

## IN VITRO AND IN VIVO-SCIENTIFIC EVALUATION ON CYTOTOXICITY AND GENOTOXICITY OF TRADITIONAL MEDICINAL PLANT *COUROUPITA GUIANENSIS* AUBL. FLOWER

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### Abstract

There has been a misconception on herbal consumption where many of these herbal drugs are assumed as non-toxic by the consumers. On that context, safety and toxicological approach should be relevantly considered and taken seriously since ignorance may lead to dangerous yet lethal consequences. Even though *C. guianensis* flower extracts has been employed in traditional medicine, there were no evidence suggesting their crude toxic compounds or dosage thresholds. Hence, this study was made in an effort to evaluate the potential of *C. guianensis* cytotoxicity on brine shrimp nauplii and Vero cell viability while genotoxicity activity was assessed on the DNA of Vero cells. *In vivo* cytotoxicity was performed using brine shrimp lethality assay (LC<sub>50</sub>) while *in vitro* cytotoxicity assay was performed utilising Vero cells by MTT assay (CV<sub>50</sub>). The genotoxicity test however, was performed using comet assay and the number of viable cells were counted based on the concentration of (CV<sub>50</sub>). The LC<sub>50</sub> concentration measured for brine shrimp lethality assay was 1210.65 µg/ml while the CV<sub>50</sub> measured for Vero cell in MTT assay was 513.22 µg/ml. There was no significant evidence of DNA damage observed with treated comet assay tail DNA (1.21 ± 1.676%) at which cell viability were recorded at (75 ± 5%). Overall findings showed that the methanolic *C. guianensis* flower extract has no significant neither in terms of cytotoxic nor genotoxic potentiality suggesting its prospectful development into a therapeutic agent. The study also calls for further safety evaluation is near future using *in vivo* animal studies.

**Keywords:** Couroupita guianensis, cytotoxicity, genotoxicity, traditional plant

## Introduction

For centuries, people has been consuming or applying compounds deriving from plant extracts with therapeutic modalities. Approximately more than 40,000 plant species have been in adopted by pharmaceutical industries marketing their intentions into treating various medical conditions [1]. In spite of the increasing demand for herbal medicine and rich tradition of employing medicinal plants for treatment of various diseases, in general one should also acknowledge that besides contributing pharmacological benefits, plants have also been implicated as an initiating factor in DNA damages, cell mutations and natural toxic elements [2]. Plants with the cytotoxic and genotoxic consequences are only found to manifest after a long period of consumption [3].

The species, *Couroupita guianensis* Aubl. (Lecythidaceae) has become rare and endangered medicinal plant due to the environment and anthropological overexploitation [4,5]. This tree stands robust, deciduous and evergreen at a growing height of 20 m where it supports clusters of cauliflorous inflorescence flowers with striking fragrance [6]. *C. guianensis* is also commonly referred to as Cannon ball tree due to its fruits' striking resemblance to a cannon ball [7]. Traditional healers associate *C. guianensis* with multifarious roles due to the fact that all parts of the tree can be utilised for medicinal purposes. The *C. guianensis* flowers in particularly, were observed in the treatment cure for scorpion bite, cold, intestinal gas formation and stomach ache. The infusions made with the mixture of flowers and leaves have been documented for treating inflammatory related matters [8]. Some of the other plant part's healing nature includes curing skin diseases, flu, stomach ache, toothache and pathogenic infections [9]. In addition to that, scientific studies have also explored *C. guianensis* and recorded properties such as antimicrobial, antifungal, antiseptic, antidepressant, antimalaria, anticancer and antihelminthic [10, 11,12, 13]. However, there seemed to be very little information on the cytotoxicity potential risk of the *C. guianensis* flowers available in literature. In our previous work, the prospect of 80% methanolic extraction of *C. guianensis* flowers

as a promising antifungal and antioxidant agent has been demonstrated [14]. Commencing from there onwards, this present study observes the methanolic extraction of *C. guianensis* flowers which tends to prospectively report its in vitro, in vivo cytotoxicity and genotoxicity potentiality. Hence, the employment of cytotoxicity and genotoxicity studies were carried out on the methanolic extraction of *C. guianensis* as a primary step for playing a successful role in identifying the hazardous effect at the basal level relevantly at the cell molecular and functionality [15].

## Methods

Collection, identification and preparation of *C. guianensis*

The flowers of *C. guianensis* were collected from Universiti Sains Malaysia and authenticated at the Herbarium of the School of Biological Sciences, Universiti Sains Malaysia, Pulau Pinang, Malaysia, where a sample was deposited (Voucher specimen: USM/HERBARIUM/11577). Prior to analysis, the flowers were rinsed and air-dried in room temperature for a period of two weeks and transformed into powder using mechanical grinder.

Preparation of *C. guianensis* methanol extract

A sample of 100 g of plant powder was soaked in 500 mL (1:5) of 80% methanol at room temperature (RT) ( $23^{\circ}\text{C} \pm 2$ ) for 7 days. The filtrate from each extraction was concentrated under vacuum on a rotary evaporator (Buchi, Switzerland) at  $40^{\circ}\text{C}$  and the concentrated extract was finally poured into Petri dishes and brought to dryness at  $40^{\circ}\text{C}$  in oven. The resultant extract paste is stored at RT in dark.

Brine shrimp hatching

*Artemia salina* eggs were hatched in a vessel with sterile artificial seawater prepared by dissolving 38 g table salt in 1 L distilled water. The vessel was kept under an inflorescent bulb and facilitated with good aeration for 48 h at RT. After hatching, nauplii released from the egg shells were collected by using micropipette. The larvae were isolated from the eggs by aliquoting them in small beaker containing the seawater.

Brine shrimp toxicity test

The bioactivity of the *C. guianensis* flower extract was monitored by the brine shrimp lethality test [16,17] to predict the presence of cytotoxic activity in the compound. The extracts were dissolved in methanol and diluted with artificial seawater. A two-fold dilution was set up to yield a series of concentrations from 2000 to 100 µg/mL. Positive control was prepared by diluting Potassium dichromate at the ranging concentrations of 100 to 900 µg/mL.

Approximately 10 nauplii was introduced into these preparations where the dead nauplii were counted after 24h. A computation was made based on the mortality percentage to determine the lethality at (LC<sub>50</sub>) of the nauplii [18]. A graph was plotted based on this following formula:

$$\% \text{ Mortality} = \frac{Sc}{St} \times 100$$

Where, Sc = number of dead nauplii

St = total number of nauplii

#### Cell line culture

Vero cell line was obtained from the Tissue Culture Laboratory of the Institute for Research in Molecular Medicine, USM. The Vero cell line was initiated from the kidney of a normal adult African green monkey on 27<sup>th</sup> March 1962, by Yasumura and Kawakita at the Chiba University, Japan [19]. Vero cells were cultured and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 µg/ml). Then the cells were cultured at 37°C in a humidified 5% carbon dioxide (CO<sub>2</sub>) incubator.

#### MTT-based cytotoxicity assay

The flower extract of *C. guianensis* were tested for *in vitro* cytotoxicity using Vero cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [20].

Hundred µL of media (DMEM) was added into each of the 96-well plates (triplicate). Then, the extracts were diluted in media to a final

concentrations of 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.125 µg/ml, 1.5625 µg/ml and 0 µg/ml. The Vero cells were plated at the density of 5 × 10<sup>4</sup> cells/ml cells/well (100 µl) into 96-well microtitre plates. Controls were prepared similarly but without the extract and the 96-well plate was incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator for 24 hours. After the incubation period, MTT (20 µl of 5 mg/ml) was added into each well and the cells were further incubated to 4 hours, until a purple precipitate was clearly visible [20]. The medium, together with MTT, were aspirated from the wells and 100 µl Dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple precipitate. The absorbance for each well was measured at 570 nm in a microtitre plate reader and the percentage of cell viability (CV) was calculated using the formula below;

$$\%CV = \frac{\text{Average absorbance of the treated wells}}{\text{Average absorbance of the control wells}} \times 100$$

Genotoxicity activity determined using Comet assay

To determine the possible genotoxicity activity of *C. Guianensis* flower, Vero cells were incubated for 24 h at 37°C/5% CO<sub>2</sub> with extract at CV<sub>50</sub> concentration, 50 µM H<sub>2</sub>O<sub>2</sub> as positive control and with culture medium as a growth control. After incubation, cell genoprotectivity was determined using OxiSelect™ Comet Assay Kit (Cell Biolabs, Inc, San Diego, CA, USA) according to the manufacturer's instructions [21]. Briefly, cells were harvested and centrifuges (1000rpm, 2 min) and washed with PBS. The cell pellet was mixed with liquefied Comet Agarose at a 1:10 ratio (v/v) and pipetted on an OxiSelect Comet Slide (75 µL/well). The cells were embedded, lysed and treated with alkaline solution to relax and denature its DNA accordingly to the manufacturer's instructions. The samples are further electrophoresed in a chamber (300 mA for 30 minutes) to separate intact DNA from its damaged parts, washed with sterile MilliQ water, treated with 70% cold ethanol for 5 minutes, air-dried, and stained with the Vista Green DNA dye.

The images of comets were taken using epifluorescence microscope at the magnification of 20X (Carl Zeiss Apo Tome, Germany). Hundred randomly selected cells (50 cells from each of the two replicate slides) were screened and the DNA damage is estimated by measuring the length of the comet tail using an ocular scale fitted in the eyepiece of the microscope.

#### Statistical analysis

All data were expressed as mean  $\pm$  standard deviation (SD) from at least three independent experiments. Statistical analysis was performed by using Statistical Package for Social Sciences (SPSS) version 20.0. Mean values were calculated and one-way ANOVA were used to compare the mean for more than two groups. A *p*-value of  $< 0.05$  was considered significant.

### Results and Discussion

It is imperative to study the safe usage of a promising herbal extract before it can be considered for drug development. The brine shrimp lethality bioassay demonstrates a rapid, cheap and simple method to assess in terms of screening, fractioning and monitoring plant bioactive compounds [22,23]. In many cases, this method has been rationally providing outcomes that correlates well with cytotoxicity, anti-cancer activity and other biological properties over the years [24]. The lethal concentration ( $LC_{50}$ ) of the test samples was determined upon 24 h by utilising a graph to plot percentage of shrimps' mortality against the sample concentration (toxicant concentration) where the best fit line was acquired from the data by regression analysis (Figure 1). The potassium dichromate served as positive control and the  $LC_{50}$  was quantified as 461.55  $\mu\text{g}/\text{mL}$  (Figure 2). The brine shrimp results presented in Figure 1 showed that the *C. guianensis* flower extract is effectively non-toxic on these shrimps. The lethality has been described here as the concentration that kills the nauplii within 24 h of extract exposure. The extract however, exhibited very low toxicity, giving a  $LC_{50}$  value of 1210.65  $\mu\text{g}/\text{ml}$ . The  $LC_{50}$  values have been disclosed to be highly toxic when they are below 249  $\mu\text{g}/\text{ml}$  while values above 1000  $\mu\text{g}/\text{ml}$  are contemplated as non-toxic [25, 26]. Based on the graph, it is apparent

that the brine shrimp lethality of *C. guianensis* flower extract is concentration dependent. In addition to that, one could also agree that a good relationship has been displayed here with the brine shrimp lethality assay to determine the safety of compounds found in *C. guianensis* flower extract, from a pharmacological standpoint. A similar study with methanolic extract of *Couroupita guianensis* Aubl. flower has been performed using Swiss albino mice where, the acute toxicity study at the dosage of 2000mg/kg, disclosed absence of mortality and concerning clinical symptoms, thus highly suggesting the non-toxic effect of this flower extract in mice [27].

The health of cells is dictated via the rates of cell viability or proliferation and upon exposure to physical or chemical detrimental agent, the cells' health and metabolism may be seen compromised [28]. As a result, to discover if a certain substance has deleterious effect on cell viability, an effective, sensitive, reliable and reproducible cytotoxicity assay is required. The MTT assay has been the very first tetrazolium salt-based assay utilised for adherent mammalian cells [20], suitable for analysing cell cytotoxicity. This simple yet old-time assay still sets as benchmark for all the new emerging cytotoxicity technique where it serves as a gold standard [29]. The reduction of the MTT formation is proportional to cell viability. In 24 h period of exposure to Vero cells, the extract showed 50% cell viability ( $CV_{50}$ ) at the concentration of 513.22  $\mu\text{g}/\text{ml}$  and further impairment of cell viability continues in a concentration dependent manner. This ostensibly excludes of any cytotoxic effect of *C. guianensis* flower extract at the *in vitro* cellular stage. Similarly, a study conducted by Pinheiro et al. [30] to assess the potentiality of *C. guianensis* as anti-inflammatory agent, also investigated the cytotoxicity effect *C. guianensis* ethanolic extract, hexane and ethyl acetate fractions on murine macrophage cell line, RAW 262.7 cells. The study concluded that neither the crude ethanol extract or its fractions demonstrated cell viability loss justifying *C. guianensis* as a non-toxic agent. However, the isolated compound known as isatin (1H-indole-2,3-dione) from *C. guianensis* flower did show cytotoxicity activity against human

promyelocytic leukemia HL60 cells at the  $CC_{50}$  value of 2.94  $\mu\text{g/ml}$ . The possibility that an isolated compound is capable of exerting potent effect relies on the amount of the chemical constituents. The crude extracts are composed by vast number of constituents including isatin that may either affect in an antagonistic or synergistic manner influencing the final effect unlike in its single presence [30]. Thus, isolated compounds in its pure existence may be more toxic than the plant whole crude compounds.

It is essential to consider the genotoxicity evaluations of a promising therapeutic agent as a part of an early safety assessment prior to its industrial development. The comet assay has been prominently utilised for testing genotoxicity at both *in vitro* and *in vivo* level [31, 32]. This alkaline, single-cell gel electrophoresis assay is applied for detecting impairments in DNA in eukaryotic cells which has also been demonstrated to be technically simple, affordable and sensitive [33, 34]. The breaks on the DNA is conveniently measured and made as sensitive marker to perceive genotoxic characteristics as induced by toxicant agents or substances [33]. The results obtained for *C. guianensis* to evaluate the degree of DNA damage in Vero cells displayed no significant effect at the  $CV_{50}$  concentration of 513.22  $\mu\text{g/ml}$  (Figure 4). The comets are observed here with distinct heads and without tails (Figure 4. A, B) whereas the positive control demonstrated using  $\text{H}_2\text{O}_2$  showed comets with distinct heads and tails (Figure 4. C). Fragmented DNAs are observed to have migrated away from the individual cells forming these tails or comet formations (Figure 5). In the present study, the number of viable cells (only cells with distinct heads) were calculated over 100 randomly selected cells and a plot was generated (Figure 6). The *C. guianensis* flower extract ( $75\% \pm 5$ ) did not indicate significant percentage change in relative to untreated cells ( $89\% \pm 6$ ) whereas a lower percentage of viable cells was observed for  $\text{H}_2\text{O}_2$  ( $38 \pm 5\%$ ). In order to affirm the DNA migration, the percentage of tail DNA was calculated and plotted (Figure 7). The tail percentage escalates in the  $\text{H}_2\text{O}_2$  treated group at the percentage of  $34.7 \pm 1.483\%$  while there were no significant differences observed

for the group treated with *C. guianensis* ( $1.21 \pm 1.676\%$ ). These data altogether confirmed that *C. guianensis* flower extract at the concentration of 513.22  $\mu\text{g/ml}$  did not exert genotoxic effect in Vero cells.

According to many literatures, genotoxic substances possess chemical and physical properties that causes deleterious effect in almost many ways in nucleic acids thus resulting in mutation- lead cancers [35, 36, 37, 38]. Moreover, the levels of DNA damage may be classified and detected using three types of tests where the first test being precision detection of breaks in DNA, followed by gene mutation revelation, and then final assessment using cytogenetic test [39]. These findings revealed that *C. guianensis* flower extract does not instigate significant injury to the cellular DNA at the concentration of  $CV_{50}$  which has ruled out the first test on the DNA breaks (since there is no DNA fragments found to constitute the comet formation). Furthermore, considering the fact that *C. guianensis* infusion has been utilised in ethnomedicine to treat multifarious diseases and wounds [40, 41] it is therefore, substantiates its safety consumption. However, further assays on the subject of genetic level safety should certainly be executed for the methanolic extract of *C. guianensis* flower.

### Conclusion

This is the very first time a study pertaining to safety evaluation of *Couroupita guianensis* flower extract has been performed. The results of the current study documented the absence of mortality, signs of cytotoxicity and genotoxicity of the *C. guianensis* flower extract confirming its ethnopharmacological practises and consumption among the rural communities. Advancement of *C. guianensis* as a therapeutic agent may augment the medical, nutraceutical and pharmaceutical industry. On that account, additional studies such as *in vivo* toxicological animal studies are still required to corroborate *C. guianensis* for the safe consumption of this plant.

### Acknowledgments

This research was funded by USM-RIKEN Joint Laboratory on Bioprobe Discovery Research Grant from Universiti Sains Malaysia, Pulau Pinang, Malaysia with grant number 304.CIPPM.6316068. Shanmugapriya was supported by the Graduate Assistant Scheme from Universiti Sains Malaysia, Pulau Pinang, Malaysia.

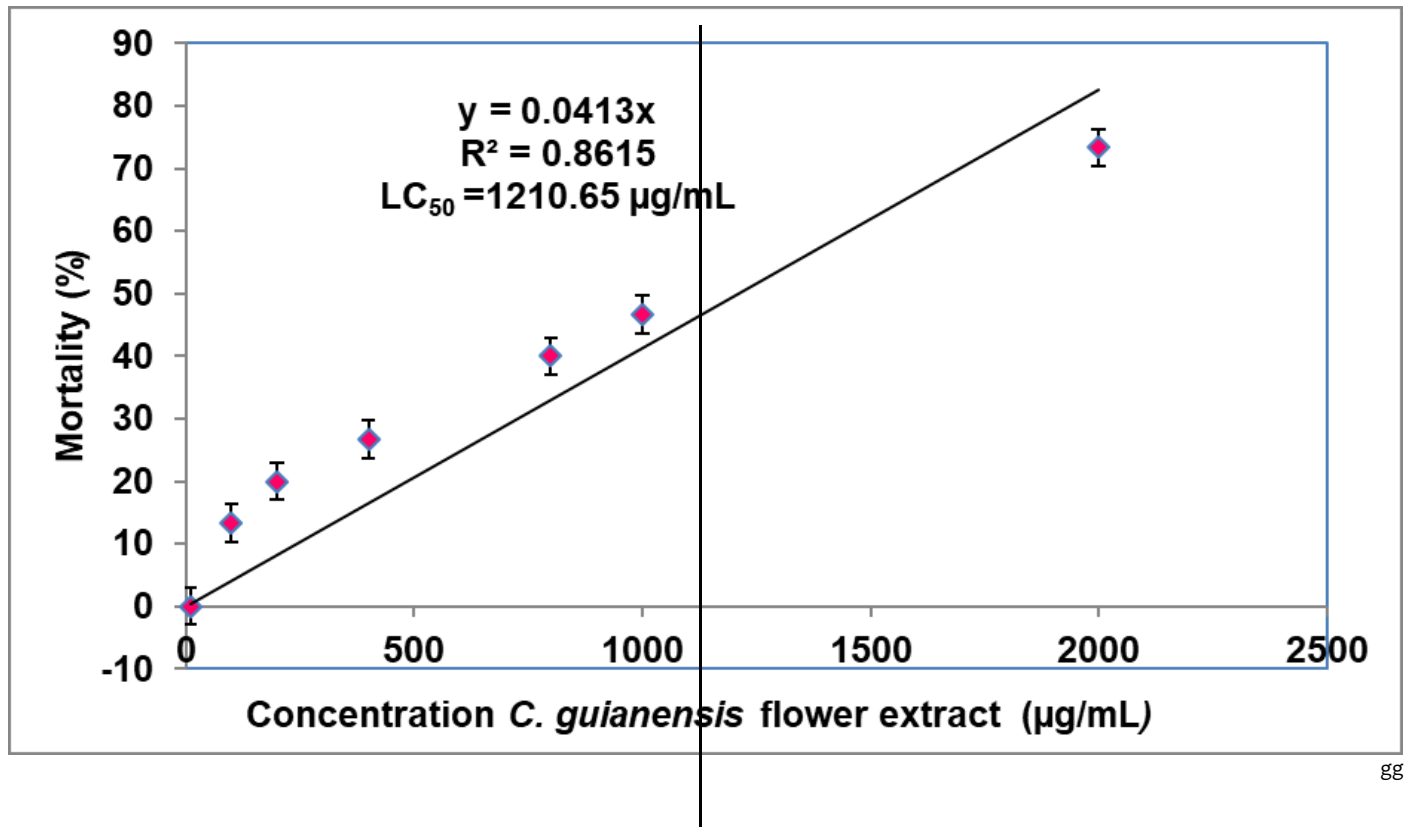
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Figure. 1. Brine shrimp lethality assay of *Couroupita guianensis* flower extract and linear regression analysis plot showing the  $R^2$  value and the equation of the line.

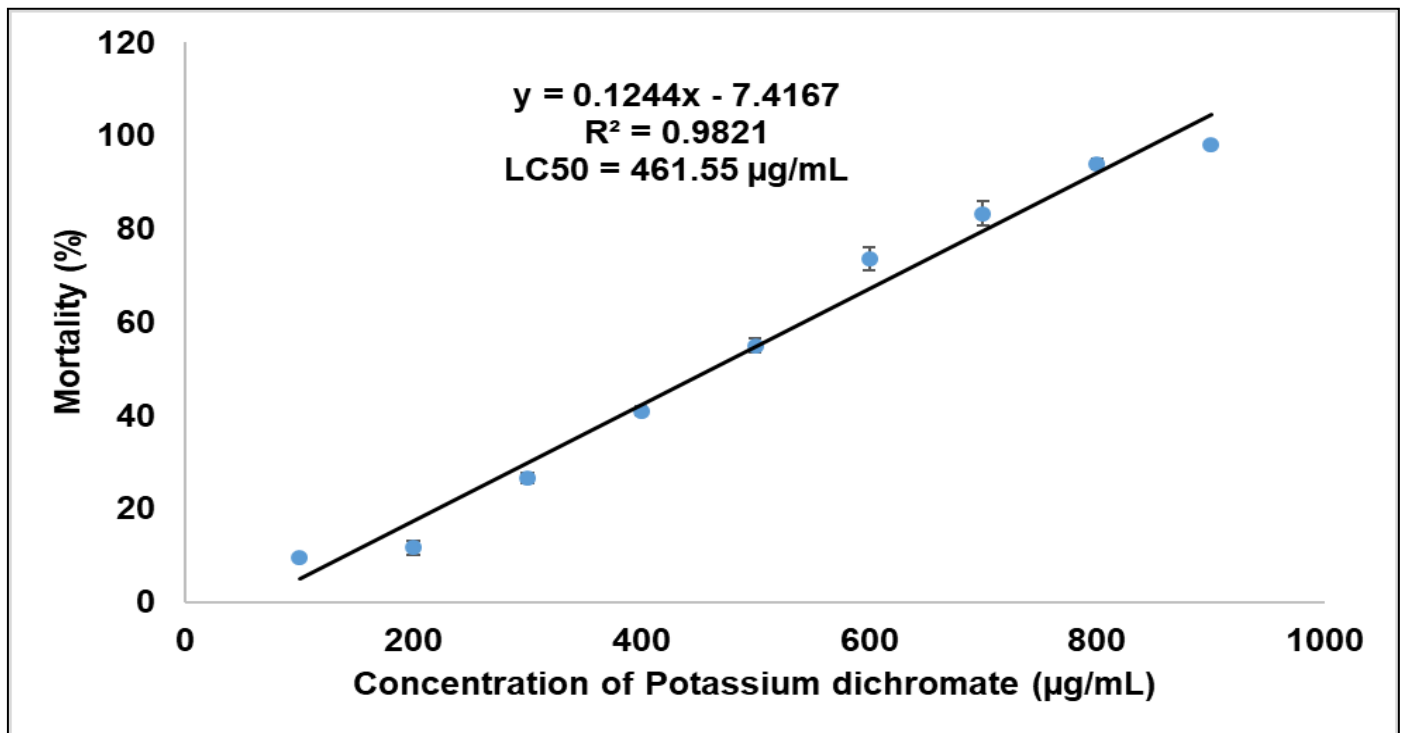


Figure. 2. Brine shrimp lethality assay of potassium dichromate as a positive control and linear regression analysis plot showing the  $R^2$  value and the equation of the line

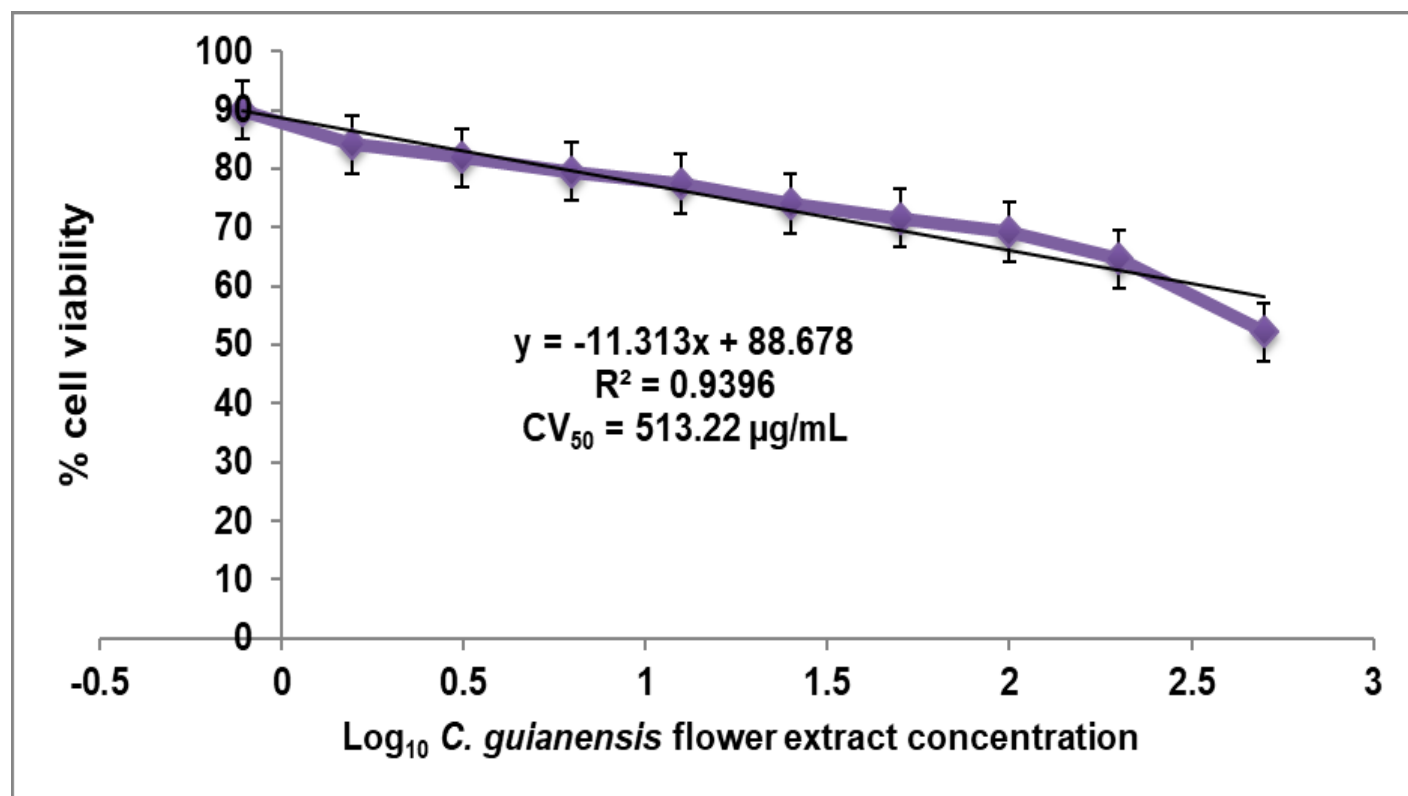
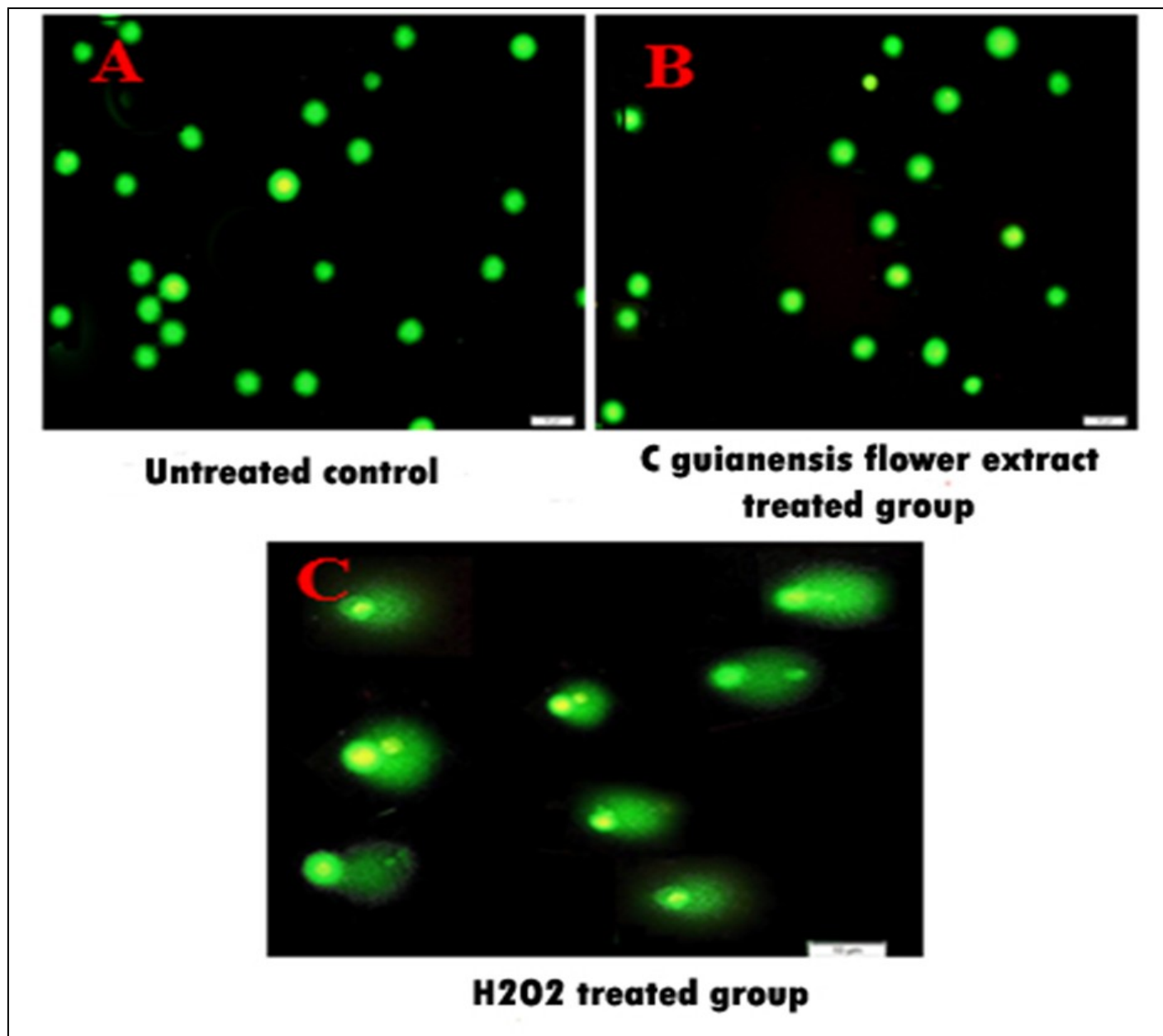
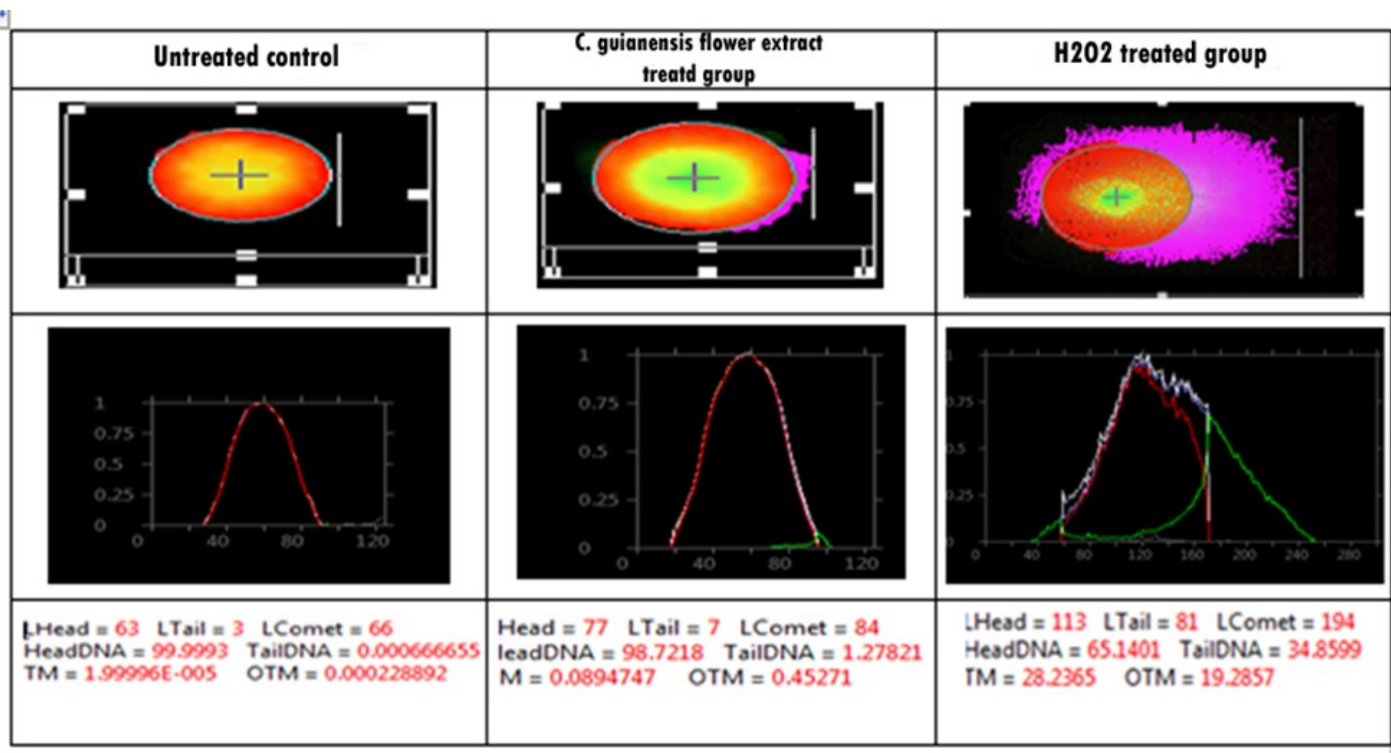


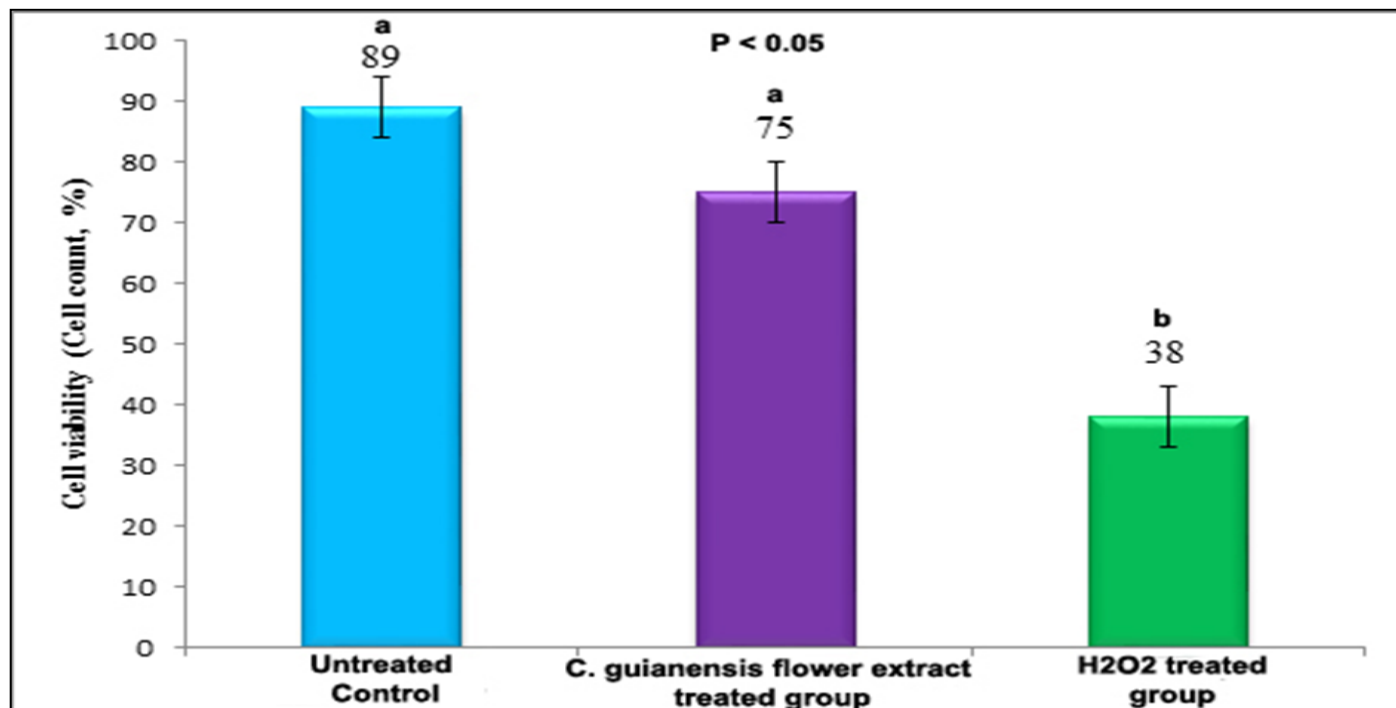
Figure. 3. Cytotoxicity of *Couroupita guianensis* flower extract against Vero cells



**Figure. 4.** Photomicrograph of Vero cell's DNA in untreated control group showing no DNA damage (A), treated with *Couroupita guianensis* flower extract group showing no DNA damage (B) and H<sub>2</sub>O<sub>2</sub>-induced DNA damage in Vero cells group showing DNA damage with comet tail (C) (Magnification: 200X)



**Figure. 5.** Image analysis of Comet assay by CASP version 1.2.2 software of Vero cells treated with *Couroupita guianensis* flower extract of 513.22 µg/mL IC<sub>50</sub> concentration, H<sub>2</sub>O<sub>2</sub> treated and untreated Vero cells for 24 hours.



**Figure 6.** Cell viability in percentage of Vero cells treated with *Couroupita guianensis* flower extract of 513.22  $\mu\text{g}/\text{mL}$   $\text{IC}_{50}$  concentration,  $\text{H}_2\text{O}_2$  treated and untreated Vero cells for 24 hours. Values are expressed as means  $\pm$  SD of triplicates. Different alphabets (a-b) indicate significant differences ( $p < 0.05$ ) using One-way ANOVA followed by Turkey's multiple comparison test.

**Figure. 7.** The percentage of tail DNA in Vero cells treated with *Couroupita guianensis* flower extract of 513.22  $\mu\text{g}/\text{mL}$   $\text{IC}_{50}$  concentration,  $\text{H}_2\text{O}_2$  treated and untreated Vero cells for 24 hours. Values are expressed as means  $\pm$  SD of triplicates. Different alphabets (a-b) indicate significant differences ( $p < 0.05$ ) using One-way ANOVA followed by Turkey's multiple comparison test.

