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PHYTOCHEMICAL ANALYSIS, CYTOTOXICITY AND GENOTOXICITY OF *PSIDIUM GUAJAVA L.* (MYRTACEAE) LEAF EXTRACTS

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ABSTRACT

Psidium guajava is one of the oldest and widely used medicinal plants in the world. The leaves, bark, roots and immature fruits are used in African ethno-medicine because of their anti-inflammatory, anti-diarrhoeal and anti-microbial properties amongst others. The aim of this work was to determine the phytochemical profile of locally sourced mature *Psidium guajava* leaves and to also evaluate the cytotoxic and genotoxic properties of aqueous extracts of *Psidium guajava* leaves. Ethanol extracts of *Psidium guajava* leaves were subjected to analysis using GC-MS. Aqueous extracts were prepared by boiling pre-weighted fresh leaves in tap water and subsequently used in the *Allium cepa* assay. Analysis revealed terpene compounds (80.6%) as the most abundant phytochemical in ethanol extracts of the leaves. The results also revealed an overall significant concentration and time-dependent reduction in mitotic index ($p \leq 0.05$). The value of EC50 was estimated to be 54.4 g/l. Examination of treated *Allium cepa* root tip cells revealed chromosomal aberrations including binucleation, bridges, distorted anaphase, clumping, vagrant chromosomes and c-mitosis. Aqueous leaf extracts of *Psidium guajava* leaves have genotoxic and cytotoxic potentials which should be seriously considered in its therapeutic applications.

Keywords : *Psidium guajava*, phytochemicals, ethno-medicine, *Allium cepa*, cytotoxic, genotoxic

INTRODUCTION

Psidium guajava (Family: Myrtaceae) is one of the 150 species of *Psidium*, most of which are well known throughout the tropics (Luber *et al.*, 2015; Ofodile *et al.*, 2013). The high phytochemical content makes several parts of the plant useful in African ethnomedicine. The leaves and bark are rich in phytochemicals such as tannins, quinones, alkaloids, saponins, terpenes and flavonoids (Egharevba *et al.*, 2010; Egga *et al.*, 2014). The antimicrobial, anti-plasmodial, anti-allergic, anti-hyperglycemic and antitumor activities of several parts of the guava plant are well documented (Rattanachaikunsopon and Phumkhachorn, 2010). Because of their astringency, the leaves, bark, root and immature fruits are commonly employed to halt gastroenteritis, diarrhoea and dysentery throughout the tropics (Tona *et al*., 2000). Crushed leaves are applied on wounds, ulcers and rheumatic places for healing, while the leaves are chewed to relieve toothache (Lin, 2002). It is also taken as emmenagogue, vermifuge (Temjenmongla and Yadava, 2003) and as treatment for leucorrhoea in women (Holetz, 2002). A combined decoction of leaves and bark is given to expel the placenta after birth (Yadava, 1996).

The use of plant models in cytogenotoxicity testing such as the *Allium cepa* assay is beneficial and acceptable because, they are inexpensive and show good correlation with other test systems. They also seldom produce false results and are very reliable; and as such, they are suitable for genotoxicity monitoring programmes (Steinkellner *et al.*, 1998). The *Allium cepa* assay has been used to determine the cytogenotoxicicty of several chemicals, industrial effluents and phytochemicals (Salaam *et al.*, 2016). Plant growth regulators such as kinethin

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and benzylaclenine are reported to produce aberrations such as micronuclei and multi-polar spindle formation, endo-reduplication and binucleation in *Allium cepa* root tip cells (Soh and Yang,1993). *Pulicaria crispa* showed a strong inhibitory effect on cell division in onion root tip cells (Shebab, 1999). Extracts of *Cymbopogon citratus* also produced chromosomal aberration including bridges, clumping, erratic chromosome movement, c-metaphase and binucleation in *Allium cepa* root tip cells (Williams and Omoh, 1996).

de Rosangela *et al.* (2003) showed that high concentrations of *Psidium guajava* leaf infusions caused anti–mitotic effects on mitosis on *Allium cepa* roots. Ofodile *et al.* (2013) also reported the presence of chromosomal aberrations including c-mitosis, vagrant, binucleation and bridges in *Allium cepa* root tip cells treated with ethanolic and water extracts of ground guava leaves after 96 h treatment. The effects of infusions of three varieties of guava leaves on *Lactuca sativa* germination, root tips and meristematic cells was investigated by Luber *et al.* (2015). They reported a significant decrease in the frequency of cell cycle alterations and aberrations such as bridges, c-metaphase, sticky chromosomes and fragmentation. This study reports the phytochemical profile of mature guava leaves in addition to cytotoxic and genotoxic effect caused by aqueous extracts of *Psidium guajava* leaves obtained by boiling, a method of extraction often adapted in Nigerian ethno-medicine, on *Allium cepa* roots.

MATERIALS AND METHODS

Plant Materials and Reagents

Mature guava leaves were obtained from a guava tree in the Faculty of Education, within the premises of University of Lagos, Lagos, Nigeria and duly identified by a plant taxonomist. A sample specimen was deposited in the herbarium of the Department of Botany, University of Lagos, Lagos, Nigeria with voucher number LUH 7355. The common onion, *Allium cepa* (purple variety) bulbs of equal sizes were obtained from Mushin market, Lagos, Nigeria. Chemicals of analytical grades were used in preparing all reagents.

Preparation of Extracts

Extracts for phytochemical analysis were prepared by grinding 5 g fresh guava leaves in 10 mL of ethanol. The resulting mixture was centrifuged at 4000 rpm for 30 min. The supernatant was subsequently used in Gas Chromatography-Mass Spectrophotometry (GC-MS). Extracts for cytotoxic and genotoxic evaluation were prepared by boiling pre-weighed (20, 40, 60, 80 and 100 g) fresh leaves in 1L of distilled water for 15 min. The extracts were filtered through a muslin cloth to trap particles. The concentrations of extracts obtained were taken as 20, 40, 60, 80 and 100 g/L. Tap water was used in extract preparation and as the control (0 g/L). Extracts were freshly prepared prior to treatments throughout the duration of the experiments.

Phytochemical Analysis

Phytochemical analysis was carried out in GCMS-QP2010 ULTRA (Shimadzu, Japan) with an AOC-20s autosampler (Shimadzu, Japan) and AOC-20i autoinjector (Shimadzu, Japan) using 1 µl of crude ethanol extracts in Rtx5MS column. GC condition consisted of the following parameters: column oven temperature, 40 °C; injection temperature, 250 °C; injection mode, splitless; gas type, helium; oven temperature programme, 40 °C, 2 min hold time, 6 min /°C, 280 °C, 2 min hold time. Mass spectrophotometry condition consisted ion source temperature 200 °C, interference temperature 250 °C and scan mode 40 m/z – 550 m/z. Chromatogram peaks were analysed against NIST/EPA/NIH mass spectral library (NIST11 MS Version 2).

Allium Assay

The assay was done according to modifications of Williams and Omoh (1996) method. Onion bulbs were used in the assay with five replicates. The outer scales and the dead dry roots of the brownish part of the onion bulbs were removed and scrapped aseptically to expose the apices of the root primordia. Glass jars were filled to the brim with tap water and the base of the bulbs (exposed apices) placed on the mouth of the filled glass jar to allow root growth. After 48 h, the bulbs were transferred to glass jars filled to the brim with freshly prepared leaf extracts. The experiment was monitored over a period of 48 h. Root growth inhibition was determined by monitoring the growth of a marked root per replicate in treatment and the control. The percentage root growth was calculated as follows:

(Mean root length of treatment / Mean root length of control) x 100
Two slides were prepared per replicate at 4, 6, 12, 18, 24, 36 and 48 h to score chromosomal aberrations. Photomicrographs of normal mitotic phases and aberrations were taken using Wild photo automat microscope with MPS 55 photo system. Mitotic index and depression were also calculated according to Salaam *et al.* (2016).

Data Analyses
Descriptive statistics and charts were done in Microsoft Excel v2007. Multivariate analysis of variance (MANOVA) and Tukey range test were used to determine the level of significance ($p \leq 0.05$) of mitotic index values over treatment and time in IBM SPSS Statistics version 20, 2018.

RESULTS

Phytochemical analysis by GC-MS revealed an abundance of terpene compounds in ethanol extracts of *Psidium guajava* leaves. Phytochemicals present were terpenes (17.7 %), sesquiterpenes (33.3 %), terpenoids (2 %), sesquiterpene alcohol (33.3 %), fatty acids (7.8 %) and sterols (5.9 %). The prominent peaks correspond to the following compounds, peak numbers in parentheses: caryophyllene (6), β-curcumene (12), β-bisabolene (17), α-bergamotene (18), β-sesquiphellandrene (20), α-farnesene (21), β-bisabolol (27), α-bisabolol (28), phytol (30), linolenic acid (32) and β-stigmasterol (33) (Figure 1).

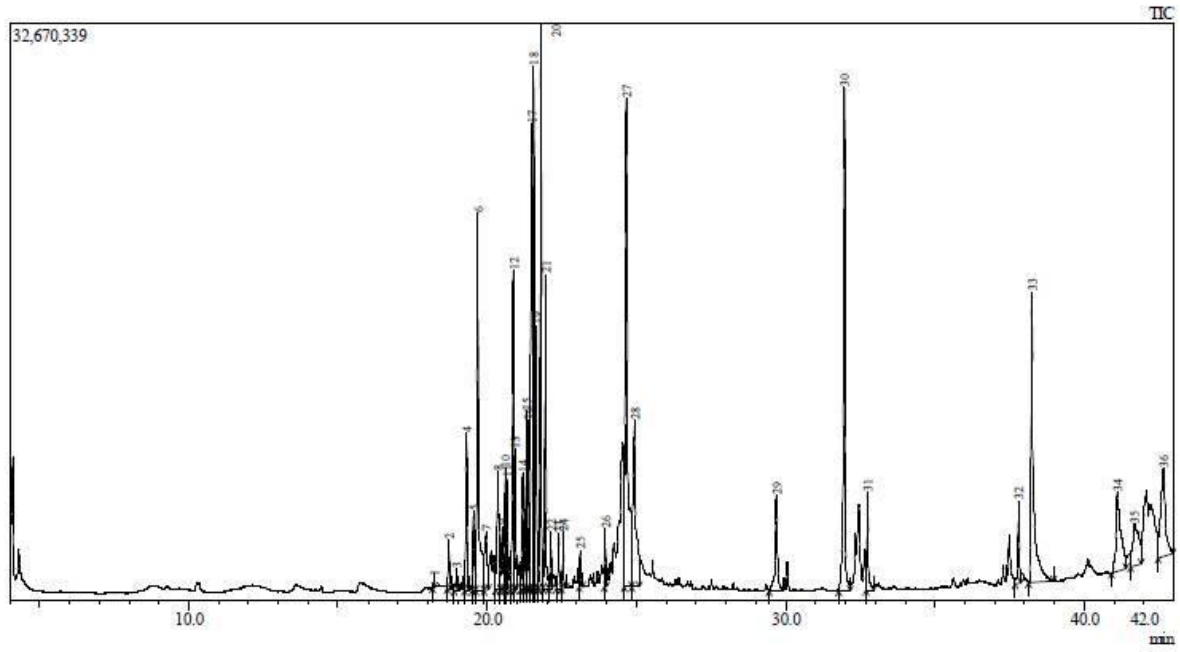
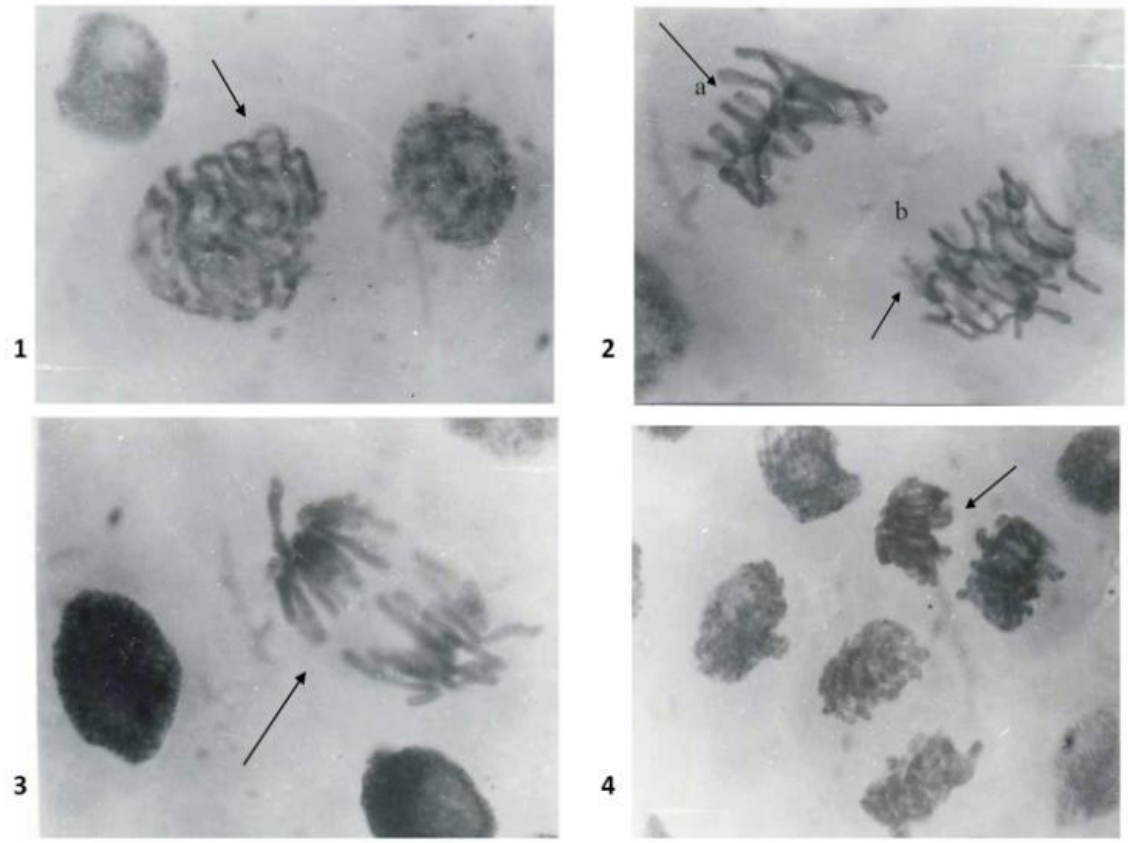


Figure 1: GC-MS Chromatogram of *Psidium guajava* Ethanol Leaf Extracts.

The untreated root tips of *Allium cepa* produced cells with normal mitotic phases i.e. prophase (Plate 1), metaphase (Plate 2a), anaphase (Plate 2b and 3) and telophase (Plate 4) with the chromosomes showing normal behaviours in each division phase.



Plates 1 - 4: Normal Mitotic Division Phases.
1 – Prophase; 2a – Metaphase; 2b – Early anaphase; 3 – Late Anaphase; 4 – Telophase.

The effects produced by the aqueous leaf extract of *Psidium guajava* on *Allium. cepa* were apparent at both microscopic and macroscopic levels affecting the root tip cells and root architecture, respectively. A decline in root growth was obsevered with increasing concentration (Table 1).

Table 1: Percentage Growth of *Allium cepa* Roots in Aqueous Extracts of *Psiduim guajava* Leaves

Concentration (g/l)	Mean Root Lengths (cm)	% Growth
0 (Control)	0.70 ± 0.18	100.0
20	0.58 ± 0.07	82.9
40	0.48 ± 0.07	68.6
60	0.30 ± 0.09	42.9
80	0.25 ± 0.05	35.7
100	0.18 ± 0.05	25.7

The value of EC50 was estimated at 54.4 g/l from the root growth curve (Figure 2).

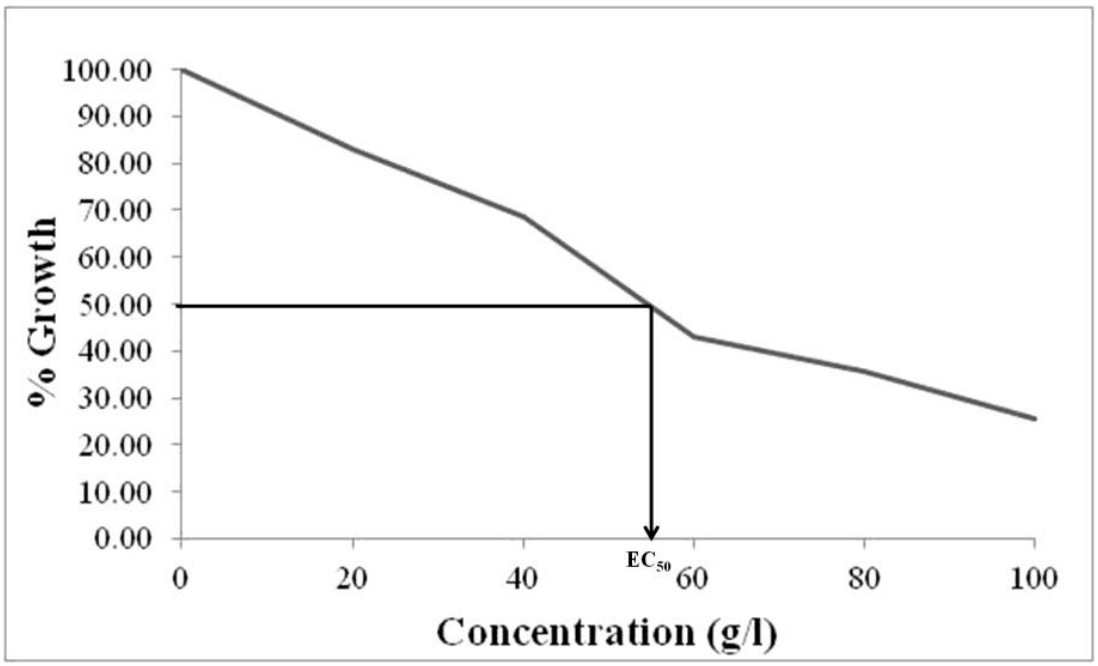


Figure 2: Growth Curve of *Allium cepa* Roots in Aqueous Leaf Extracts of *Psidium guajava*.

A change in root colour from the normal white to varying degrees of brown (light brown to dark brown) was observed with increasing concentration and treatment time. A deviation from the straight to bent shape was observed at 24 h onwards in 40 g/L, 18 h onwards in 60 and 80 g/L and 6 h onward in 100 g/L. An overall significant and concentration-time dependent reduction in mitotic index was observed in all treatments (Figure 3). Similarly, a sharp mito-depression was recorded in all treatments between 4 h and 18 h.

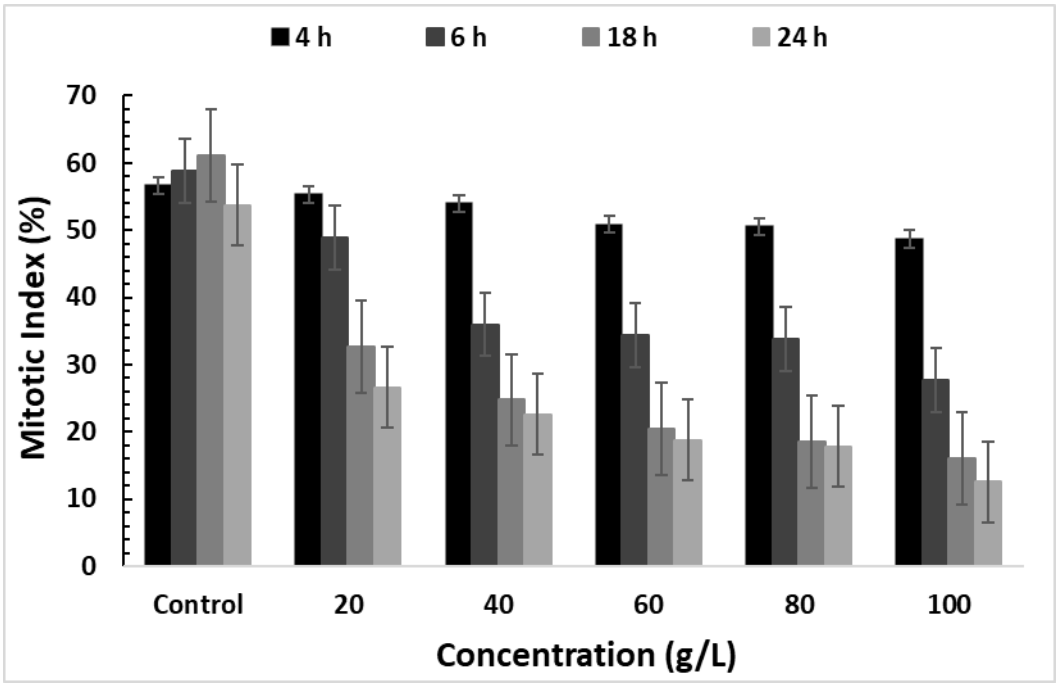
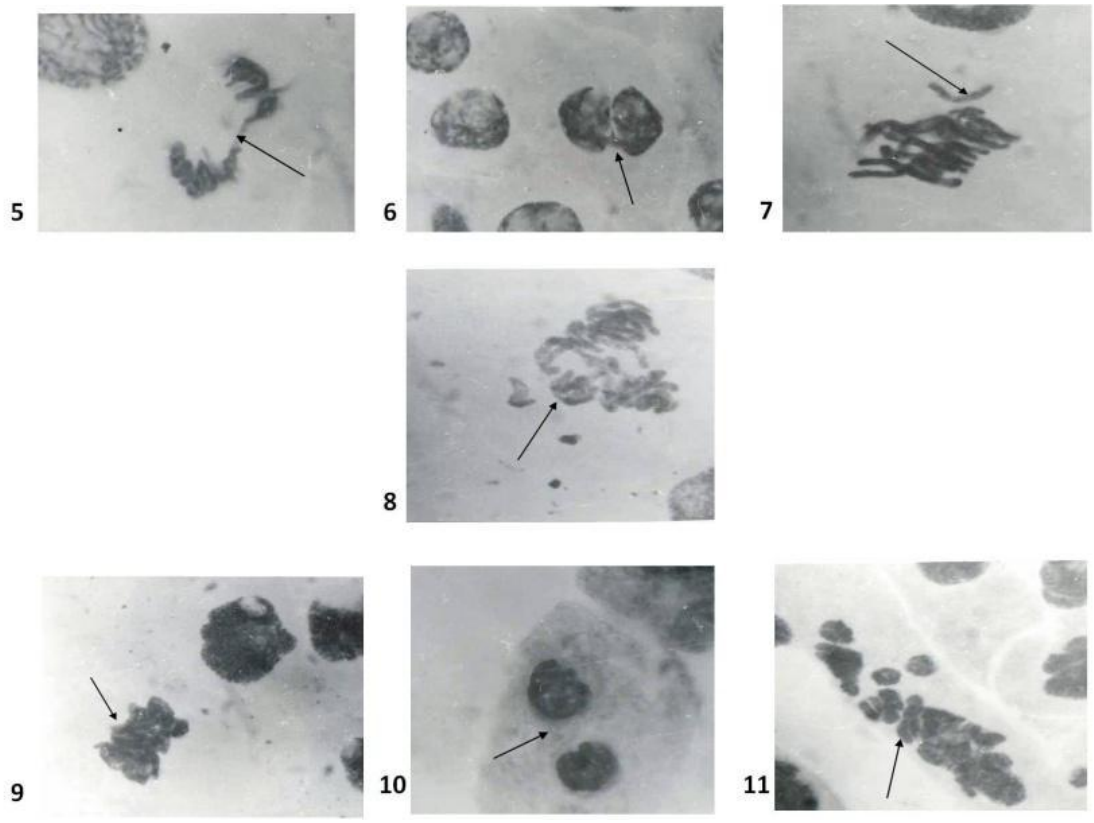


Figure 3: Effect of Concentrations of *Psidium guajava* Aqueous Leaf Extracts on Mitotic Index of *Allium cepa* Root Tip Cells.

Examination of slides revealed several chromosomal aberrations including bridges (Plates 5 and 6), vagrant chromosomes (Plate 7), distorted anaphase (Plate 8), clumping (Plate 9), binucleation (Plate 10) and c-mitosis (Plate 11) in treated *Allium cepa* root tip cells.

Complete mitotic arrest was observed in 100 g/L after 48 h. There were no dividing cells and chromosomes appeared clumped. Anaphase and telophase bridges were frequently seen in cells of root tips treated with 100 g/L at 4 h. Vagrant chromosome was seen in roots treated with 100 g/L of leaf extract for 4 h. Anaphase distortion was observed in 60 g/L of treatments at 4 and 18 h. Clumping was observed at 60 g/L treated cells and in 100 g/L treatment at 4 and 6 h. Binucleated cells were seen in slides prepared from roots treated with 60 g/L and 80 g/L of leaf extract but most frequently at 24 h. Colchine mitosis (c- mitosis) was seen in roots treated with 60 g/L of extract for 6 h. The frequency of chromosomal aberrations can be summarized thus: bridge formation > binucleation > clumping in examined cells. The aberrations were also frequently observed only in treatment with high concentrations of extract (60, 80 and 100 g/L).



Plates 5 - 11: Chromosomal Aberrations Induced by Treatment with Aqueous Leaf Extract of *Psidium guajava* (shown by the arrow).
5 – Anaphase bridge; 6 – Telophase bridge; 7 – Vagrant; 8 – Distorted anaphase; 9 – Clumped chromosomes; 10 – Binucleate cell; 11 – C-mitosis.

DISCUSSION

Phytochemical screening of the ethanol extracts of mature *Psidium guajava* leaves showed the abundance of compounds such as caryophyllene, β-curcumene, β-bisabolene, α-bergamotene, β sesquiphellandrene, α-farnesene, β-bisabolol, α-bisabolol, phytol, linolenic acid, β-stigmasterol. Some of these compounds like β- caryophyllene, phytol, α and β bisabolol, stigmasterol are associated with the medicinal properties involved in its antitumor, antimicrobial, anti-inflammatory and gynaecological uses, respectively (Kadir and Barry, 1991; Amiel *et al.*, 2012; Ghaneian *et al.*, 2015; Martin *et al.*, 2015; Sriraman *et al.*, 2015). The presence of phytol detected in the ethanol extracts has been implicated in hepatotoxicity in mice (Mackie *et al.*, 2009). Other compounds such as stigmasterol, β- caryophyllene and α-bergamotene have also been shown to be potentially cytotoxic, when used in doses beyond safety recommendations (Martin *et al.*, 2015; Sriraman *et al.*, 2015). Some of these compounds may be associated with the cytotoxic and genotoxic effects observed in the study.

The microscopic examinations of the treated onion root tips revealed that aqueous extract of *Psidium guajava* affects the chromosome and the spindle apparatus of *Allium cepa*. These microscopic examinations are important because they allow for the assessment of chromosomal damages and cell division disturbances, thus providing additional information as to the severity of the toxic effects of the leaf extracts (Luber *et al.*, 2015). The formation of binucleated cells in treated root tip cells are most probably the result of cell plate formation suppression by *Psidium guajava* aqueous leaf extracts. Suppression of cell plate formation results in inhibition of cell division without chromosome or nuclear division being affected. This increases the risk of polyploidy and rate of multinucleate cell formation in successive divisions (Kihlman, 1966; Salaam *et al.*, 2016). Binucleation amongst other aberrations was also reported by Ofodile *et al.* (2013) upon exposure of *Allium cepa* roots to ethanol extracts of *Psidium guajava* leaves.

The c-mitosis effect observed in this study was probably due to the inhibition of spindle formation. When spindle formation is inhibited, both cell and nuclear divisions are prevented. Furthermore, there is no interaction between the centromere and the spindle hence, no arrangement on an equatorial plate (Kihlman, 1966; Salaam *et al.* 2016). Clumping of metaphase chromosomes induced by *Psidium guajava* extracts has also been reported by Williams and Omoh (1996) with *Cymbopogon citratus* on *Allium cepa* roots. Clumping occurs due to fragmentation of chromosomes which creates sticky ends aggregating into thick lumps in the cell (Salaam *et al.*, 2016). The presence of clumping and c-mitosis due to the aqueous leaf extracts of *Psidium guajava* suggests a cytotoxic effect and an aneugenic mechanism (Luber *et al*., 2015). Clumping at high concentrations recorded in this study is contrary to similar observation at the low concentration of extracts by Ofodile *et al.* (2013). This discrepancy might be due to the differences in extraction methods used by these authors. Anaphase distortion is characterized by the disorderly separation of chromosomes which is caused by the partial disruption of the spindle fibers by the extracts (Soy and Yang, 1993).

A vagrant chromosome is usually seen in a cell at metaphase and its presence indicates that there is no interaction of the centromere of such a chromosome with the spindle. This increases the risk of anueploidy as such chromosome will be lost during the next division cycle (Williams and Omoh 1996; Salaam *et al.*, 2016). The presence of telophase and anaphase multi-bridges suggests a clastogenic effect which is an indication of mutagenicity of the extracts. Clastogenic effects are the results of breaks in chromosomes thus, the bridges could have been caused by breaks in chromosomes followed by rejoining of the broken ends (Luber *et al.*, 2015). The concentration time-dependent reduction in mitotic index shows that the extracts have a strong inhibitory effect on cell division in *Allium cepa* roots. Furthermore, the anti-mitotic effect observed is apparent from decrease in root growth rate. These findings agree with the works of de Rosangela *et al.* (2003) with infusions of *Psidium guajava* leaves on *Allium cepa* roots. They showed that the reduction in mitotic index also increased with increase in concentration and duration of treatment. This effect had been shown to occur with water extracts of *Pulicaria crispa* on *Allium cepa* roots (Shebab, 1999). The various chromosomal aberrations induced by aqueous leaf extracts of *Psidium guajava* on *Allium cepa* roots indicate that it is cytotoxic and genotoxic. Its indiscriminate use should, therefore, be discouraged. Further experiments with other test systems to determine how this toxicity affects the physiology and functionality in animals are encouraged.

CONCLUSION

Extracts of *Psidium guajava* leaves are rich in several beneficial phytochemicals but also possess cytotoxic and genotoxic potentials which should be seriously considered in its ethnomedicinal uses in the treatment and management of diseases.

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