RESEARCH ARTICLE

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Andrographolide, an Antioxidant, Counteracts Paraquat-Induced Mutagenesis in Mammalian Cells

Preechaya Tajai[1]*, Tawit Suriyo[2,3], Nuchanart Rangkadilok[2,3], Bogdan I Fedeles[4], John M Essigmann[4], Jutamaad Satayavivad[2,3]

Abstract

Paraquat (1,1'-dimethyl, 4,4'-bipyridinium dichloride; PQ), a commonly used herbicide worldwide, is both toxic and mutagenic. The mutagenic effect of PQ stems from its ability to redox-cycle, generating oxidative stress and subsequently oxidative DNA damage, which miscodes when replication is attempted. Andrographolide (AP_1), the major constituent in the leaves of the herbaceous plant *Andrographis paniculata*, is a diterpenoid with reported antioxidant activity. The present study employed the mammalian cell line AS52 to investigate the protective effect of AP_1 against PQ-induced mutagenesis. AP_1 induced cytotoxicity in AS52 cells in a dose-dependent manner (IC_{50} = 15.7 μM), which allowed the selection of a non-lethal dose for the mutagenesis studies. While PQ was mutagenic in AS52 cells as evidenced by the increased levels of 6-TGr mutants, AP_1 by itself did not increase the mutation frequency. However, co-treatment with AP_1 (1-5 μM) or the antioxidant N-acetylcysteine (2 mM) almost completely counteracted the mutagenicity of PQ (10-100 μM) in AS52 cells. Taken together, these findings suggest that AP_1, and likely by extension, *A. paniculata* extracts, are effective antioxidants that can protect against PQ-induced mutations, and thus could be a promising alternative treatment for PQ poisoning.

Keywords: Andrographolide- antioxidant- pesticide- paraquat- environmental mutagen- carcinogen

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Introduction

Paraquat (1,1'-dimethyl, 4,4'-bipyridinium dichloride; PQ) is one of the most widely used herbicides especially in developing countries including Thailand (Cocheme and Murphy, 2008; Blanco-Ayala et al., 2014; Tajai et al., 2018). Its toxic effects are believed to stem from its ability to generate intracellular reactive oxygen species (ROS) through redox cycling and disrupt the mitochondrial electron transport chain (Cocheme and Murphy, 2008; Blanco-Ayala et al., 2014). An imbalance in the redox state of cells results in extensive mitochondrial damage and cell toxicity (Speit et al., 1998; Cocheme and Murphy, 2008; Blanco-Ayala et al., 2014). Epidemiological studies suggest that PQ has mutagenic and carcinogenic properties (Jee et al., 1995; Wesseling et al., 1996). The mutagenic and genotoxic properties of PQ have also been documented in *in vitro* studies (Speit et al., 1998; Jovtchev et al., 2010; Tajai et al., 2018). By generating ROS that can induce oxidative DNA lesions such as the mutagenic base 8-oxoguanine (8OG), PQ can induce G to T transversion mutations (Tajai et al., 2018). Our previous report showed that the mutagenicity of PQ increases significantly in our genetically engineered AS52 cell culture system that lacks the DNA repair proteins required to repair 8OG. This result suggests that genomic accumulation of 8OG is the main driver of PQ-induced mutagenesis (Tajai et al., 2018).

Several studies recommend antioxidant therapy for the treatment of PQ poisoning (Kim et al., 2003; Blanco-Ayala et al., 2014; Ortiz et al., 2016). In support of this view, our previous work demonstrates that the co-treatment with antioxidants such as N-acetylcysteine (NAC) alleviates the mutagenicity of PQ (Tajai et al., 2018). Many other natural or artificial antioxidants, such as silymarin, quercetin, ellagic acid, and L-ascorbic acid have been proposed as potential treatments for PQ poisoning. These antioxidants act either by directly scavenging ROS or by activating oxidative stress response pathways, such as upregulating the expression of Nrf2 (nuclear factor erythroid-2-related factor 2) transcription factor and increasing the levels of antioxidant enzymes (e.g., catalase, superoxide dismutases, glutathione-S-transferases) (Blanco-Ayala et al., 2014).

Andrographis paniculata has been traditionally used as an alternative medicine especially in the Southeast Asian countries including Thailand (Yoopan et al., 2007; Suriyo

*[1]Faculty of Pharmacy, Payap University, Chiang Mai 50000, Thailand. [2]Laboratory of Pharmacology, Chulabhorn Research Institute, Bangkok 10210, Thailand. [3]Center of Excellence on Environmental Health and Toxicology, Ministry of Education, Bangkok 10210, Thailand. [4]Departments of Biological Engineering and Chemistry, and Center for Environmental Health Sciences, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, USA. *For Correspondence: tapreechaya@gmail.com*

et al., 2014). Given its broad range of pharmacological activities, *A. paniculata* has been used for treating various diseases such as upper respiratory tract infection, fever, common cold, and inflammation (Yoopan et al., 2007; Suriyo et al., 2014; Wong et al., 2016). The major constituent in *A. paniculata* leaves and stems extracts is andrographolide (AP_1), a diterpenoid reported to exhibit anti-inflammatory (Sheeja et al., 2006; Low et al., 2015), antioxidant (Sheeja et al., 2006; Chen et al., 2012; Low et al., 2015), and anticancer activities (Sheeja and Kuttan, 2007; Al-Henhena et al., 2014; Suriyo et al., 2014; Monger et al., 2017). In terms of mechanism, AP_1 has been shown to scavenge free radicals and inhibit xanthine oxidase and lipid peroxidation (Lin et al., 2009). Moreover, AP_1 potently up-regulates the mRNA expression of Nrf2 (Wong et al., 2016), which subsequently drives the antioxidant properties of AP_1. Given these biological activities, we hypothesized that AP_1 can protect mammalian cells from the mutagenic effects of PQ.

In this study, AS52 cells are employed to investigate the protective effect of AP_1 against PQ-induced mutations. AS52 cells, a Chinese hamster ovary (CHO) cell line, are a versatile tool for measuring mutagenesis in mammalian cells. This cell line is engineered to carry the bacterial xanthine-guanine phosphoribosyltransferase (*gpt*) gene, while lacking the native homologous *hprt* gene. Using a forward mutagenesis assay, *gpt* mutants in AS52 cells can be selected by their ability to grow in the presence of 6-thioguanine (6-TG) (Tajai et al., 2018).

Materials and Methods

Cell culture and chemicals

AP_1 (purity 98%) was isolated from *A. paniculata* and purified in-house according to our previous report (Pholphana et al., 2013). AS52 cells were kindly provided by Dr. Gerald N. Wogan (Massachusetts Institute of Technology, USA). AS52 cells were maintained in Ham's F-12 medium (Gibco, USA) supplemented with 10% heat-inactivated (56ºC for 30 minutes) fetal bovine serum (FBS) (Merck Millipore, Germany), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine (Gibco) in a humidified 5% CO_2 incubator at 37ºC. AS52 cells were cultured in the presence of mycophenolic acid (MPA) (Sigma-Aldrich, USA) for 7 days in order to remove the spurious or pre-existing 6-thioguanine resistant (*6-TGR*) mutants. The MPA-containing medium was the complete medium (above) supplemented with 10 µg/ml MPA, 250 µg/ml xanthine (Sigma-Aldrich), 22 µg/ml adenine (Sigma-Aldrich), 11 µg/ml thymidine (Sigma-Aldrich), and 1.2 µg/ml aminopterin (Sigma-Aldrich). Thereafter, cells were maintained in complete medium plus 11.5 µg/ml xanthine, 3 µg/ml adenine and 1.2 µg/ml thymidine for 3 additional days (Tindall et al., 1986; Tindall and Stankowski, 1989; Wattanawaraporn et al., 2012; Tajai et al., 2018). All experiments in this study were carried out within 10 passages after the MPA treatment. Paraquat, 6-thioguanine, hydrogen peroxide, and N-Acetyl-L-cysteine were purchased from Sigma-Aldrich.

Determination of cell viability by the TM assay

AS52 cells were seeded in 96-well plates (2,500 cells/well) and allowed to attach for 24 h. Subsequently, cells were treated with various concentrations of AP_1 (1-50 µM) for 24 h. Cell viability was determined by adding to each well 10 µl of TM cell viability reagent (Invitrogen, USA) and incubating at 37°C for 30 minutes as recommended by the manufacturer. Fluorescence intensity was recorded at 560 nm excitation/590 nm emission using a SpectraMax M3 microplate reader. The data were then expressed as the percentage of cell viability relative to the control group (untreated cells).

gpt mutation assay

AS52 cells were seeded in 6-well plates ($5x10^5$ cells/well) and cultured for 24 h to enable cell attachment. Then, cells were treated with different concentrations of PQ (10, 25, 50, and 100 µM) for 24 h, hydrogen peroxide (H_2O_2) (100 µM) for 1 h as positive control (Kim et al., 2003), AP_1 (1, 5 µM) for 24 h, NAC (2 mM) for 24 h, and the combinations of PQ + AP_1 and PQ + NAC for 24 h. After treatment, cells were trypsinized and cytotoxicity was assessed by trypan blue exclusion. Treated cells were then maintained in complete medium for 7 days in order to allow full expression of the *gpt* mutant phenotype. Thereafter, treated cells ($5x10^5$ cells) were suspended in 100 ml of complete medium containing 10 µM 6-TG to select 6-TG resistant (6-TGR) colonies and seeded at a density of $5x10^4$ cells/100 mm dish (10 dishes/group). Simultaneously, treated cells ($2.5x10^3$ cells) were suspended in 50 ml of complete medium without 6-TG (non-selective conditions) to estimate plating efficiency (PE) and seeded at a density of 500 cells/100 mm dish (5 dishes/group). After incubation for 2 weeks, colonies were stained with 0.5% crystal violet in 25% methanol in water and only colonies with ≥ 50 cells were counted (Tindall et al., 1986; Tindall and Stankowski, 1989; Wattanawaraporn et al., 2012; Tajai et al., 2018). Plating efficiency (PE) and mutation frequency (MF) were calculated as follows:

PE = Number of colonies/Number of seeded cells

MF = Number of 6-TGR colonies/(Number of seeded cells x PE)

Statistical analysis

The data from at least three independent experiments were presented as the mean ± SEM. Student's t test was used to determine the statistical significance of differences in cell viability and mutation frequencies between experimental and control groups. Analyses were done using GraphPad Prism 7 (Graphpad Software, Inc.) and $p \leq 0.05$ was considered statistically significant.

Results

PQ generates ROS such as superoxide anion ($O_2\bullet-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH) through redox cycling. An imbalance between the rate of free radical production and the rate of their elimination by the cellular defense mechanisms manifests as oxidative stress, which results in oxidative damage to DNA that

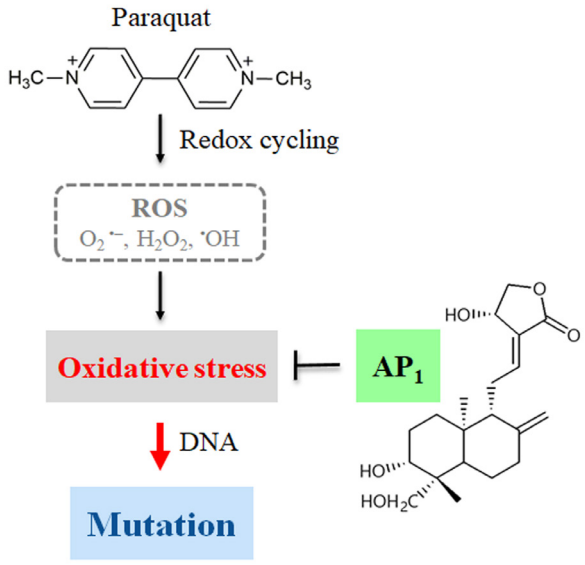

Figure 1. A Schematic of the Mechanism of PQ-Induced Mutations and the Protective Effect of Antioxidants. The present work demonstrates that AP_1 is sufficient to alleviate the mutagenic properties of PQ.

can lead to mutations (Speit et al., 1998; Cocheme and Murphy, 2008; Tajai et al., 2018). Our previous report demonstrated that the formation of ROS and the genomic accumulation of oxidative DNA lesions (8OG) are the main drivers of PQ-induced mutagenesis (Tajai et al., 2018). The key finding of the present study is that supplementation with antioxidants such as AP_1 and NAC diminishes the oxidative stress levels and consequently the level of mutagenesis induced by PQ (Figure 1).

AP1 induced cytotoxicity in AS52 cells.

AP_1 induced cytotoxicity in AS52 cells in a dose-dependent manner (Figure 2). Cells were treated with various concentrations of AP_1 (1-50 µM) and their viability evaluated after 24 h. AP_1 treatment led to a significant decrease in cell survival (IC50 = 15.71 µM);

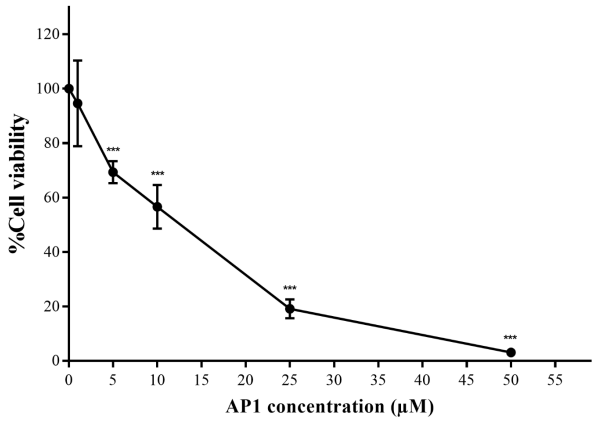

Figure 2. The Cytotoxicity of AP_1 in AS52 Cells. Cells were treated with various concentrations of AP_1 (1-50 µM) for 24 h, after which the cell viability was measured with the TM assay. The results are presented as mean ± SEM, N = 3. ***p < 0.001 compared with the control (untreated group).

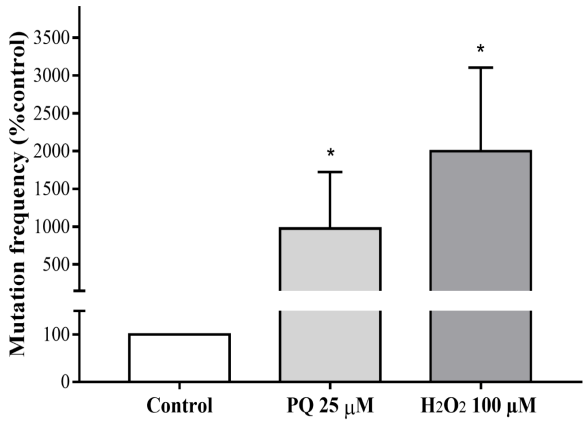

Figure 3. The Mutagenicity of PQ in AS52 Cells. PQ was mutagenic as evidenced by the increased fraction of 6-TGr mutants. Cells were treated with PQ 25 µM for 24 h, or H_2O_2 100 µM for 1 h (positive control). The results are presented as mean ± SEM, N = 3. *p < 0.05 compared with the control (untreated group).

at the highest dose of 50 µM viability was only 3.1% (Figure 2).

PQ increased mutation frequency in AS52 cells

The mutagenic ability of PQ was evaluated by counting the 6-TG resistant (6-TGR) colonies relative to the total number of colony-forming units plated. AS52 cells were treated with 25 µM PQ for 24 h. For a positive control, a 1 hour treatment with H_2O_2 (100 µM) was performed. As shown in Figure 3, PQ exposure led to a significant increase in mutation frequency. The positive control also generated a statistically significant increase in the number of 6-TGR colonies (Figure 3), consistent with previously reported data (Kim et al., 2003).

AP1 did not increase mutation frequency in AS52 cells

When the mutagenesis assay was repeated with AP_1 and NAC compounds alone, no significant differences in mutation frequencies between the treated groups (AP_1 and NAC) and controls were observed. Neither AP_1 at 1

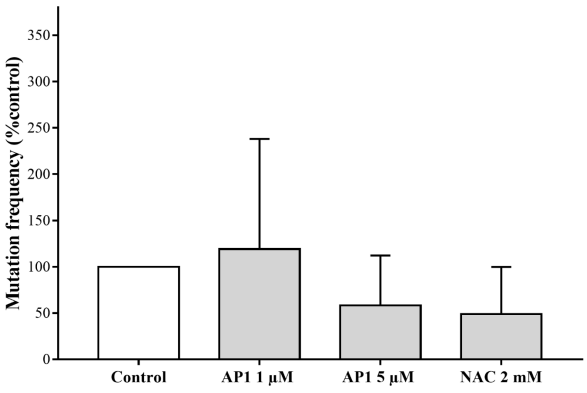

Figure 4. The Mutagenicity of the Antioxidants AP_1 and NAC in AS52 Cells. Cells were treated with AP_1 (1 and 5 µM) and NAC (2 mM) for 24 h. No significant differences in mutation frequency were observed between the treated cells and control. The results are presented as mean ± SEM, N = 3. *p < 0.05 compared with the control (untreated group).

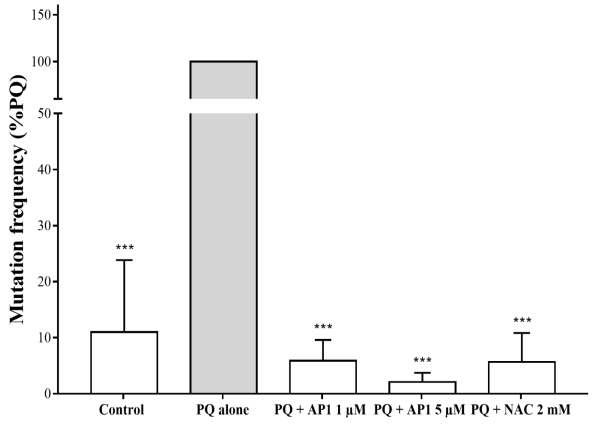

Figure 5. Antioxidants AP_1 and NAC Counteract PQ-Induced Mutagenesis. AS52 cells were treated with PQ 25 µM, the combination of PQ 25 µM + AP_1 1 µM, PQ 25 µM + AP_1 5 µM, and PQ 25 µM + NAC 2 mM for 24 h. Both antioxidants significantly decreased the number of 6-TGr mutants caused by PQ. No significant differences in mutation frequency were observed between the combination of PQ + AP_1 and control (untreated group). The results are presented as mean ± SEM, N = 3. ***p < 0.001 compared with the PQ treatment group (%PQ).

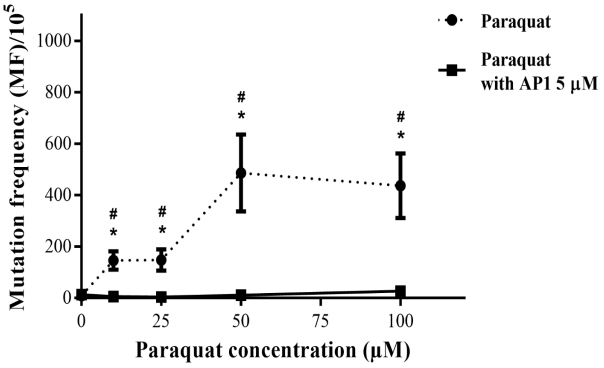

Figure 6. AP_1 5 µM is Anti-Mutagenic Over a Wide Range of PQ Concentrations. AS52 cells were treated with PQ (10-100 µM) for 24 h and the combination of PQ (10-100 µM) + AP_1 5 µM for 24 h. AP_1 5 µM significantly decreased the number of 6-TGr mutants caused by PQ alone. The results are presented as mean ± SEM, N = 3. *p < 0.05 compared with the control (untreated group). #p < 0.05 compared with the PQ + AP_1 co-treatment.

µM and 5 µM, nor NAC at 2 mM increased the mutation frequency compared with the untreated group (Figure 4).

AP1 and NAC decreased the mutagenesis of PQ in AS52 cells

Antioxidants AP_1 and NAC significantly decreased the mutagenesis of PQ. NAC, a prodrug of the natural amino acid L-cysteine, can act both directly as a free radical scavenger, reducing the available concentration of ROS, and as a cysteine source which helps restore and boost the levels of glutathione, the main cellular antioxidant. AS52 cells were treated with PQ 25 µM, the combination of PQ 25 µM + AP_1 1 µM, PQ 25 µM + AP_1 5 µM, and PQ 25 µM + NAC 2 mM for 24 h. While the exposure of AS52 cells to 25 µM of PQ generated a significantly increased mutational frequency compared to baseline (Figure 3), the co-treatment with AP_1 and NAC completely abrogated the PQ-induced mutagenesis. The mutation frequency in the antioxidant co-treated cells was 20-100 fold lower than the PQ treatment group (Figure 5), a level indistinguishable from the mutation frequency in untreated AS52 cells.

The best antioxidant adjuvant (AP_1 at 5 µM) was beneficial over a wider range of PQ concentrations. When exposing AS52 cells to PQ alone at concentrations ranging from 10-100 µM for 24 h, the mutation frequency increased from ~9 6-TGR colonies per 10^5 cells plated (the detection limit of the assay) to 486 6-TGR colonies per 10^5 cells plated. Co-treatment with AP_1 at 5 µM completely abrogated PQ-induced mutations over the entire dose range of PQ (Figure 6). Taken together, these observations support the hypothesis that the antioxidant AP_1 is effective at counteracting PQ mutagenesis in mammalian cells.

Discussion

The central goal of this study was to investigate the protective effect of AP_1 against PQ-induced mutagenesis. The previously documented antioxidant and anti-inflammatory properties of AP_1 have suggested that this diterpenoid could be effective at preventing the toxic and mutagenic properties of a redox cycling molecule such as PQ, but direct evidence to that effect has been lacking.

The present study showed that AP_1 was cytotoxic to AS52 cells in a dose-dependent manner, an observation consistent with previously reported data documenting the cytotoxic effect of both *A. paniculata* extracts and purified AP_1 (Suriyo et al., 2014; Banerjee et al., 2016; Monger et al., 2017; Islam et al., 2018; Khan et al., 2018). High doses of AP_1 have been found to be cytotoxic to most mammalian cell lines (Islam et al., 2018), through a mechanisms that involves disruption of mitochondrial membrane potential (Banerjee et al., 2016), cell cycle arrest (Suriyo et al., 2014; Banerjee et al., 2016) and apoptosis (Suriyo et al., 2014; Banerjee et al., 2016; Monger et al., 2017) due to immune system mediated effects (Islam et al., 2018). These observations highlight the importance of properly dosing AP_1 for therapeutic purposes.

Our results here extend our previous study where we demonstrated that the mutagenicity of PQ is caused by its ability to generate oxidative stress and subsequent oxidative DNA damage such as the mutagenic base 8OG (Tajai et al., 2018). In agreement with our previously reported data, we show here that PQ is mutagenic in AS52 wild-type cells. PQ mutagenesis presumably reflects the formation of oxidative DNA lesions, because, as we previously reported, the PQ-induced mutation frequency increases significantly in engineered cells that lack the DNA repair proteins necessary for fixing oxidative DNA damage (Tajai et al., 2018). Additionally, the PQ-induced mutagenesis is alleviated by the presence of antioxidants. Our previous work (also confirmed here) demonstrated that NAC co-treatment counteracts mutagenicity of PQ (Tajai et al., 2018). The present work demonstrates for the

first time that supplementation with AP_1 is also effective at counteracting PQ-induced mutagenesis in the AS52 cells. The mechanism of AP_1 likely involves both the direct scavenging of ROS and the activation of the Nrf2 pathway that stimulates the production of antioxidant enzymes. AP_1 has been shown to have free radical scavenging activities (Lin et al., 2009) and to potently upregulate Nrf2 mRNA expression (Wong et al., 2016; Seo et al., 2017; Wong et al., 2018).

There is increasing evidence that chronic exposure to PQ is associated with an increased cancer incidence (Jee et al., 1995; Wesseling et al., 1996), presumably due in part to the mutagenic properties of PQ. Many studies have proposed compounds with antioxidant activity as alternative treatments for PQ poisoning (Kim et al., 2003; Podder et al., 2012; Kim et al., 2013; Zerin et al., 2013; Blanco-Ayala et al., 2014; Ortiz et al., 2016). Our present work adds AP_1 to that list. From a toxicological standpoint, we show here that *in vitro* AP_1 by itself was not mutagenic in AS52 cells at doses effective against PQ exposure. Thus, AP_1 appears to be safe within the window of efficacy. However, long term *in vivo* studies are needed to define and confirm its safety profile.

In conclusion, this investigation indicates that andrographolide (AP_1), and by extension, AP_1-containing extracts from *A. paniculata* could be useful as alternative treatments for PQ poisoning due to their potent antioxidant activity that alleviates the PQ-induced oxidative stress and mutagenesis.

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Abbreviations

8OG, 8-oxoguanine (or 7,8-dihydro-8-oxoguanine); AP_1, andrographolide; *gpt*, xanthine-guanine phosphoribosyltransferase; NAC, N-acetyl-L-cysteine; PQ, paraquat; ROS, reactive oxygen species.

Statement conflict of interest

The authors state that they have no conflicts of interest.

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