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Genotoxic and antimicrobial studies of the leaves of *Psidium guajava*

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Abstract

Background: The guava, *Psidium guajava* is one of the most gregarious of fruit trees, of the Myrtaceae family. The leaf of *P. guajava* is a common herb used in the treatment of diarrhea in Nigeria and this has generated special interest in the probable antimicrobial and genotoxic effects of the leaf. However the mode of action of the leaf extracts has not been reported, hence the genotoxicity study.

Material and Methods: Antimicrobial activity of the aqueous and ethanolic extracts of the leaves of *Psidium guajava* on *Aspergillus fumigatus, Candida albicans, Salmonella* spp., and *Staphylococcus aureus* were investigated using agar-well method and also subjected to phytochemical screening and Gas chromatography-Mass spectroscopy analysis. General toxicity and genotoxic effects of the aqueous leaf extracts (0.01 g/mL, 0.03 g/mL, 0.06 g/mL and 0.08 g/mL) of *P. guajava* on *Allium cepa* root tips were also investigated using aceto-orcein squash technique.

Results: Results showed that both aqueous and ethanolic extracts of guava leaf inhibited the growth of the bacteria and fungi tested. The ethanolic extract showed stronger inhibition than the aqueous extract against the organisms. A total of forty one compounds were identified in guava leaves using GC-MS analysis and these substances were found to be essential oils. The cytological effects at low concentration included mainly c-mitosis, vagrant chromosomes, chromosome bridges, and binucleate cells with EC_{50} of 0.02 g/mL.

Conclusions: The antimicrobial activity of the essential oils from the extracts of leaves of *P. guajava* could be partly due to alterations associated with the cell division as deduced from the results.

Keywords: Antimicrobial, genotoxicity, guava microorganisms, mitotic index, *Psidium guajava.*

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INTRODUCTION

Guava is a plant in the Family Myrtaceae, genus *Psidium,* which contains about 100 species of tropical shrubs and small trees. *Psidium guajava* L. has a long history of folk medicinal uses in Nigeria and worldwide as a cough sedative, an anti-diarrheic, antioxidant, in the management of hypertension, obesity and in the control of diabetes mellitus (Karawya et al. 1999, Abdelrahim et al. 2002, Ojewole 2006, Ayub et al. 2010). The leaf of the plant has also been reported to be antimalarial (Rattanachaikunsopon and Phumkhachorn 2010), antiinflammatory and antitumor generation (Chen et al. 2007). Tea made from its leaves or buds is indicated for anti-diarrhea and also antiseptic (Teixeira et al. 2003). The leaves are rich in flavonoids, in particular, quercetin and much of guava's therapeutic activity is attributed to these flavonoids (Metwally et al. 2010, Rattanachaikunsopon and Phumkhachorn 2010).

The *Allium cepa* L root system is extremely useful in biological testing and the rot tips are often the first to be exposed to chemicals spread in nature, in soil and water. Observation of the root tip system therefore constitutes a rapid and sensitive method for environmental monitoring (Rank 2003, Majer et al. 2005). Cytotoxicity and environmental pollution (El-shahaby et al. 2003) have been assessed by the in vivo onion *(A. cepa)* root tip cell test, which is known to give similar results with in-vitro animal cytotoxicity tests (Teixeira et al. 2003). The test have been shown to have a good correlation with tests in other living systems, hence, results obtained from *Allium* test are usually handled with

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care, because, it could serve as an indicator of toxicity of the test materials (Fiskesjo 1997). Increasing drug resistance by microorganisms has resulted in constant search for antibiotics from biological materials in recent times (Ofodile et al. 2005, Ofodile et al. 2011). Previous reports have attributed the activity of *P. guajava* to their phytochemical attributes. Settheetham and Ishida (1995) reported genotoxic effect of guava leaf on human cells in vitro using established human cell line in Thailand but there seem to be limited report on the genotoxicity of the leaf using *Allium cepa* test. This paper reports the antimicrobial activity and in¬ vivo onion root tip tests to investigate the genotoxic potentials of the extracts of the leaves of *P. guajava.*

MATERIALS AND METHODS

Collection and identification of plant material

Guava leaves were collected from the Botanical garden of Yaba College of Technology, Yaba, Lagos. The fresh plants were rinsed and dried completely for about five days at room temperature before using them for this study.

Preparation and extraction of plant material

The plant leaves were ground with an electric blender and ground sample (100 g) was added to 200 mL of distilled water and extracted at room temperature for 24 h and was filtered using Whatman No 1 filter paper under aseptic condition to obtain the water extract. The filtrate obtained was concentrated using a rotary evaporator. The extract was then collected in fresh sterile universal bottles and was stored in the refrigerator at 4°C. For ethanol, extract ground leave sample (100 g) was soaked with 200 mL of ethanol and was extracted for 24 h and filtered using Whatman No.1 filter paper. The filtrate was concentrated by evaporation of solvent at room temperature and placed in the holding chamber of the Soxhlet extractor. The extract was collected into a fresh sterile universal bottle and stored at 4°C. The water extract was reconstituted in water to obtain concentrations (50 mg/500 uL, 75 mg/500 ML and 100 mg/500 uL) while the ethanolic extract was constituted in ethanol to obtain concentrations (50 mg/500 uL, 75 mg/500 uL and 100 mg/500 uL). For the genotoxicity studies, the fresh leaves were weighed and washed with tap water and blended with 500 mL of tap water. The blended materials were then filtered with a sieve to obtain the filtrate, which were kept in the refrigerator until use. This preparation is considered as the stock solution. Concentrations of the stock solution were estimated using gravimetric methods. Serial dilutions were made from the stock solution for the experiment at the various required concentrations.

Antimicrobial assay

Extracts obtained were evaluated for antibacterial and antifungal activity according to agar diffusion method used by Doherty et al. (2010). Fifteen millilitres of sterile nutrient agar and potato dextrose agar each was poured into Petri dish and was allowed to solidify. One millilitre of the dilution factor (10^{-6}) of organisms was poured on the surface of the plates and then spread using a spreader to distribute the bacteria and fungi evenly on the media. The media was then allowed to stay for some minutes. A cork borer (8 mm diameter) was sterilized by flaming and used to prepare wells on opposite sides of the plates. The wells created were then filled with the plant extracts, the plates were allowed to stand for 1 h for the pre-diffusion of the extracts and the incubation was done at 37°C for 24 h.

The *Allium* test

Twenty *A. cepa* bulbs were divided into five groups (4 bulbs in each group, the last group being the control). The bulbs were then rooted in the extract for 96 h. After 48 h five root tips from each bulb were harvested and fixed in ethanol; glacial acetic acid (3:1) and slides for microscopic studies were prepared from these fixed rot-tips using the acero-orcein squash technique. After 96 h five (5) root tips from each onion bulb were measured and their root lengths recorded (Fiskesjo 1985).

Preliminary phytochemical tests

Preliminary phytochemical screening was carried out according to the method of Harbone (1973) and the method used by Ofodile et al. (2009). The water and ethanolic extracts of the leaves of the plant were also screened for the presence of alkaloid,

tannins, saponins, phlobatannins, glycosides, anthraquinones, saponins and steroid.

Gas chromatography - Mass spectroscopy (GC-MS) analysis

The instrument used during this analysis was the GC-MS model Agilent Technologies 7890A and MS Agilent Technologies 5975C at the Instrumentation Laboratory of the Department of Chemistry, University of Lagos. For each extract, 5 g of the sample was placed in a separating funnel and 25 mL of hexane was added, the mixture was vigourously shaken.

The mixture was concentrated by exposure to air for 5 h to concentrate the analyte, then the water in the sample was eliminated by using anhydrous sodium sulphate in a Pastuer pipette with cotton wool as the separating medium into the vial bottle ready for GC analysis whereby the sample is then injected into the sample compartment while the Helium gas which is the mobile phase pushes sample into column and the column being the stationary phase does separation at different retention time. Initial temperature condition was 60°C to hold for 2 min, at the temperature of 40°C to the final temperature of 240°C to hold for 2 min. Injection volume was 1 uL, pressure: 12.936, psiseptum purge flow: 5 mL/min, mode: splitters using column: HP 5-MS, Length: 30 m, I.D: 0.320, Film: 0.25.

RESULTS

Results of the antimicrobial activity of the aqueous and ethanolic extracts of the leaf of *P. guajava* are shown on Tables 1, 2 and 3 while genotoxic effect of the leaf are shown on Tables 4 and 5.

The lowest mean zone of inhibition of aqueous extracts of guava leaf was against *Candida albicans* at the concentration of 50 mg/500 uL while it was most potent at 100 mg/500 uL and against *Salmonella* spp. The aqueous extract of *P. guajava* showed slightly stronger activity against the bacteria than the fungal organisms. Activity of the crude water extract also increased with time and the effect on the organisms were comparable to the activity of chloramphenicol on the organisms Table 1.

The ethanolic extract of the leaf completely inhibited the growth of *Staphylococcus aureus* at 100 mg/500 uL. *Aspergillus fumigatus* was the least susceptible organism to this extract and at the concentration of 50 mg/500 uL and its activity against *S. aureus* and *Salmonella* spp. were comparable to the control substance. Activity of the ethanolic extract on the organisms also increased with time and the crude extract was more potent against bacteria than the fungi tested. The ethanolic and the crude water extracts of guava leaf showed stronger antibacterial than antifungal activity for the period of the experiment Table 2.

Preliminary phytochemical screening of crude extract of the leaves of guava revealed the presence of saponins, tannins, steroid and glycosides. The presence of phytoconstituents is reported in Table 3. The result of the Gas chromatography and mass spectroscopy (GC-MS) of the methanol extract of the leaf of guava showed 41 compounds. The chromatogragh showed that the compound Cyclohexene, 1-methyl-4-(5-methyl-1-methylene-4-hexenyl) detected at the retention time of 20.837 mins was the most abundant substance with 13.021% followed by Cyclohexene, 3-(1, 5-dimethyl-4-hexenyl)-6-methylene at the retention time of 21.299 mins with 11.331%. The least abundant compound detected at the retention time of 23.113 mins and was characterised as 2-Acetylbenzoic acid with 0.34810%.

The results of the macroscopic parameters studied are shown in Table 4. The pattern observed here showed a concentration-dependent root growth inhibition. Data from microscopic studies are shown in Table 5 which showed a mitotic index of 6.4% for the control. The M.I. for the other concentrations were 6.0% (0.01 g/mL), 2.0% (0.03 g/mL) 4.2% (0.06 g/mL) 2.4% (0.08 g/mL) and 11% (11 g/mL). Chromosome abberations observed ranged from vagrant chromosomes, c-mitosis, anaphase bridge binucleate cells and sticky chromosomes. The photomicrographs obtained from the microscopic studies are presented in Fig. 1. Chromosome abberations seemed to decrease with increasing concentration of the extract of *P. guajava.*

Table 1. The mean zone of inhibition of aqueous extract of guava leaf against fungi.

Testorganisms	Concentration of ethanol extract (mg/uL)	Mean zone of inhibition with guava leaf (mm)			Mean zone of inhibition with chloramphenicol (mm)		
		Day			Day		
		1	2	3	1	2	3
Aspergillus fumigatus	50/500	2.0	4.0	5.0	22.0	25.0	29.0
	75/500	4.0	7.0	8.0	23.0	28.0	33.0
	100/500	7.0	8.0	10.0	28.0	34.0	37.0
Candida albicans	50/500	8.0	9.0	14.0	20.0	23.0	27.0
	75/500	11.0	13.0	16.0	26.0	27.0	30.0
	100/500	13.0	16.0	19.0	26.0	29.0	31.0
	Concentration of aqueous extract (mg/uL)						
Aspergillus fumigatus	50/500	17.0	15.0	18.0	14.0	14.0	17.0
	75/500	13.0	10.0	13.0	15.0	16.0	19.0
	100/500	10.0	10.0	11.0	14.0	17.0	20.0
Candida albicans	50/500	8.0	9.0	12.0	12.0	15.0	16.0
	75/500	10.0	11.0	14.0	11.0	14.0	18.0
	100/500	14.0	14.0	18.0	13.0	18.0	18.0

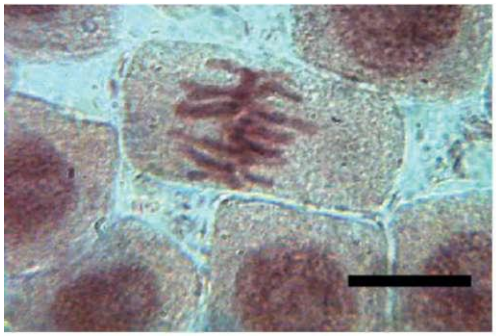
Table 2. The mean zone of inhibition of ethanol extract of guava leaf against bacterium.

Testorganisms	Concentration of ethanol extract (mg/uL)	Mean zone of inhibition with guava leaf (mm)			Mean zone of Inhibition with chloramphenicol (mm)		
		Day			Day		
		1	2	3	1	2	3
Staphylococcus aureus	50/500	18.0	19.0	22.0	19.0	26.0	26.0
	75/500	20.0	25.0	29.0	26.0	28.0	30.0
	100/500	*T.I	T.I	T.I	26.0	26.0	27.0
Salmonella species	50/500	10.0	10.0	12.0	23.0	24.0	24.0
	75/500	18.0	20.0	21.0	24.0	25.0	28.0
	100/500	18.0	21.0	24.0	27.0	29.0	32.0
	Concentration of aqueous extract (mg/uL)						
Staphylococcus aureus	50/500	11.0	13.0	15.0	14.0	15.0	18.0
	75/500	12.0	15.0	18.0	15.0	17.0	20.0
	100/500	16.0	19.0	20.0	15.0	18.0	21.0
Salmonella species	50/500	11.0	11.0	13.0	13.0	14.0	16.0
	75/500	18.0	18.0	20.0	12.0	15.0	18.0
	100/500	18.0	21.0	22.0	13.0	17.0	18.0

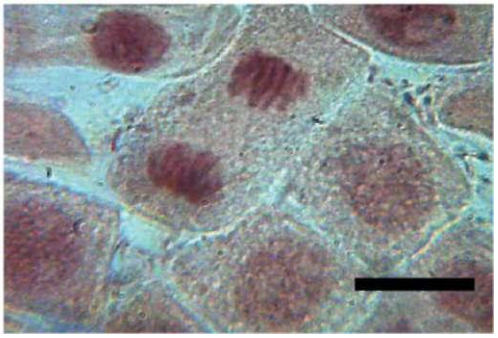
*T. I = Total Inhibition

DISCUSSION

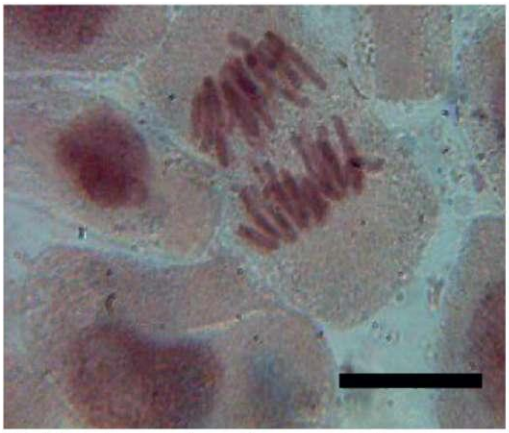
The results showed greater activity of the ethanol extract than the aqueous extracts this could be explained by the difference in compound extracted by the two solvents. In this case the more active component was extracted by ethanol. Ofodile et al. (2005) reported activity of three solvent extracts where the most active compounds were from the least polar substance. The increased inhibitory activity of the leaf extracts with time (Tables 1 and 2) could suggest bactericidal and fungicidal attributes in the extracts tested but Rattanachaikunsopon and Phumkhachorn (2007)



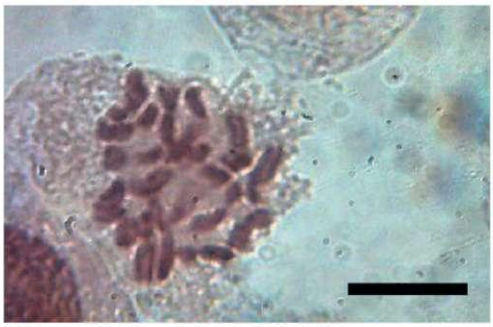
(a) Regular metaphase From control



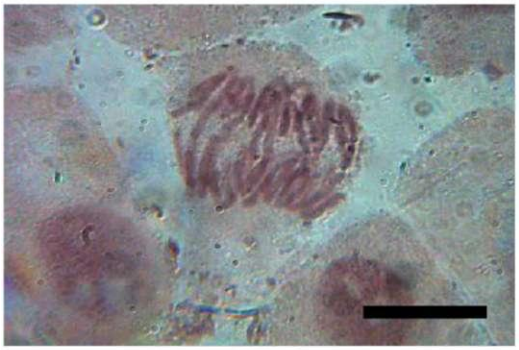
b) Regular telophase From control



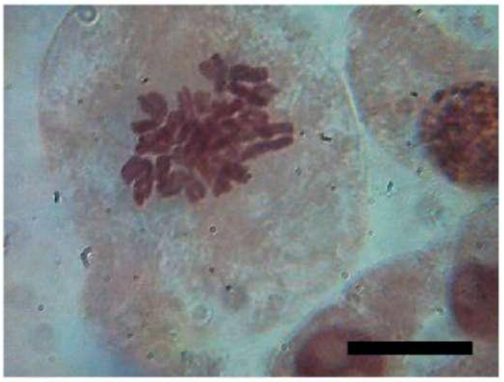
(c) Regular anaphase from control



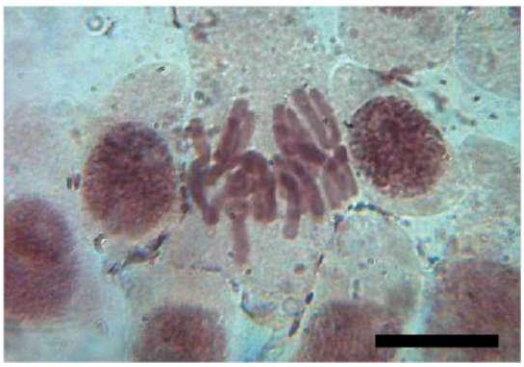
(d) c-metaphase from 0.01 g/mL



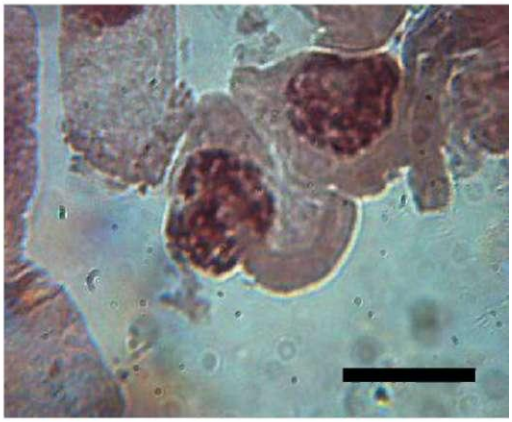
(e) Anaphase bridge from 0.01 g/mL



(f) Vagrant chromosome from 0.01 g/mL



(g) Vagrant chromosome from 0.03 g/mL



(h) Sticky telophase chromosomes from 0.06 g/mL

Fig. 1. Microscopic effects of aqueous extracts of *Psidium guajava* on *Allium* cepa root tip (Mag. x 40). Scale Bar is 50 |jm.

Table 3. Preliminary phytochemical screening of water and ethanolic extracts of the leaves of *Psidium guajava.*

Phytoconstituents	Crude extract inference	
	Water	Ethanol
Alkaloids	-	-
Saponins	+	+
Tannins	+ +	++
Phloba tannins	++	+
Anthraquinines	+	++
Glycosides	+	+
Steroid	+	+

+= presence; -= absence

Table 4. Root lengths of *Allium cepa* for different treatments of *Psidium guajava* leaf extract on Day 4.

Concentration of extract (g/mL)	Sample size	Mean root lengths ±SEM
Control 1	20	1.90±0.35
0.03	20	0.51 ±0.02
0.06	20	0.42±0.02
0.08	20	0.37±0.03
1.0	20	0.22±0.02

reported bateriostatic effect of flavonoids isolated from the leaves of this plant. The stronger antibacterial effect of the extracts of the leaf than antifungal could be because the sapponins, tannins, anthraquinines, glycosides and steroids in the leaves are more bactericidal than fungicidal. Reports on the antimicrobial activity of *P. guajava* leaf extracts were attributed to the presence of tannins, triterpenoids and flavonoids in the leaves (Q'adan et al. 2005, Arima and Danno 2002). Tested organisms, *S. aureus, Salomonella* sp. and *A. fumigatus* (Shelef et al. 1980, Rattanachaikunsopon and Phumkhachorn 2007) have been implicated in food borne poisoning and Candida albicans is a known dermatophytes (Ofodile et al. 2009). This implies that the extracts can be used as food preservative to improve the shelf life and safety of foods. Smith and Suliana (1975) and Baby and Mini (2010) also found some essential oils isolated from guava leaves to be antibiotic using different parameters.

The data obtained in these experiments, indicate that the leaf extracts of *P. guajava* at the concentrations tested resulted in a significant dose-dependent root growth inhibition. There was a good correlation between the macroscopic and cytological parameters. However, the types of chromosome aberrations varried somewhat with the concentrations of the sample. At lower concentrations, (0-01 g/mL and 0.03g/mL) c -mitosis, (and to some extent, bridges, binucleate, and vagrant chromosomes were the most common aberrations, whereas at higher concentration, (0.06, 0.08) sticky chromosome were the most common (although this was also seen at lower concentrations). Binucleate chromosomes were also found at 0.08 g/mL. Similar observation have been made by other workers who have used the *Allium* test to establish genotoxicity in other plant extracts (Nwakanma et al. 2009, Oyedare et al. 2009, Adegbite and Sanyaolu 2010). Experiments with other test systems such as *Crinum jagus* root tips have also given similar results (Nwakanma and Okoli 2010). These results suggest that the test extracts posses inhibitory and mitodepressive effects on the cell division of *A. cepa.* The inhibitory and mitodepressive activities of these extracts may probably be part of the mechanism and the mode of actions utilized in the treatment of illness. Evidence in support of this has been documented on some plant extracts with anticancer therapy (Sheng et al. 2000, Kuras et al. 2006). Some herbs may act as "immunomodulators" - stimulating the immune system to fight against cancer cells. Others may have a direct cytotoxic action - however this does not

Table 5. Chromosome aberration of the *Allium cepa* root tip cells treated with different concentrations of *Psidium guajava* leaf extracts.

Concentration (g/mL)	Total number oF cells counted	Number oF dividing cells	Dividing cells				Stickiness	C mitosis	Bridges Fragment	Vagrant	Binuclei	••••iiwi.r ™%P,°se	Attached	index (%)
			p	M	A	T								
Control	500	32		10	7	10								6.4
0.01	332	20		3	0	13	13							6.0
0.03	243	5		1	0	4	4							2.0
0.06	141	6		0	1	2	3							4.2
0.08	201	5		0	0	5	5							2.4
11.0	181	2		0	0	2	2							1.10

P= prophase, M= metaphase, A= anaphase, T= telophase

necessarily mean they should immediately be used; as an agent that has been shown to be toxic to cancer cells may also be toxic to healthy cells.. Oleuropein from *Olea europaea* was reported to affect and disrupt purified actin filaments, providing direct antitumor effects due to cell disruption (Zaid et al. 2012). According to Soltys et al. (2011, 2012), Cyanamide mode of action during inhibition of onion *(A. cepa)* root growth involves disturbances in cell division and cytoskeleton formation and inhibition of tomato *(Solanum lycopersicum* L.) root growth by cyanamide is due to altered cell division.

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Psidium guajava Yapraklarının Genotoksik ve Antimikrobiyal Açıdan İncelenmesi

Özet

Giriş: Guava, *Psidium guajava,* Myrtaceae ailesinin meyve ağaçları içerisinde en çok topluluk halinde yaşayanlarından biridir. *P. guajava* yaprağı, Nijerya'da ishal tedavisinde yaygın olarak kullanılmaktadır ve bu durum yaprağın muhtemel antimikrobiyal ve genotoksik etkisi konusunda özel bir ilgi oluşturmuştur. Ancak, yaprak ekstrelerinin etki mekanizması hakkında yayın yapılmamıştır ve bu yüzden genotoksik araştırmaya ihtiyaç duyulmuştur.

Materyal ve Metot: *Psidium guajava* yapraklarının sulu ve etanol ekstrelerinin; *Aspergillus fumigatus, Candida albicans, Salmonella* spp. ve *Staphylococcus aureus* üzerine antimikrobiyal aktivitesi agar-kuyu metodu kullanılarak araştırıldı ve ayrıca fotokimyasal fitokimyasal tarama ve Gaz kromatografisi-Mass spektroskopi analizlerine tabi tutuldu. Aynı zamanda, *P. guajava* sulu yaprak ekstrelerinin *Allium cepa* kök uçları üzerine genel toksisitesi ve genotoksik etkileri de (0.01 g/mL, 0.03 g/mL, 0.06 g/mL ve 0.08 g/mL) aceto-orcein ezme yöntemi ile araştırıldı.

Bulgular: Sonuçlar, guava yapraklarının sulu ve etanol ekstrelerinin her ikisinin de test edilen bakteri ve fungusların büyümesini engellediğini göstermiştir. Etanol ekstresi, organizmalara karşı sulu ekstreden daha kuvvetli etki sergilemiştir. GC-MS analizi kullanılarak, guava yapraklarında toplamda kırk bir bileşik belirlenmiştir ve bu maddelerin esensiyal yağlar olduğu bulunmuştur. Düşük konsantrasyonlardaki sitolojik etkileri EC_{50} 0.02 g/mL'de; c-mitoz, vagrant kromozomlar, kromozom köprüleri ve iki nükleuslu hücreler şeklindedir.

Sonuç: *Psidium guajava* yaprak ekstresindeki esensiyal yağların antimikrobiyal aktivitesinin sebebi, bulgulardan anlaşıldığı gibi, hücre bölünmesi ile ilgili değişiklikler olabilir.

Anahtar Kelimeler: Antimikrobiyal, genotoksisite, guava mikroorganizmaları, mitotik indeks, *Psidium guajava.*