ANTIBACTERIAL ACTIVITY OF AQUEOUS AND ETHANOLIC LEAVES EXTRACT OF *Psidium guajava*

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Abstract: This work was carried out to evaluate the phytochemical and antibacterial activities of *Psidium guajava* (Guava) leaf extract against clinical bacteria species including *Staphylococcus aureus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli, Streptococcus pyogenes.* The phytochemicals found in *Psidium guajava* included flavonoids, saponins, anthraquinones, glycosides, tannins, alkaloids and steroids using ethanol and aqueous as solvent of extraction. The ethanol extract was observed to be effective against the test isolates with zones of inhibition ranging from 9.4 ± 0.03 mm in *Klebsiella pneumoniae* to 18.0 ± 0.30 mm in *Escherichia coli*, at concentration 100 mg/ml while reduced activity was observed at lower concentrations. The aqueous extract was found to be less effective against the test isolates compared to the ethanol extract, with zones of inhibition ranging from 7.5 ± 0.03 mm in *Klebsiella pneumoniae* to 14.0 ± 0.30 mm in *Escherichia coli* at 100 mg/ml. Minimum inhibitory concentration (MIC) values ranged from 6.25 mg/ml against *Escherichia coli* to 50 mg/ml against *Klebsiella pneumoniae* in both extracts. Minimum bactericidal concentrations (MBC) ranged from 25 mg/ml against *Escherichia coli* and *Bacillus subtilis* to 100 mg/ml against *Klebsiella pneumoniae* in the ethanoic extract. MBC for aqueous extract ranged from 25 mg/ml in *Escherichia coli* to 100 mg/ml in *Staphylococcus aureus.* Antibiotic sensitivity pattern of the test bacterial isolates revealed varying degree of resistance and the organisms were observed to show multiple drug resistance to the drugs. From this study, *Psidium guajava* aqueous and ethanolic extracts may be promising alternative or replacement for commonly used antibiotics.

Keywords: Antibiotics, antimicrobial, resistance, sensitivity, inhibition, phytochemical

Introduction

In the present time, microorganisms have become resistance to many antibiotics due to increased use of drugs, which is decreasing efficiency of conventional medicines (Tangpu and Yadav, 2006). So, it has become necessary to find out new antimicrobial agents. Prevention of pathogenic and spoilage microorganisms in food is usually achieved by using chemical preservatives. Chemical preservatives are responsible for many carcinogenic and teratogenic attributes as well as residual toxicity. With growing concern of microbial resistance towards conventional preservatives, consumers tend to be suspicious of chemical additives and thus the exploration of naturally occurring antimicrobials for food preservations has received much attention in recent years. Presently, medicinal plants have become effective source of medicines. Traditional medicines are safe treatment for the infections originated from microbial and non-microbial origin (Ekpendu *et al*., 2004). Some antibiotics do not have capability to treat diseases because of drug resistance capacity of pathogens (Cohen, 1992). The use of herbal treatment is one of the possible ways to treat diseases caused by multi drug resistant bacteria. Though many pharmaceutical industries have produced a number of antibiotics for several years, in many cases it was observed that the cultures were showing resistance against the medicines (Ross, 2003). *Psidium guajava* is evergreen shrub native to tropical America that has neutralized in South East Asia. Various parts of guava plant have been reported to have wide range of activity against human ailments (Olajide *et al*., 1999; Ross, 2003). Over 20 bioactive compounds have been reported to be present in leaves, stems, bark and roots of *P. guajava* (Meckes *et al*., 2005). Guava leaves were used to treat diarrhoea and stomach pains. The leaves were used in USA as antibiotic in the form of poultice or decoction for wounds, ulcers and toothache.

Guava leaf contains broad spectrum of phytochemicals including minerals, enzymes, proteins (Kaneria and Chanda, 2011), sesquiterpenoid alcohols and triterpenoid acids (Haida *et al*., 2011) alkaloids, glycosides, steroids, flavanoids, tannins, saponins. Guava is very rich in antioxidants and vitamins and also high in lutein, zeaxanthine and lycopene (Ryu *et al*., 2012). Guava contains carotenoids and polyphenols, the major classes of antioxidant pigments giving them relatively high potential antioxidant value among plant foods (Metwally *et al*., 2010). In addition three flavonoids (quercetin, avicularin, and guaijaverin) have been isolated from the leaves of *Psidium guajava* (Huang *et al*., 2011). The leaves of guava are rich in flavonoids, particularly quercetin. The bark of guava tree contains considerable amounts of tannins (11-27%); hence it is used for tanning and dyeing purposes. Leucocyanidin, luectic acid, ellagic acid and amritoside have been isolated from the stem bark. Five constituents, including one new pentacyclic triterpenoid: guajanoic acid and four known compounds beta-sitosterol, uvaol, oleanolic acid and ursolic acid, have been recently isolated from the leaves of *P. guajava* by Bontempo *et al*. (2012).

Guaijaverin has high potential antiplaque agent by inhibiting the growth of the *Streptococcus mutans*. Avicularin and guaijaverin work as urease inhibitors (against *Helicobacter pylori* urease) (Shanmugam *et al*., 2012). In several studies, guava showed significant antibacterial activity against common food borne diarrhea-causing bacteria such as *Staphylococcus* spp., *Shigella* spp., *Salmonella* spp., *Bacillus* spp., *E. coli*, *Clostridium* spp., and food spoilage bacteria such as *Pseudomonas* spp. (Hidetoshi and Danrio, 2002; Abdeirahirn *et al*., 2002). Guava is also used medicinally in many parts of the world as anti-inflammatory and antiseptic as well as in the treatment of diabetes, hypertension, pain, fever, respiratory disorders, gastroenteritis, diarrhea and dysentery (Gutiérrez *et al*., 2008). In recent years, the human population has become increasingly interested in medicinal plants due to their less toxicity, cost effectiveness, easy accessibility and presence of nutraceuticals. As a result, many researchers have over the last decades worked on the medicinal properties of different plants in different parts of the world (Barbalho *et al*., 2012). This quest has been aroused particularly due to

emergence and re-emergence of drug resistant pathogens in the community and clinical settings. Therefore, this research work was carried out to analyze the phytochemicals present in *Psidium guajava* and to evaluate the antibacterial activities of the ethanolic and aqueous extract of *Psidium guajava* leaves.

Materials and Methods

Sample collection and preparation

Guava leaves were collected from the tree around Ugbowo area in Benin City, Edo State. The leaves were identified in the Department of Plant Biology and Biotechnology, University of Benin. The leaves were dried and powdered. Fifty grams (50 g) of the powered leaves was weighed into bottle and 500 ml of distilled water was added. The same was done for ethanol. This was to carryout aqueous and ethanol extract respectively. The plant materials were soaked in the respective solvent for 24 h and then filtered. The respective filtrate was concentrated to get the crude extract from which different concentrations were prepared. All extracts were stored at 4°C when not in use.

Alkaloid test

Exactly 5 g each of the guava extract and 5 ml of honey was stirred with 5 ml of 1% aqueous hydrochloric acid on a steam bath. 1ml of the filterate was treated with few drops of Draggen doffs reagent. Blue black turbidity serves as preliminary evidence of alkaloids.

Saponin test

Five gram (5 g) each of extract and 5ml of honey was shaken with distilled water in a test tube. Frothing which persist on warming was taken as preliminary evidence for saponin.

Tannins

Five gram (5 g) each of extract and 5 ml of honey was stirred with 100 ml distilled water and filtered. Ferric chloride reagent was added to the filtrate. Formation of blue-black or blue-green precipitate indicated the presence of tannin.

Phlobotannin test

Disposition of red precipitate when an aqueous extract of the test sample was boiled with 1% hydrochloric acid served as evidence for the presence of phlobotannin.

Flavonoid test

Five milliliter (5 ml) of diluted ammonia solution was added to aqueous filtrate of the samples followed by the addition of concentrated H_2SO_4. A yellow colouration observation was taken as an evidence for the presence of Flavonoids.

Cardiac glycosides (Keller-Killiani test)

Five gram (5 g) of each of the extract and 5 ml of honey was dissolved in 2 ml glacial acetic acid containing a drop of ferric chloride solution. 1ml of concentrated H_2SO_4 was added. A browning of the interface indicated the presence of deoxy-sugar characteristic cardenolids. A violet ring may appear below the brown ring, while in the acetic acid layer, a green ring may form, just gradually spread throughout this layer.

Steroids

Two milliliter (2 ml) of acetic anhydride was added to 0.5 g of extract and 2 ml of sulphuric acid was added by the sides of the test tube which was then observed for colour change from violet or blue-green.

Terpenoids (Salkowski test)

To 0.5 g of the extract, 2 ml of chloroform was added, concentrated H_2SO_4 (3 ml) was carefully added to form a layer. A reddish brown colouration of the interface indicated the presence of terpenoids.

Test microorganisms

Pure cultures of *Esherichia coli, Bacillus subtilis, Staphylococcus aureus, Klebsiella pneumoniae* and *Streptococcus pyogenes* were collected from Microbiology laboratory, University of Benin Teaching Hospital (UBTH). Each isolates was subjected to standard morphological and biochemical technique for identification in Microbiology laboratory, University of Benin, Benin City. The morphological and biochemical tests included gram staining, motility test, oxidase test, catalase test, coagulase test and indole test.

Preparation of culture media

The media used were nutrient agar and nutrient broth and were prepared according to manufacturer's instruction.

Antimicrobial susceptibility testing

Preparation of different concentrations

Following the method of Ekwenye and Elegalam (2005), concentration of l00 mg/ml of the extract was prepared by dissolving 0.1 g of the extract in 1ml of sterile water. Then concentrations of 50, 25, 12.5 and 6.25 mg/ml were prepared from the stock concentration (l00 mg/ml) by double dilution procedure.

Bacteria inoculum preparation

The inocula were prepared by inoculating the test organisms in nutrient broth and incubating them for 24 h at 37°C. After incubation, 1 ml of the cultures was inoculated onto solidify nutrient agar at 45°C using a Pasteur pipette.

Evaluation of antibacterial activity

The antibacterial and antifungal activities of the test sample were done using the Kirby- Bauer method also known as disk diffusion method. The organisms were inoculated into the Petri dishes containing nutrient agar, potatoes dextrose agar was used for the fungus. The disks were then placed appropriately on the surface of the agar plate by using a sterile forceps. The plates were inverted and placed in an incubator at 35ºC within 15 min after disks were applied.

The plates were incubated aerobically and examined after 24 h of incubation. Each plate was examined, and the diameter of the zones of complete inhibition was measured to the nearest centimeter using a ruler.

Determination of minimum inhibitory concentration (MIC) and MBC

The MIC of the plant extracts was determined for each of the test organisms at varying concentrations of 100, 50, 25, 12.5, 6.25 and 3.125 mg/ml). One milliliter (1 ml) of each of the concentration was added in a test tube, 1 ml of nutrient broth was added and then a loopful of the test organism previously diluted to 0.5 McFarland turbidity standard was introduced to the tubes. A tube containing nutrient broth only was seeded with the test organism to serve as control. All the tubes were then incubated at 37ºC for 24 h and then examined for growth by observing for turbidity. The minimum bactericidal concentration (MBC) of the plant extract on the clinical bacterial isolates was carried out according to Ajaiyeoba *et al.* (2003). Briefly, 1 ml bacterial culture was pipetted from the mixture obtained in the determination of MIC tubes which did not show any growth and sub-cultured on to nutrient agar plate. The plate was incubated at 37ºC for 24 h. After incubation the concentration at which there was no single colony of bacteria was taken as MBC**.**

Antibiotics susceptibility pattern

Antimicrobial disc tests of the isolates were performed using the following antibiotic discs: tetracycline (20 µg), ampiclox (30 µg), zinnacef (20 µg), amoxicillin (30 µg), rocephin (25 µg), ciprofloxacin (l0 µg), streptomycin (30 µg), erythromycin (l0 µg), gentamycin (I0 µg), septrin (30 µg), chloramphenicol (25 µg), perfloxacin (l0 µg), and ofloxacin (30 µg) and antibiotics resistance was interpreted by diameter of inhibition zones around the antibiotic discs.

Results and Discussion

The phytochemicals that were present in in the leaves of *Psidium guajava* include flavonoids, saponins, anthraquinones, glycosides, tannins, alkaloids and steroids (Table 1). This finding is in agreement with the findings of Arima and Danno (2002) who also isolated these compounds from *Psidium guajava* leaves. These compounds have been reported to mediate important processes such as antioxidant, anti-inflammatory, analgesic and antimicrobial properties. Hence, the presence of these compounds in *Psidium guajava* may suggest the medicinal claim of this plant that plays functional role in therapeutic world.

Table 1: Phytochemical analysis of aqueous and ethanolic leaves extracts of *Psidium guajava*

Phytochemicals	*Aqueous Extract*	*Ethanol Extract*
Flavonoids	+	+
Tannins	-	+
Cardiac glycosides	+	+
Reducing sugars	+	+
Terpenoids	+	+
Saponins	+	+
Anthraquinones	+	+
Alkaloids	+	+
Steroids	+	+

+= Present; -= Absent

Table 2: Antibacterial activity of ethanolic leaves extract of *Psidium guajava*

Test organisms	Concentrations (mg/ml)				
	100	50	25	12.5	6.25
S. aureus	14.1±1.3	10.0±0.05	7.0 ±0.01	0.0 ±0.00	0.0±0.00
K. pneumoniae	9.4 ±0.03	6.0 ± 0.01	0.0 ±0.00	0.0 ±0.00	0.0±0.00
B. subtilis	12.0±0.10	10.5±0.04	8.4 ±0.10	4.0 ±0.06	0.0±0.00
E. coli	18.0±0.30	14.0±1.10	11.0±0.09	7.0 ±0.00	0.0±0.00
S. pyogenes	15 ± 0.10	12.2±0.25	11.0±0.30	10.3±0.02	6.0±0.01

The ethanolic and aqueous extract of *Psidium guajava* were assayed for their antibacterial activity using disk diffusion method and were found to be effect against the test bacteria (Table 2). The ethanol extract was observed to be effective against the test isolates with zones of inhibition ranging from 9.4 ± 0.03 mm in *K. pneumoniae* to 18.0 ± 0.30 mm in *E. coli*, at concentration 100 mg/ml while reduced activity was observed at lower concentrations with marked resistance at concentration of 12.5 mg/ml and below. This finding corroborates the work of Kaneria and Chanda (2011) who reported sensitivity of these bacteria to *Psidium guajava* leaves extract at similar concentrations.

The aqueous extract was found to be less effective against the test isolates compared to the ethanol extract with zones of inhibition ranging from 7.5 ± 0.03 mm in *K. pneumoniae* to 14.0 ± 0.30 mm in *E. coli* at 100 mg/ml. while lower antibacterial activity was observed at lower concentrations of the aqueous extract (Table 3). Joseph and Priya (2011) reported higher zone of inhibition in organic solvent compared to aqueous solvent. The antimicrobial activity of *Psidium guajava* leaves and other plant extracts have been reported to be dependent on both the solvent of extraction and the concentration of the extract used (Haida *et al*., 2011; Evbuomwan *et al*., 2017). In this work, ethanol extract was found to be more potent than the aqueous extract.

Table 3: Antibacterial activity of aqueous leaves extract of *psidium guajava*

Test organisms	Concentrations (mg/ml)				
	100	50	25	12.5	6.25
S. aureus	10 ± 0.02	8 ± 0.05	7.5±0.01	0.0±0.00	0.0±0.00
K. pneumonia	7.5 ± 0.03	5.2 ±0.01	0.0±0.00	0.0±0.00	0.0±0.00
B. subtilis	10.0±0.00	9.2.0±0.00	6.3±0.00	0.0±0.00	0.0±0.00
E. coli	14.0±0.30	12.0±1.10	7.0±0.09	5.0±0.00	0.0±0.00
S. pyogenes	15 ± 0.10	13.0±0.25	9.0±0.30	6.0±0.02	6.0±0.01

Table 4: MIC and MBC of ethanolic and aqueous leaves extracts of *Psidium guajava*

Test organisms	MIC (mg/ml)		MBC (mg/ml)	
	Ethanol	Aqueous	Ethanol	Aqueous
S. aureus	25	25	50	100
K. pneumoniae	50	50	100	ND
B. subtilis	12.5	25	50	50
E. coli	6.25	12.5	25	25
S. pyogenes	6.25	62.5	25	50

ND- not determined

The minimum inhibitory concentration values ranging from 6.25 mg/ml against *E. coli* to 50 mg/ml against *K. pneumoniae* in both extract (Table 4). The minimum bactericidal concentration was found to be higher in both extract, with values ranging from 25 mg/ml against *E. coli* and *B. subtilis* to 100 mg/ml against *K. pneumoniae* in the ethanolic extract. While the MBC for aqueous extract ranged from 25 mg/ml in *E. coli* to 100 mg/ml in *S. aureus*. Similar finding was earlier reported by Buvaneswari *et al*. (2011). This implies the antibacterial efficacy of *Psidium guajava* leaf extract.

Table 5: Antibiotic susceptibility testing (positive control)

Organisms	CPX	ST	SXT	E	PEF	CN	APX	Z	AM	RO	Res. Index
S. aureus	S	R	R	R	R	S	R	R	S	R	0.7
B. subtilis	R	R	S	R	S	S	S	R	R	R	0.6
S. pyogenes	R	S	R	R	R	S	S	R	S	R	0.6
Gram–ve	TE	NB	AX	OF	C	CPXF	AM	N	CN	CPX	
E. coli	R	R	R	R	S	R	R	R	R	S	0.8
K. pneumoniae	S	R	S	S	S	R	R	R	R	R	0.6

CPX-Ciprofloxacin, RO-Rocephin, ST-Streptomycin, TE-tetracycline, SXT-Septrin, E-Erythromycin, C-Chloramphenicol, PEF-Pefloxacin, CN-Gentamycin, N-Nalidixic, APX-Apmpiclox, AM-Amoxacillin, Z-Zinnacef, S-Sensitivity, R- Resistance Res. Idx- resistance index

Antibiotic sensitivity pattern of the test bacterial isolates was assayed using conventional antibiotic discs and the organisms were observed to show multiple drug resistance to the drugs (Table 5). All bacteria were found to be multidrug resistant. However, *Escherichia coli* was the most resistant bacterium with resistance index of 0.8 while the least resistant were *Bacillus subtilis*, *Streptococcus pyogenes* and *Klebsiella pneumoniae* with resistance index of 0.6. The most effective drugs were gentamycin and carbenicillin. The susceptibility of multidrug resistant pathogens to extracts of *Psidium guajava* might imply an alternative or replacement for existing antibiotics.

Conclusion

Guava (*Psidium guajava*) has been shown in this study to be a repository of phytochemicals which are responsible for its high antimicrobial properties observed in this work. Therefore, this plant should further be researched upon for its therapeutic potentials and possible synergistic or combinatorial activity with other plants or conventional antibiotics.

Conflict of Interest

The authors declare that there is no conflict of interest.

References

Abdeirahirn SI, Almadboul AZ, Omer MEA & Elegami A 2002. Antimicrobial activity of *Psidium guajava* L. *Fitoterapia*, 73: 713-715.

Ajaiyeoba EO, Onocha PA, Nwozo SO & Sama W 2003. Antimicrobial and cytotoxicity evaluation of *Buchholzia coriacea* stem bark. *Phytotherapy*, 74: 706-709

Arima H & Danno G 2002. Isolation of antimicrobial compounds from guava (*Psidium guajava L*) and their structural elucidation. *Biosci., Biotech. & Biochem.,* 66: 172-179.

Barbalho SM, Farinazzi-Machado FMV, Alvares GR, Brunnati ACS & Otoboni AM 2012. *Psidium guajava* (Guava): A plant of multipurpose medicinal applications. *Medicine & Aromatic Plants*, 1(4): 104-111.

Bontempo P, Doto A, Miceli M, Mita L & Benedetti R 2012. *Psidium guajava* L. Anti-Neoplastic Effects: Induction of Apoptosis and Cell Differentiation. *Cell Proliferation*, 45: 22-31.

Buvaneswari S, Raadha CK, Krishnaveni N & Jayashree S 2011. *In-vitro* antimicrobial activity of *Psidium guajava* against clinically important strains. *Phytotherapy*, 1(1): 14-22.

Cohen ML 1992. Epidemiology of drug resistance: Implications for a post-antimicrobial era. *Science*, 257: 1050-1055.

Ekpendu TO, Akshomeju AA & Okogun JI 1994. Antinflamatory antimicrobial activity. *Letters of Appl. Microbio.*, 30: 379-384.

Ekwenye UN and Elegalam NN 2005. Antibacterial activity of ginger *(Zingiber officinale* Roscoe) and Garlic *(Allium sativum* L.) Extracts on *Escherichia coli* and *Salmonella typhi. Int. J. Molecular Medicine & Advance Sci.*, 1(4): 411-416.

Evbuomwan L, Obazenu EI & Inetianbor J 2017. Antimicrobial activity of *Zingiber officinale* against multidrug resistant microbial isolates. *Health Sciences Research*, 4(6): 76-81.

Gutiérrez RMP, Mitchell S & Solis RV 2008. *Psidium guajava*: A review of its traditional uses, phytochemistry and pharmacology. *Journal of Ethnopharmacology*, 117: 1-27.

Haida KS, Baron A & Haida KS 2011. Phenolic compounds and antioxidant activity of two varieties of guava and rue. *Review Brasillica CiêncSaúde*, 28: 11-19.

Hidetoshi A & Danrio G 2002. Isolation of antimi-crobial compounds from guava (*Psidium guajava* L.) and their structural elucidation. *Biosci. Biotech. & Biochem.*, 66: 1727- 1730.

Huang CS, Yin MC & Chiu LC 2011. Antihyperglycemic and antioxidative potential of *Psidium guajava* fruit in Streptozotocin- induced diabetic rats. *Food and Chemical Toxicology*, 49: 2189-2195.

Joseph B & Priya RM 2011. Phytochemical and biopharmaceutical aspects of *Psidium guajava* (L.) essential oil: A review. *Res. J. Medicinal Plant*, 5: 432-442.

Kaneria M & Chanda S 2011. Phytochemical and Pharmacognostic Evaluation of Leaves of *Psidium guajava* L. (Myrtaceae). *Pharmacognosy*, 23: 32-41.

Bauer AW, Kirby WM, Sherris JC and Turck M 1966. Antibiotic susceptibility testing by a standardized single disk method". *American J. Clinical Pathol.*, 45(4): 493–6.

Lozoya X, Meckes M, Abouaaid M. Tortoriello J, Nozzolillo C & Arnason JT 1994. Querectin glycosides in *Pisdium guajava* L. leaves and determination of a Spasmocytic principle. *Archive of Medical Res.*, 25: 11-18.

Meckes M, Calzada F, Tortoriello BJ, Gonzalez JL and Martinez M 2005. Terpenoids isolated from *Psidium guajava* hexane extract with depressant activity on central nervous system. *Phytotherapy Research*, 10: 600-609.

Metwally AM, Omar AA, Harraz FM & El Sohafy SM 2010. Phytochemical investigation and antimicrobial activity of *Psidium guajava* L. leaves. *Pharmacognosy Magazine*, 6: 212-218.

Olajide OA, Awe SO & Makinde JM 1999. Pharmacological studies on the leaf of *Psidium guajava*. *Filotherapia*, 3: 70-25.

Ross IA 2003. *Psidium guajava.* In: Medicinal Plant of the World. Thesis.

Ryu NH, Park KR, Kim SM, Yun HM & Nam D 2012. A hexane fraction of guava leaves (*Psidium guajava* L.) induces anticancer activity by suppressing AKT/mammalian target of rapamycin/ribosomal kinase in human prostate cancer cells. *J. Medicinal Food* , 15: 231-241.

Shanmugam MK, Ong TH, Kumar AP, Lun CK and Ho PC 2012. Ursolic acid inhibits the initiation, progression of prostate cancer and prolongs the survival of TRAMP mice by modulating pro-inflammatory pathways. *PLoS One*, 7: 324-336.

Tangpu TV & Yadav AK 2006. Anticestodal efficacy of *Psidium guajava* against experimental *Hymenolepis Diminuta* infection in rats. *Indian J. Pharmacol.*, 38: 29-32.