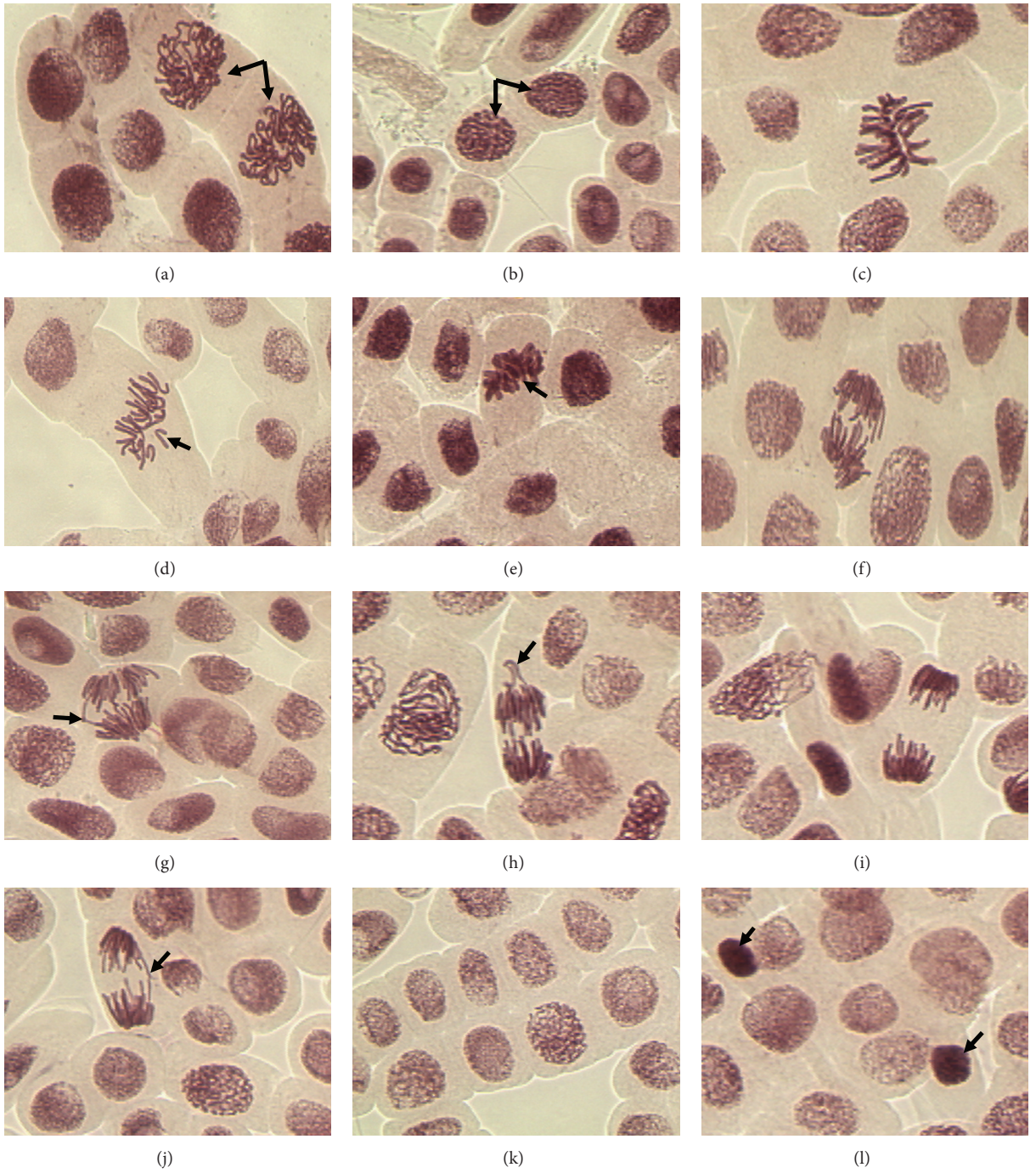

Figure 8: Chromosome aberrations observed in *A. cepa* meristematic cells exposed to extracts of *P. longifolia* leaf. (a) Spindle disturbance at prophase; (b) stickiness in prophase; (c) normal metaphase; (d) C-mitosis in metaphase; (e) stickiness in metaphase; (f) normal anaphase; (g) bridges in anaphase; (h) vagrant in anaphase; (i) normal telophase; (j) bridge in telophase; (k) normal interphase; and (l) micronucleus in interphase. Magnification 400x.

The mitotic index was significantly decreased to 0.170 when the meristem cells were exposed to Fenton reagent alone without any pretreatment with leaf extract. The changes in the organization and morphology of the chromosomes were observed in the meristem cells exposed to the Fenton reagent. The results of the Fenton reaction lead to the generation of abundant OH radicals, which eventually results in cellular division. However, such phenomenon was reverting back to normal in the extract treated (100 μg/mL) cell together with the Fenton reagent for which the MI was 0.386. Moreover, when 10 μg/mL quercetin was tested in combination with Fenton reagent (30 mM H_2O_2, 100 μM ascorbic acid, and

100 μM $FeCl_3$), the total number of chromosome aberrations was reduced (1.07%), with the MI index value of 0.401 [20]. Chromosome aberrations were observed in all stages of mitosis. Table 5 shows the type and frequency of chromosome aberrations in the extract and Fenton reagent treated root tips cells, particularly stickiness, bridges, vagrant chromosome, and c-metaphase. The sticky chromosomes and chromosome bridges were the most common chromosome aberrations observed at 500 μg/mL of leaf extract as well as in the Fenton reagent treated cells. The changes in the organization and morphology of the chromosomes in the root tips exposed to the *P. longifolia* leaf extract were observed (Figure 8). Six types of chromosome aberrations were recorded in the anaphase-telophase cells (Figures 8(a)–8(l)).

In third world countries, medicinal plants are commonly used as popular medicine. However, their indiscriminate and uncontrolled use can cause more harm to public health than good; thus, the knowledge about these plants, from their cellular levels to their action on living organisms, is important. The plant test system of *A. cepa* is an ideal bioindicator for the first screening of genotoxicity, helping with studies that prevent damage to human health [39]. Therefore, the data can be extrapolated for all animals and humans. Since, at a concentration of 100 μg/mL, *P. longifolia* leaf extract treated cells showed normal mitotic cell division without any apparent toxic effects, it was determined as the optimal concentration and chosen as a safe concentration.

4. Conclusion

In conclusion, the results in *in vitro* tests demonstrated that *P. longifolia* leaf was devoid of significant genotoxic effect under our experimental conditions. The *A. cepa* results showed that the methanol extract of *P. longifolia* leaf, applied in lower concentrations, can be important for keeping the genetic stability of the organism. The most effective concentration of methanol extract of *P. longifolia* leaf was 100.00 μg/mL. The methanol extract of *P. longifolia* leaf may contain genoprotective compounds against DNA damage. Further investigations in acute oral toxicity study revealed that *P. longifolia* leaf extract was safe after oral administration as a single dose to female albino Wistar rats with up to 5000 mg/kg body weight. In addition, further cytogenetic studies dealing with clastogenicity and genotoxicity of this extract with more comprehensive chronic oral toxicity study assessment in animal models may reveal further interesting results for its usage for human welfare.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

Subramanion L Jothy was supported by MyPhD Fellowship from the Ministry of Higher Education, Government of Malaysia, Malaysia. This work was partly supported by UM-MoHE HIR Grant (grant no.: UM-MoHE HIR H-18001-C0020) From University of Malaya, Malaysia.

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