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Research Article

CYTOLOGICAL AND PHYSIOLOGICAL EFFECTS OF *COUROUPITA GUIANENSIS* LEAF EXTRACTS ON *PISUM SATIVUM* SEEDS

Mendhulkar V.D., Yadav Neetu, *Yadav Anu and Nisha K.
Department of Botany, Institute of Science, 15, Madam Cama Rd, Fort, Mumbai, Maharashtra- 400032
**Author for Correspondence*

ABSTRACT
Couroupita guianensis is commonly used in traditional medicine to treat variety of diseased conditions including cold, stomachache, gastritis, piles, dysentery, and scorpion poison. The aqueous and chloroform extracts of 30% and 50% concentration were tested on root meristems of *P. sativum*. Distilled water was used as negative control. Our findings indicated reduction in mitotic index at higher concentrations of *C. guianensis* extract. High degrees of chromosome aberrations were also observed including stickiness, bridges, vacuolated nucleus and laggard formation. Effects on physiological and metabolites quantification also have been observed to reveal possible link between genotoxic and mito-depressive impact of plant extract in *Pisum sativum*. The phytoconstituents are responsible for exhibiting the cytogenetic impact of the extract; therefore some qualitative assays were performed in the present study.

Keywords: *Genotoxicity; Pisum Sativum; Mitotic Index; Chromosome Aberrations*

INTRODUCTION
The scope for screening of active plant product for the clinical application and properties has gained remarkable significance due to the increasing trend of using natural products as drugs. This trend also has given an insight to the clinical biology to discover new drugs with varying combination and formulation (Manimegalai and Rakkimuthu, 2012). Medicinal plants are the highest productive source of novel compounds and medicines of natural origin. According to World Health Organization, more than 80% of the world's population relies on traditional drug for their primary healthcare demands (Bodekar *et al*., 1997). Despite the profound therapeutic advantages possessed by some of the medicinal plants, some constituents of medicinal plants are found to be potentially toxic, mutagenic and carcinogenic (Yuet *et al*., 2012). One important plant that is used in traditional medicine is *Couroupita guianensis*, a tree belonging to the Lecythidaceae family. This plant has several medicinal properties. It is used for treating skin conditions. The flowers of the plant are used to cure cold, intestinal gas formation and stomachache and are also used for immune modulatory activity and larvicidal activity against vector. Leaves of *C. guianensis* are widely used as an analgesics medicine by the Brazilian rural population. It is said to possess antifungal, antibiotic and analgesic qualities. It is used extensively as an ingredient in the many preparations which cure gastritis, scabies, bleeding, piles, dysentery, and scorpion poison and to treat hypertension, tumors, pain, inflammatory processes, cold, stomach ache, skin diseases, malaria, wounds and toothache (Manimegalai and Rakkimuthu, 2012). Recent studies have shown that long-term exposures to traditional medicinal plants might be associated with increases in the rates of morbidity and mortality (Yuet *et al*., 2012). Since, *C. guianensis* plant parts are used as medicine, it becomes important to investigate the genotypic effect of this plant extract. The aim of this study was to evaluate the genotoxicity of *C. guianensis* extract using *Pisum sativum* as the experimental model.

MATERIALS AND METHODS
1.1. Plant Sample
Fresh plant leaves of *Couroupita guianensis* were collected from the Institute of science botanical garden in December 2013.
1.2. Preparation of the Plant Extract
The healthy leaves of the selected plant were collected and thoroughly washed in running tap water and air dried for 7-10 days. The dried material was mechanically powdered, sieved using meshes and stored in

an airtight container. Aqueous extracts of the leaves were obtained by mixing 5g of the leaf powder with 100 ml of distilled water. The content was boiled in microwave oven for 2 minutes and then filtered using Whatman No.1 filter paper and stored in amber colored bottles in refrigerator. Organic extract of the leaves was obtained by mixing 5g of the leaf powder with 50ml of chloroform solution and subjected to the sonication in a sonicator for 1 hour and then filtered using Whatman No.1 filter paper. Chloroform was evaporated by subjecting the extract to hot water bath and then the remains were dissolved in minimal quantity of water, stored in amber colored bottles with tight lids and stored in refrigerator.

1.3. Pisum Sativum Root tip Assay

1.3.1. Pre-Treatment: Physiologically uniform and healthy seeds of *Pisum sativum* were used to study genotoxic effect of plant extract in the present study. The two sets of 100 pre-conditioned seeds were treated with aqueous and chloroform extract of 50% and 30% concentrations for one hour.

On completion of treatment period the seeds were washed with distilled water and placed in Petri dish on moist blotting paper. The seeds were allowed to germinate in Petri plates under laboratory conditions. The observation on shoot and rot length was made for 7 days to observe effect of extract on their vital physiological parameters.

1.3.2. ***Cytological Parameters:*** For mitotic screening the treated seeds were allowed to germinate separately in sterilized petri plates. Total of 100 seeds per treatment were used. The root tips were fixed in Conroy's fluid for 30 minutes, washed in running tap water and preserved in 70% alcohol. The root tips were hydrolyzed in 1N HCl at 60°C and stained with 2% acetocarmine. The dividing cells were observed and recorded. Mitotic index is determined using the formula: Total no. of dividing cells/ Total no. of cells observed X 100. Olympus light microscope with digital camera was used to get the clear image of the chromosome aberrations.

1.4. Phytochemical Analysis

The aqueous and organic extracts obtained from the dried leaves powder, were tested for the qualitative presence of the phytochemicals, Tannins, Saponins, Flavonoids and Steroids.

2.4.1 ***Tests for Tannins & Saponins:*** 5 drops of 0.1% Ferric chloride was added to 5ml of the plant extract. The presence of tannin was indentified with the appearance of blue black color (Trease and Evans, 2002).

For saponins, 10 ml of the plant extract was added with 3 ml of distilled water and shaken well to obtain froth. 5 drops of Olive oil were added in the froth with vigorous shaking. The formation of emulsion indicates the presence of saponins (Sofowora, 1993).

2.4.2 ***Tests for Flavonoids & Steroids:*** 3ml of the plant extract was mixed with 1ml of 10% NaOH. Yellow coloration was obtained indicating the presence of crude flavonoids (Trease and Evans, 2002). Quantitative analysis of total flavonoid content was determined using Aluminium Colorimetric method. For calibration, quercetin was used as a standard material. Various concentrations of standard quercetin solution were used to make a standard calibration curve (Kalita *et al*., 2013).

1 gram of plant powder was mixed with 10 ml of chloroform, followed by boiling and filtration. To the above 2 ml of the filtrate add 2 ml acetic anhydride and a few drops of concentrated H_2SO_4. Presence of blue-green ring in the solution confirms the presence of steroids (Sofowora, 1993).

RESULTS AND DISCUSSION

3.1. *Mitotic Index in P. Sativum after Treatment with C. Guianensis Leaf Extracts*

The cytological effects of *C. guianensis* extract were studied in different concentrations of plant extract in root tips of *Pisum sativum* and the results obtained are tabulated in Table-1. Exposure of *C. guianensis* extract inhibited the mitotic cell division at higher concentration when compared to the mitotic index of control group.

The aqueous extract of the *C. guianensis* indicated MI values 20.02 and 18.13 corresponding to the 30% and 50% concentrations, respectively. The treated samples with chloroform extract of similar concentrations exhibited 16.46 and 13.54 MI, respectively. This may indicate that *C. guianensis*

chloroform crude extract exerted a genotoxic effect at 50% concentration more pronouncedly. The mitotic indexes in treated cells were lower compared to the distilled water (negative control) which was 25.33.

Mitotoxicity was noted in all the stages of mitotic cell division (Table 1). Chromosomal anomalies like sticky chromosomes and chromosome bridges were of common occurrence (Figures A, B, C and D). Other chromosomal abnormalities observed were laggard, clumped chromosomes and vacuolated nucleus (Figures F and G respectively). Few indications of aberrant cells were also observed in control root tips which may be attributed to the natural process.

Higher plants such as *P. sativum* are accepted as admirable genetic models to evaluate genotoxic effects. Results of the current study reflected the utility of root tips cells of *P. sativum* for monitoring the genotoxic effects of *C. guianensis* plant extracts. *P. sativum* assay enabled the assessment of different genetic endpoints, which are mitotic index and chromosome aberration. Mitotic index is used as an indicator of cell proliferation biomarkers which measures the proportion of cells in the mitotic phase of the cell cycle. Hence, the decrease in the mitotic index in *P. sativum* somatic cells could be interpreted as retardation in mitosis or cellular death.

The depression of mitotic index reflects negative impacts on somatic growth at initial stages as a primary effect in the development of plant (Mendhulkar *et al*., 2005). Low mitotic index may be reflecting a direct genotoxic effect of *C. guianensis* extract. Several types of chromosome aberrations were considered in the different phases of cell division to evaluate chromosomal structural variations. Analysis of chromosome aberrations not only allowed estimation of genotoxic effects, but also enabled evaluation of their clastogenic and aneugenic actions (Rank and Nielsen, 1997).

Therefore the mitotic index was analyzed in this study to determine the genotoxicity of *C. guianensis* extract in *P. sativum*. The cells of *P. sativum* root tips after treatment with extracts of *C. guianensis* showed retardation in mitotic index. There were significant differences between treated groups and control group in mitotic index (Table 1). The mitotic activity of *C. guianensis* chloroform extract was significantly decreased at 50% concentration (Table 1), as compared to the control. The reduction of the mitotic index might be explained as being due to the obstruction of the onset of prophase, the arrest of one or more mitotic phases, or the slowing down the rate of cell multiplication (Christopher and Kapoor, 1988).

The mito-depressive effect suggests that *C. guianensis* extract had some effects on cell division of *P. sativum*. This may be due to abnormal conditions of the cells induced by the treatments. The abnormalities of chromosomes could be due to the blockage of DNA synthesis or inhibition of spindle formation, thereby causing structural chromosomal changes. *C. guianensis* extract sometimes does not allow the initiation of spindle formation or DNA biosynthesis (Akinboro and Bakare, 2007).

Chromosome aberrations provided important information and may be considered an efficient test to investigate the genotoxic potential of the treatments analyzed (Carita and Marin-Morales, 2008). The chromosome aberrations observed in all the concentrations of the studied treatments includes chromosome stickiness, bridges, laggards and vacuolated nucleus. These aberrations were due to the effect of the extract on the spindle formation and thus resulted in cell division disturbances.

Chromosome bridges indicating the clastogenic effect caused by chromosome breaks (Leme and Marin-Morales, 2009).

The chromosomal stickiness was a common occurrence in this study (Figures C & D). A remarkable correlation between the frequencies of stickiness and the bridges was observed. This supports the hypothesis that stickiness may result from improper folding of chromosome fibers which makes the chromatids connected by means of sub-chromatid bridges (McGill *et al*., 1974; Klasterska *et al*., 1976). However, this stickiness may be interpreted as a result of depolymerisation of DNA, partial dissolution of nucleoproteins, breakage and exchanges of the basic folded fiber units of chromatids and the stripping of the protein covering of DNA in chromosomes (Mercykutty and Stephen, 1980). In many studies sticky chromosomes indicated a highly toxic, irreversible effect, probably leading to cell death (Fiskesjo, 1985).

Another remarkable abnormality was chromosome bridges. Chromosome bridges were commonly observed during anaphase (Figures A & B). The bridges noticed in the cells were probably formed by

breakage and fusion of chromatids or sub-chromatids (Shehab and Adam, 1983). Chromosome bridges may be caused by stickiness of chromosomes which blocks their separation and free movements and thus they remained connected at both the ends (Kabarity *et al*., 1974).

A low frequency of laggards (Figure G) and vacuolated nucleus (Figure F) was also observed. Their presence may be attributed to the partial asynapsis failure of the spindle apparatus to organize and function in a normal way (Grant, 1982). Some cells are enlarged and maintain continuity by cell wall through cellular channel and it facilitates the transfer of cytoplasmic content from one cell to other due to the occurrence of cytomixis (Patil and Mendhulkar, 1993).

Table 1: Mitotic index in *Pisum sativum* root tips treated with various concentrations of the *C. guianensis* leaf extract

S.N.	Conc. (in %)	Total number of cells screened	No. of dividing cells			Total number of dividing cells	Mitotic index
			Prophase	Metaphase	Anaphase		
1.	Control	750	20	91	79	190	25.33
2.	Aqueous extract - 30%	674	6	76	53	135	20.02
3.	Aqueous extract - 50%	612	2	60	49	111	18.13
4.	Chloroform extract - 30%	650	4	56	47	107	16.46
5.	Chloroform extract - 50%	598	3	42	38	81	13.54

Genotoxicity and cytotoxicity are biometric processes and any impact of such crude extract is definitely related to the quality and quantity of phytoconstituents present in the plant extract.

Table 2: Phytochemical analysis of crude extracts of *C. guianensis*

Sr. No.	Phytochemicals	Aqueous extract	Chloroform extract
1.	Flavonoid	+	+
Total flavonoid content of extract(µg of QE/mg of extract)		99.54	99.52
2.	Tannin	+	+
3.	Saponin	+	+
4.	Steroid	-	-

+: indicates presence. -: indicates absence.

3.2. *Radical and Shoot Length*

The shoot length started 3 days after the emergence of plumule and linear pattern of radical growth was observed on 7th day similar to control samples with some variation but of unremarkable nature. The seed samples treated with 30% aqueous extract exhibited radical length almost similar to control but the treated samples with 30% & 50% chloroform mediated leaf extract of *C. guianensis* indicated relatively more impact compared to other studied samples (Figure 2). However, shoot length parameter was remarkably

affected in all the treatments compared to control (Figure 3) at 7th day. Treatments with aqueous extract 30%, 50% and chloroform extract 30% and 50%, revealed remarkable impact on retardation of shoot length at marginally equal level.

3.3. *Phytochemical Analysis*

Phytochemical analysis of the leaf extract of *C. guianensis* was done using the methods described above. Presence of Saponins, Flavonoids and Tannins was confirmed in the aqueous and chloroform extracts. Steroid was absent in both aqueous and chloroform leaf extracts. Total flavonoid content was quantitatively estimated using the Aluminium Colorimetric method and the results are tabulated in Table-2.

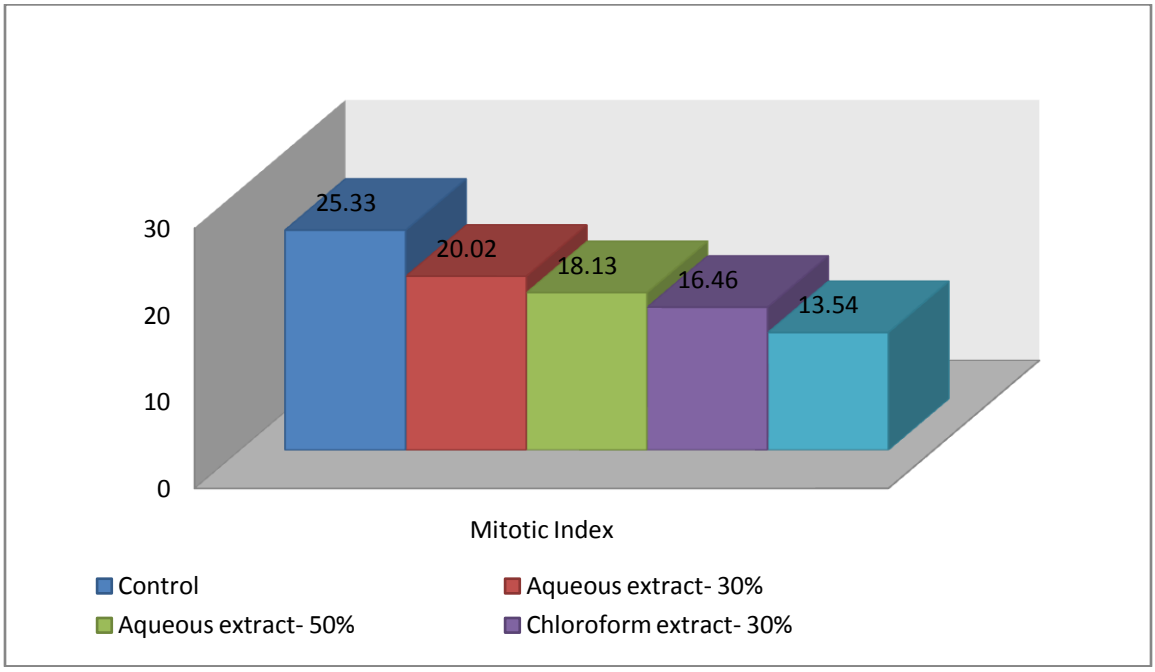


Figure 1: Effect of *C. guianensis* leaf extracts on mitosis in *P. sativum* root tips

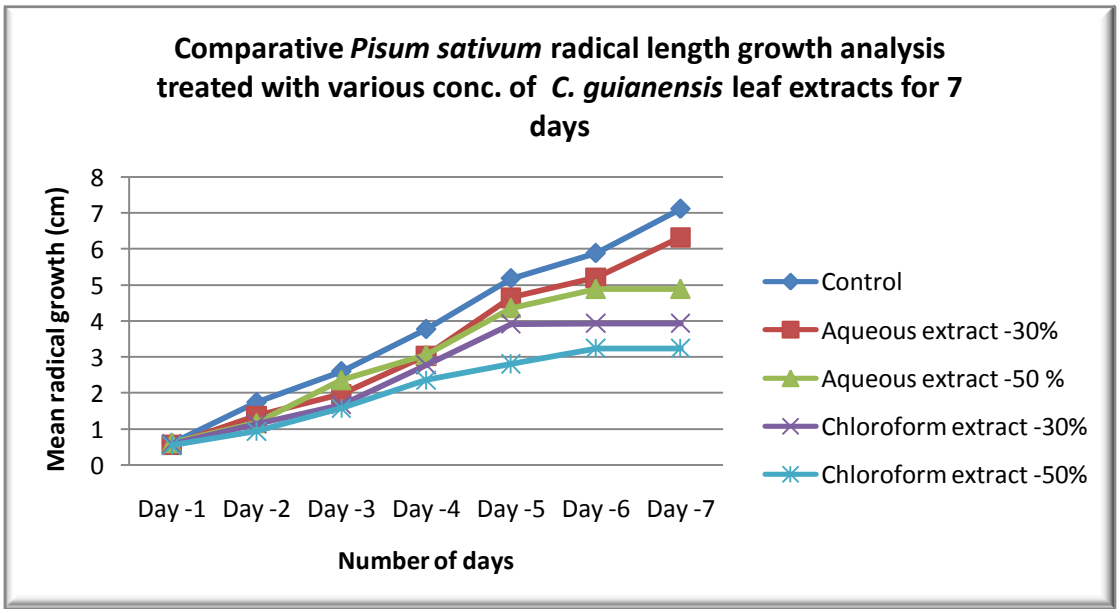


Figure 2: Effect of different concentrations of *C. guianensis* leaf extracts on the radical growth in *P. sativum* seeds

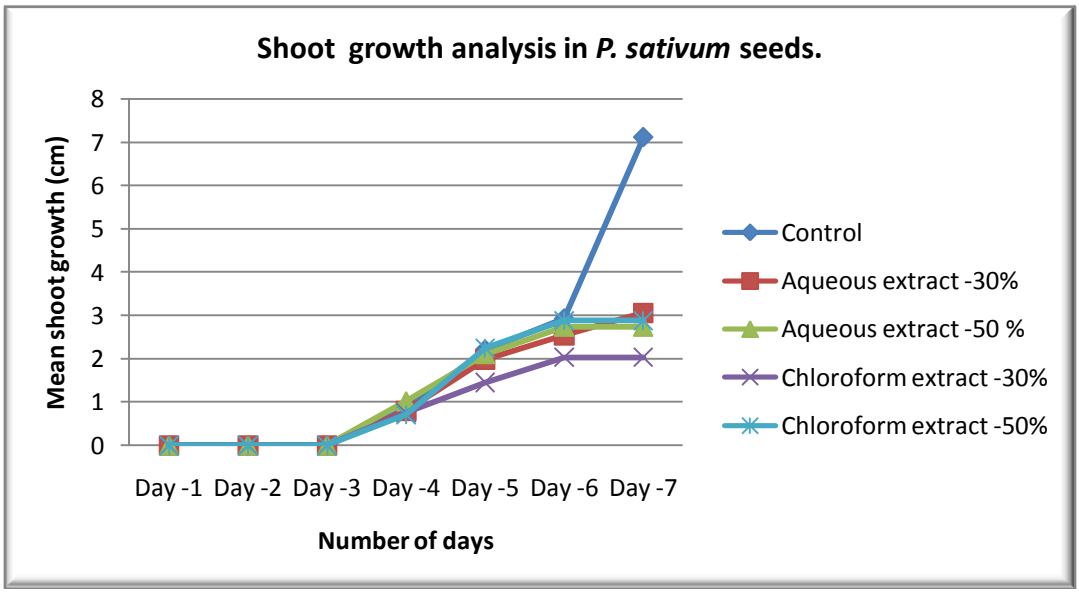
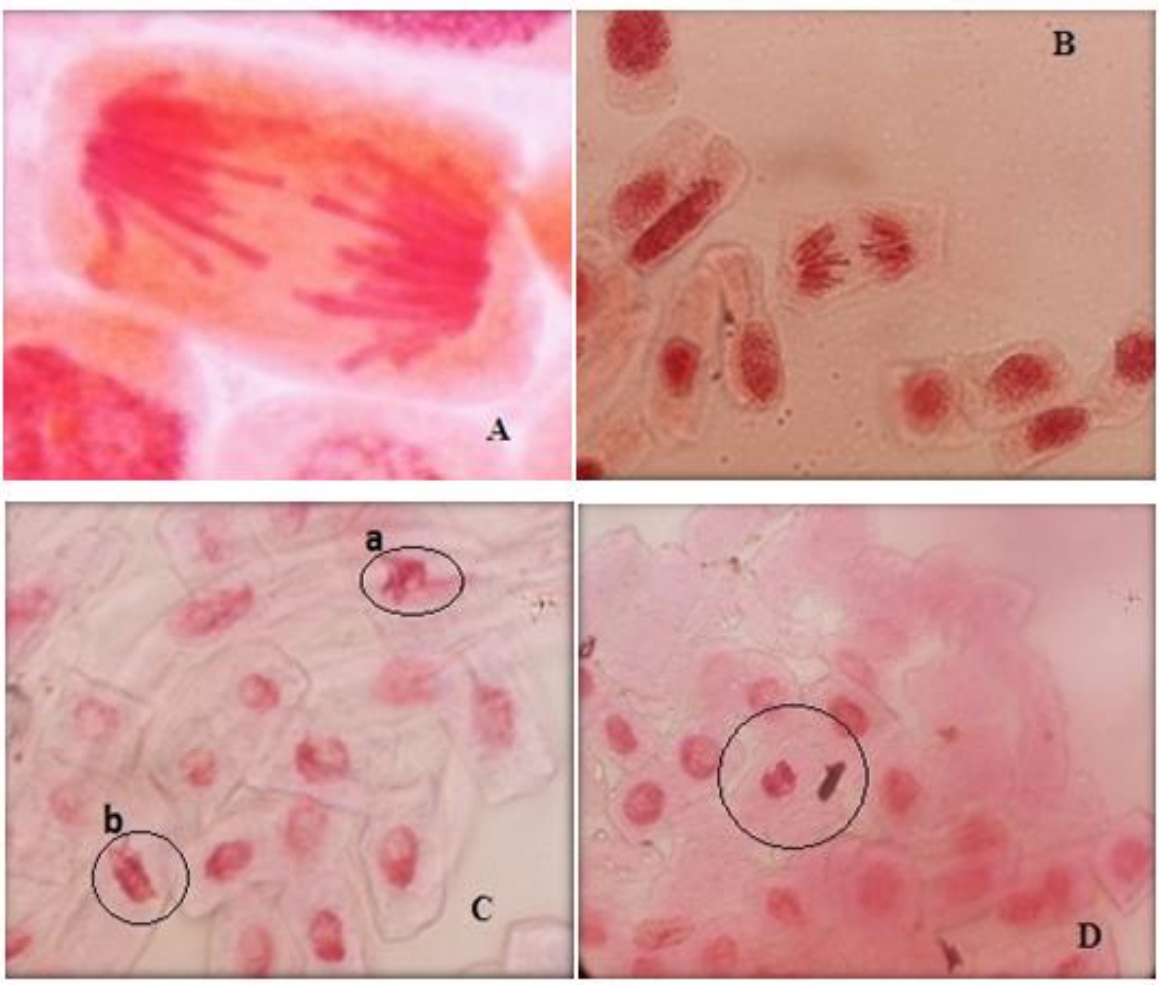


Figure 3: Effect of different conc. of *C. guianensis* leaf extracts on the *P. sativum* shoot length growth



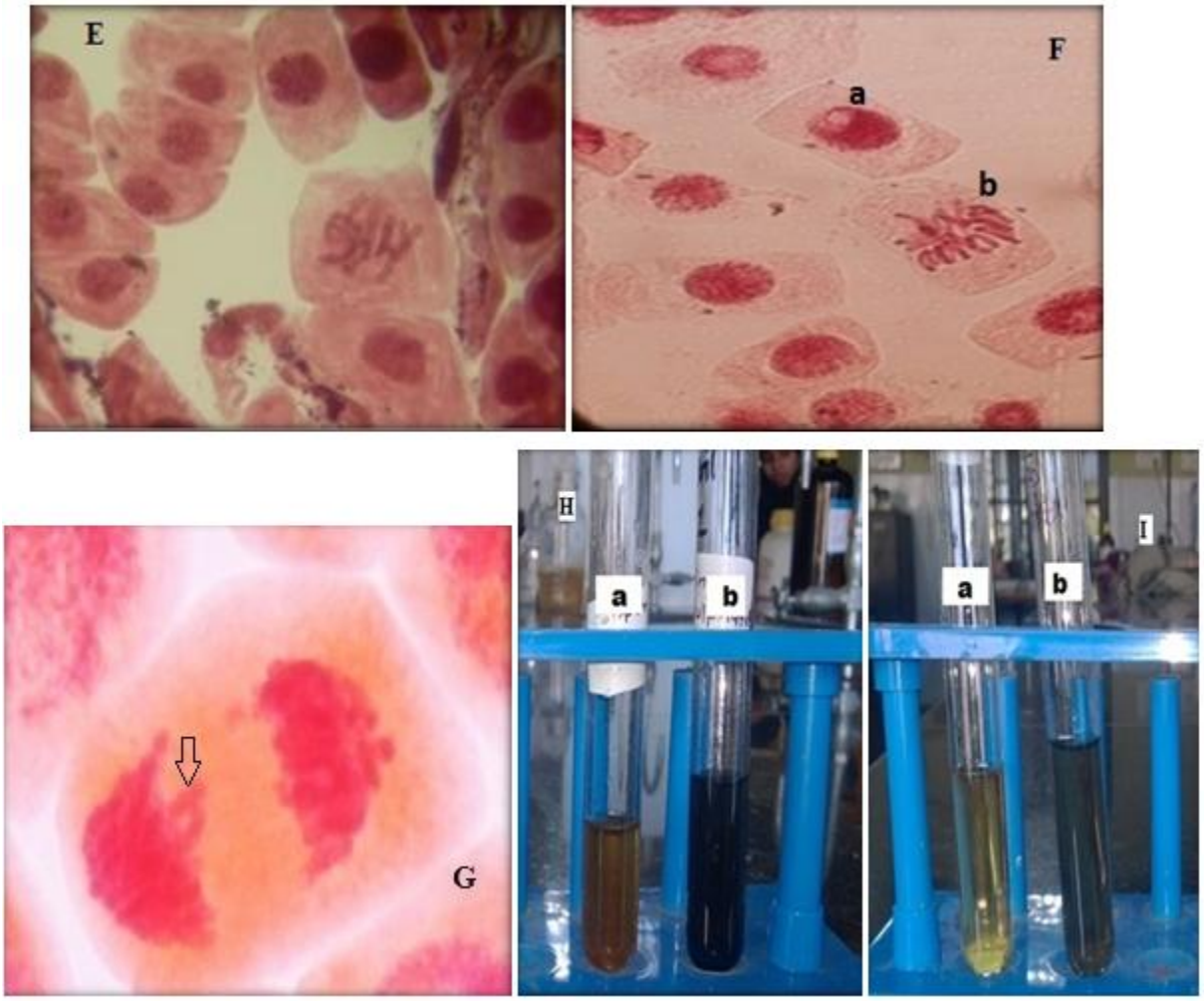


Photo plate 1: Chromosome aberrations in *P. sativum* root tips treated with *C. guianensis* leaf extracts. A- Broken Chromosome Bridge. B- Chromosome Bridge in Anaphase. C- (a) Clumped metaphase, (b)- Sticky chromosome. D- Sticky chromosome. E- Chromosomal overlapping in Metaphase. F- (a) Vacuolated nucleus, (b) Overlapping chromosomes in Metaphase stage. G- Chromosomal Laggard in late Anaphase. H- Chloroform extract of *C. guianensis* leaves (a) Presence of yellow coloration indicates the presence of Flavonoids. (b) Presence of blue black color indicates the presence of Tannins. I- Aqueous extract of *C. guianensis* leaves. (a) Presence of yellow coloration indicates the presence of Flavonoids. (b) Presence of blue-black color indicates the presence of Tannins

To this context, in the present study quantitative assay was done for flavonoid content and it was found to be 99.54µg/mg and 99.52µg/mg in the aqueous extract and chloroform extract, respectively. Qualitative tests confirmed the presence of saponins, tannins and flavonoids in the plant extract.

Conclusion

From the present study it appears that when applied in high doses, *C. guianensis* leaf extract shows cytotoxic and genotoxic activity. In this study, we have used crude extracts of *C. guianensis* leaf. Studies with crude plant extracts are appropriate because traditional medicinal herbs are generally used as crude extracts. The crude extract is a complex mixture of biologically active compounds. Retardation of mitotic index and insertion of chromosomal structural changes are the clear indications of the ability of some constituents in the leaf extract to exhibit its impact at cytotoxic and genotoxic level. However, some of

the constituents in the extract contain metabolic components like flavonoids, saponins, tannins which can be cytotoxic and/or genotoxic; others can be cytoprotective and/or antigenotoxic. The results of this study suggest that, although *C. guianensis* has beneficial effects as a medicinal plant, it can cause serious problems and damage to cells when used improperly. In addition, further cytogenetic studies dealing with clastogenicity and genotoxicity of this extract with more comprehensive genotoxicity assessment in animal model may reveal further interesting results for its usage for human welfare.

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