

Cytotoxicity and genotoxicity of *Allamanda* and *Plumeria* species

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ABSTRACT: Species of *Allamanda* and *Plumeria* have been used as medicinal plants for a long time. This study investigates the cytotoxicity and genotoxicity of their extracts on lymphocytes and HeLa cells using cell viability tests and comet assay. *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra* were collected and hexane-extracted. The maximum concentrations of crude extracts were 315, 625, 357, and 303 mg/ml, respectively. For cytotoxicity on lymphocytes treated with the maximum final concentration of the four extracts, the cell viabilities were 79%, 96%, 70%, and 60%, respectively. In HeLa cells, the IC₅₀ was 13.5, 3.75, 21.5, and 14.5 mg/ml, respectively. These extracts showed significant genotoxicity ($p < 0.01$) on both lymphocytes and HeLa cells. These findings suggest that *Allamanda* and *Plumeria* should not be used as medicinal plants.

KEYWORDS: comet assay, medicinal plants

INTRODUCTION

Chemical contents and phytochemical properties of *Allamanda* and *Plumeria* have been extensively reported. Some *Allamanda* species contain glabridin, new lignin, kaempferol, naringenin, allamandicin¹, squalene, α -tocopherol, vitamin E, and 9,12,15-octadecatrien-1-ol². They showed algicidal properties³, anti-proliferative effect against leukaemic and endothelial cells⁴, anti-mitotic activity towards sea urchin eggs, reinforcing the anti-tumour potential⁵, and tyrosinase inhibitory activity¹.

Some *Plumeria* species contain iridoids, triterpenoids, plumieride, ursolic acid, plumeric acid, plumericin, isoplumericin, stigmast-7-enol lupeol carboxylic acid, and fulvoplumericin. They exhibited algicidal, antibacterial, bacteriostatic, cytotoxic, anaesthetic, cardiotoxic, antipyretic, antitumor, anti-mutagenic, gastroprotective, anti-inflammatory, antioxidant, and antiarthritic activities. More interestingly, *P. rubra* containing fulvoplumericin acts as an inhibitor of human immunodeficiency virus type 1 (HIV) reverse tran-

scriptase. *P. alba* showed hepatoprotective action, *P. bicolor*, showed high activity against *Leishmania donovani*^{6,7}.

Additionally, *Allamanda* and *Plumeria* species have been used for the treatment of many diseases such as rheumatism, diarrhoea, blennorrhoea, venereal disease, leprosy, psychosis, diuresis, laxative, and inducing vomiting⁶⁻⁸. Although some poisonous have been reported, such as when used in excess, it becomes a strong laxative and causes excessive vomiting and sometimes death⁸, algicidal poisons³, strong cytotoxic effects on leukaemic and endothelial cells⁴, and anti-mitotic activity towards sea urchin eggs⁵. The symptoms of poisoning include a slow and irregular pulse, which can be followed by vomiting, and finally, toxic shock.

The plants must be therefore thoroughly tested for toxicity before their use in humans can be approved. Thus this study aims to study representative species of each genus for cytotoxicity by haemocytometer cell counting and genotoxicity by comet assay⁹⁻¹² on human lymphocytes and the cervical cancer cell line, HeLa.



Fig. 1 The plant samples used in this study: (a) *Allamanda cathartica*, (b) *A. violacea*, (c) *Plumeria obtusa*, and (d) *P. rubra*, showing their leaf shapes and flowers.

MATERIALS AND METHODS

Plant materials

Species identification of *Allamanda* and *Plumeria* species was completed using the literature^{2,13} by Prof. Dr Arunrat Chaveerach, Department of Biology, Faculty of Science, Khon Kaen University. The plant names have been verified using the Plant List (www.theplantlist.org). Mature leaves of the plant species, *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra* (Fig. 1) were collected in April 2015 for preparation of chemical extracts.

Preparation of chemical extracts

Air-dried leaf samples were ground into a powder, then 25 g of each sample was mixed with 125 ml hexane (analytical grade) in a flask, wrapped with aluminium foil, and stored for three days at room temperature. The samples were then filtered through filter papers. Hexane was removed from the filtrates by a rotary evaporator (Buchi Rotavapor

R-210, Switzerland), then they were redissolved with 10% dimethyl sulphoxide (DMSO). These solutions (crude extracts) were 10-fold serial diluted for four levels with sterile water prior to the toxicity tests.

Lymphocytes preparation

Blood samples (24 ml) were collected from two healthy human subjects into heparinized blood collection tubes using sterile techniques. Lymphocytes were isolated by adding red blood cell lysis buffer, and then centrifuged to produce haemolysis. The collected lymphocytes were re-suspended in PBS. Cell counts were completed with a haemocytometer with trypan blue staining. The cell suspension was prepared at a concentration of $4-6 \times 10^5$ cells/ml of RPMI-1640 (Gibco, Life Technologies, USA) supplemented with 10% fetal bovine serum (FBS), 100 $\mu\text{g}/\text{ml}$ penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin for further cytotoxic and genotoxic tests.

Cell line culturing

The HeLa (human cervical carcinoma) cell line used in this study was kindly provided by Prof. Dr Thomas Liehr (Institute of Human Genetics, Jena, Germany). Cells were cultured in DMEM (Gibco, Life Technologies, USA) supplemented with 10% FBS, 100 µg/ml penicillin and 100 µg/ml streptomycin in a humidified CO₂ incubator at 37 °C and 5% CO₂ for 2 days. Cells were washed with phosphate buffered saline (PBS) and harvested by trypsinization. Cell counts were completed with a haemocytometer with trypan blue staining. The cell suspension was prepared at a concentration of 4–6 × 10⁵ cells/ml of the medium for further cytotoxic and genotoxic tests.

Cytotoxicity testing by cell counting

The cytotoxic effects of the plant extracts were defined as a loss of membrane activity when treated with trypan blue dye^{14,15}. The cell suspension was divided into 1.5 ml microtubes (500 µl per tube) and the plant extract dilutions were added at 50 µl per tube. Thus the extract dilutions were again ten-time diluted, therefore, the highest final concentration of DMSO was 1%. The cells were then cultured in a humidified CO₂ incubator at 37 °C and 5% CO₂ for 4 h (for lymphocytes) or 24 h (for HeLa cells). The untreated cells (negative control) were incubated in culture medium only. Positive control cells were incubated with 100 µM of H₂O₂ for 15 min. Additionally, DMSO (1% final concentration) was tested to assure its toxicity. Each experiment was performed in triplicate including the controls. Means were used for analysis. The viability was calculated as the average of treated cells divided by the average of negative control cells. The concentration of the extract that produced 50% cell death (IC₅₀) was calculated from the correlation between cell viability and extract concentration.

Genotoxicity of plant extracts on lymphocytes and HeLa cell lines

The cells were treated in the same way as cytotoxicity test using the extract at a concentration of IC₅₀. The comet assay was performed according to the method described by Singh et al¹⁶ with slight modifications. Briefly, cell pellets were obtained by centrifugation and then re-suspended in 40 µl of PBS. One hundred microlitres of 0.5% low melting point agarose (LMA) was mixed with the cell suspension. The mixture was dropped onto slides that had been precoated with 1% normal melting

point agarose. Next, cover slips (22 mm × 50 mm) were placed on top of the slides and stored at 4 °C. After 5–10 min, the cover slips were removed, and the slides were submerged in a lysis solution (8 M NaCl, 0.6 M EDTA pH 8, 0.2 M Tris, 0.1% Triton X-100) for at least 1 h. The slides were then soaked in electrophoresis buffer (6 mM EDTA pH 10, 0.75 M NaOH) for 40 min. Next, electrophoresis was performed for 25 min at 26 volts and 300 milliamperes at 4 °C. After electrophoresis, the slides were immediately neutralized with 0.4 M Tris buffer (pH 7.5) for 5 min. The neutralization process was repeated three times for 5 min each. The slides were then stained with 1 µg/ml ethidium bromide (60 µl per slide) overnight at 4 °C in the dark, and images were obtained using an image analysis system (Isis, www.metasystems-international.com/isis) attached to a fluorescence microscope (Nikon, Japan). Images of 150 cells per experiment were analysed using IMAGEJ (imagej.nih.gov/ij) and statistically analysed using GRAPHPAD PRISM (www.graphpad.com/scientific-software/prism).

RESULTS

The plant extracts

The plants extracts of *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra* redissolved with DMSO have the maximum concentration at 316, 625, 357, and 303 mg/ml, respectively. The concentrations of the serial dilutions of these extracts are shown in Table 1.

Cytotoxicity of plant extracts on lymphocytes and HeLa cells

Non-dilution, 10-, 100-, and 1000-fold dilutions were performed for cytotoxic and genotoxic testing on normal human cells, lymphocytes and HeLa cells. This brings the maximum final concentration to 31.6, 62.5, 35.7, and 30.3 mg/ml when applied to the cultured media (Table 1). The experiments

Table 1 Final concentration of the plant extracts used for cell treatment, percentage of lymphocytes viability at maximum concentration, and IC₅₀ for HeLa cells.

Plant	Concentration (mg/ml)	Lymphocytes viability (%)	IC ₅₀ HeLa (mg/ml)
<i>A. cathartica</i>	31.6	79	13.5
<i>A. violacea</i>	62.5	96	3.75
<i>P. obtusa</i>	35.7	70	21.5
<i>P. rubra</i>	30.3	60	14.5

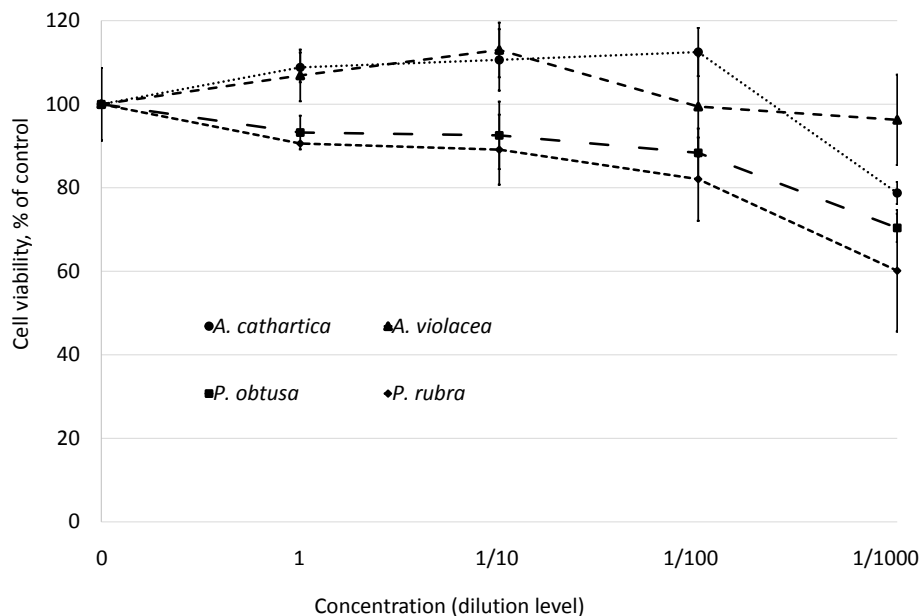


Fig. 2 Viability of lymphocytes when treated with the plant extracts, *A. cathartica*, *A. violacea*, *P. obtusa*, *P. rubra* which did not show an IC_{50} .

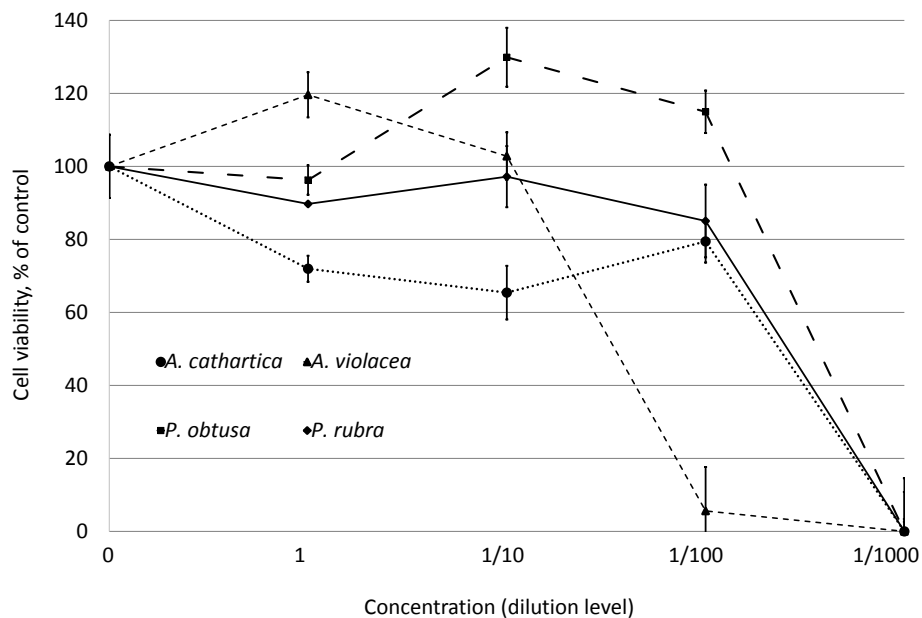


Fig. 3 Viability of HeLa cells when treated with the plant extracts; the concentrations at IC_{50} are 13.5 mg/ml for *A. cathartica*, 3.75 mg/ml for *A. violacea*, 21.5 mg/ml for *P. obtusa*, and 14.5 mg/ml for *P. rubra*.

indicated a clear dose-dependent cytotoxic effect of the four species.

The percentage of cell viability when treated with 1% DMSO showed non-significant difference from the negative control for both cell types. For lymphocytes, at the maximum concentration of each extract the cell viabilities were 79%, 96%, 70%,

and 60% (Table 1) which were all higher than 50% (Fig. 2). The IC_{50} values of the extracts on lymphocytes could not be calculated for this experiment. For HeLa cells, the IC_{50} values of the plant extracts from *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra* were 13.5, 3.75, 21.5, and 14.5 mg/ml, respectively (Fig. 3).

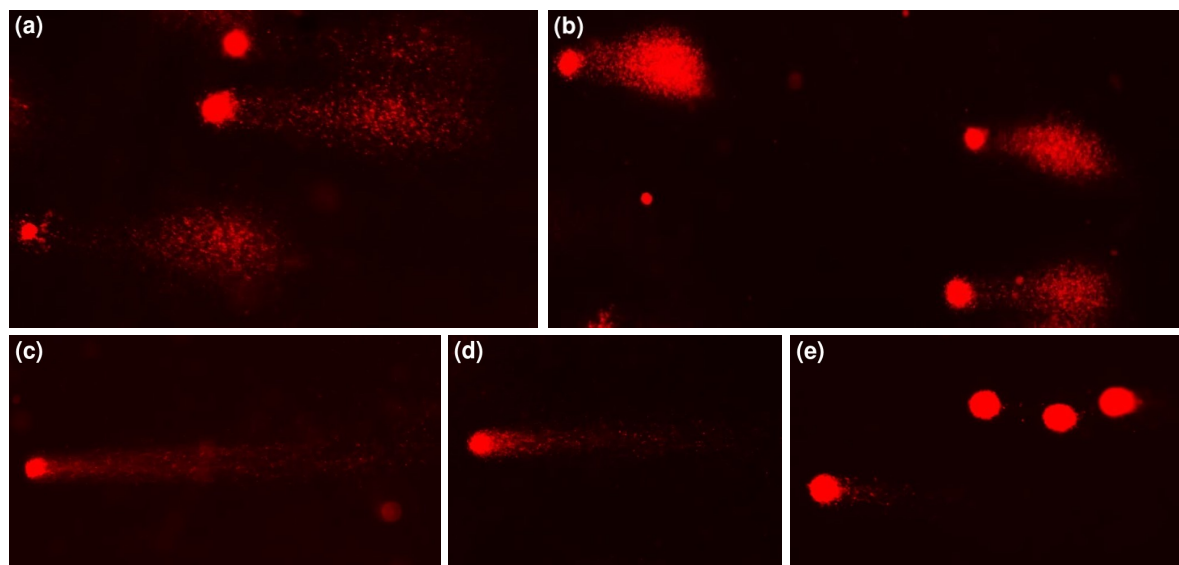


Fig. 4 Comet assays of lymphocytes with long tails and broken heads (a–d) when treated with maximum concentration of the plant extracts indicating significant DNA damage and (d) without plant extract indicating no DNA damage (200×): (a) 31.6 mg/ml of *A. cathartica*, (b) 62.5 mg/ml of *A. violacea*, (c) 35.7 mg/ml of *P. obtusa*, (d) 30.3 mg/ml of *P. rubra*, and (e) negative control.

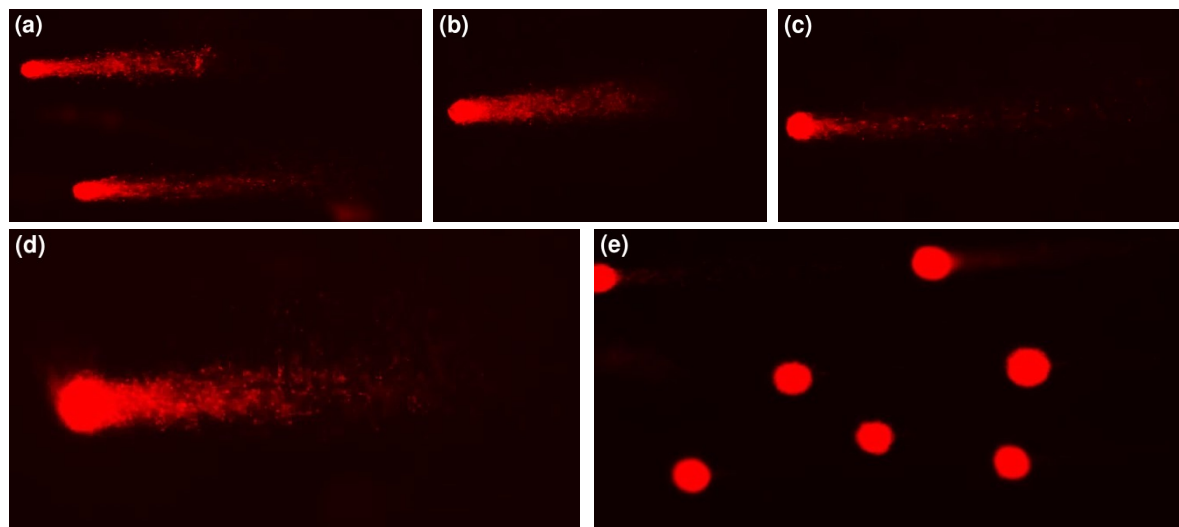


Fig. 5 Comets of HeLa cells with long tails and broken heads (a–d) when treated with the plant extracts at a concentration of IC_{50} indicating significant DNA damage and (d) without plant extract indicating no DNA damage (40×): (a) 13.5 mg/ml of *A. cathartica*, (b) 3.75 mg/ml of *A. violacea*, (c) 21.5 mg/ml of *P. obtusa*, (d) 14.5 mg/ml of *P. rubra*, and (e) without plant extract (negative control).

Genotoxicity of plant extracts on lymphocytes and HeLa cells

The maximum final concentrations of each species, *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra*, were selected for use in the comet assay on lymphocytes. The concentrations were 31.6, 62.5, 35.7, and 30.3 mg/ml, respectively. The four sample

species extracts showed significant effects on DNA damage ($p < 0.01$), shown by long tails and broken heads (Fig. 4a–d) compared to the negative control (Fig. 4e). For the HeLa cells, the four plant extracts at IC_{50} concentrations of 13.5, 3.75, 21.5, and 14.5 mg/ml were used for testing. DNA damage was statistically significant ($p < 0.01$) (Fig. 5a–d) when compared to the negative control (Fig. 5e).

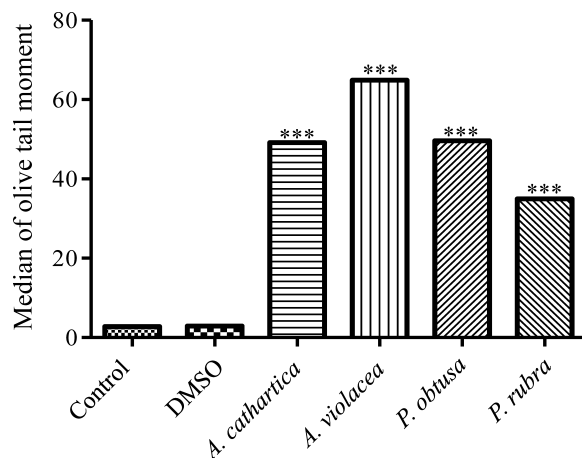


Fig. 6 Median of tail moment values from comet analysis of lymphocytes treated with the four plant extracts, *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra*, the negative control, and 1% DMSO. (***: statistically significant at $p < 0.01$ level compared to the control).

Additionally, DMSO (1%) used as dissolving agent showed non-significant DNA damaged to the lymphocytes. The median of tail moment of lymphocyte DNA treated with the four plant extracts, the control and DMSO were plotted on a graph (Fig. 6) for comparison.

DISCUSSION

Allamanda and *Plumeria* genera have five and two species, respectively, that are found in Thailand^{2,13}. They were all selected to be representative for the other species remaining in the genus *Allamanda*. All of these species are commonly used as medicinal plants, yet their toxicity is not clear, and the focus is usually on their benefits. This study revealed that the *Allamanda* and *Plumeria* species should not be used for medicinal properties or any other any usage. Although the cytotoxicity levels show high percentages of viability in lymphocytes that exceed 50%, including 79%, 96%, 70%, and 60%, respectively, when treated with the maximum final concentration of the four plant extracts, *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra*, at 31.6, 62.5, 35.7, and 30.3 mg/ml for 4 h, they showed high genotoxicity level. The final concentrations, 31.6, 62.5, 35.7, and 30.3 mg/ml of each species, *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra*, respectively, were selected for use in the comet assay on lymphocytes. The four sample species extracts showed significant effects on DNA damage ($p < 0.01$), as indicated by long tails and broken heads

compared to negative controls. This result suggests that *A. cathartica*, *A. violacea*, *P. obtusa*, and *P. rubra* are toxic to human, which helps explain why their use in excess resulted in the example reported by Pupattanapong⁸. As the same taxonomic category of the two genera and a family of Apocynaceae, *Allamanda* and *Plumeria* species are probably toxic for humans. The advantage seems to be that it could be used for treatment in women with cervical carcinoma because they can effectively kill human carcinoma cell lines, HeLa cells at both cytotoxicity and genotoxicity levels. The cytotoxicity showed low concentration for killing 50% HeLa cells at 13.5, 3.75, 21.5, and 14.5 mg/ml, indicating calculated oral LD₅₀ of 3.63, 2.26, 4.32, and 3.73 g/kg, respectively, according to the formulation of Walum¹⁷. When these doses were tested on HeLa cells for 4 h, they revealed statistically significant DNA damage ($p < 0.01$) compared to the control. However, for trying a human carcinoma treatment in patients, the extracts would need to be corrected for the toxic elimination in the normal human cells. Alternatively, the extracts should be tested for treatment in the same way as chemotherapy or radiation cancer treatment depending on cancer stage, timing, and concentration.

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