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In vitro Antibacterial Activity of *Psidium guajava* L. (Guava) Leaf Extracts against Clinical Isolates of Some Multi drug Resistant Enteric Bacteria causing Diarrhea in Children

Ali M[1*], Ahmed I[2], Yusha'u M.[3] and Shehu A.A[2]
[1]Department of Microbiology, Federal University Gusau
[2]Department of Microbiology, Aliko Dangote University of Science and Technology, Wudil
[3]Department of Microbiology, Bayero University Kano
*Corresponding author: Muhammad Ali, Department of Microbiology, Federal University Gusau. Email: alimuhd4real@gmail.com

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Abstract

The uses of herbal treatment are one of the possible ways to treat diseases caused by multi drug resistant bacteria. The study was aimed to screen for phytochemical and to determine antibacterial activity of aqueous and ethanol extract of Psidium guajava (L.) leaf against clinical isolates of some multi drug resistant enteric bacteria causing diarrhea in Children isolated from patients attending Murtala Muhammad Specialist Hospital Kano. Agar well diffusion method was used to determine antibacterial activity of the extract while dilution method was employed to determine the Minimum inhibitory concentration (MIC) of the extract. Preliminary phytochemical screening of the extract revealed the presence of alkaloids, flavonoids, phenol, terpenoid, anthraquinone, saponin and tannin except Steroid. The leaf has highest percentage of flavonoid (6.75%), followed by alkaloid (5.5%), phenol (3.2%), saponin (3.1), terpenoid (2.25%), tannin and anthraquinone (2.1%) each. The overall sensitivity of the isolates to the extract indicated that Klebsiella spp was the most sensitive with average zone of inhibition of 13.25 mm, followed by E. coli with 13.0 mm, Salmonella spp with 12.25 mm while least sensitivity was shown by Shigella spp with 11.38 mm. it is concluded that the leaf of Psidium guajava contained bioactive components that possess antibacterial activity.

Keywords: *Antimicrobial Activities, Diarrhea, Inhibition, Phytochemical, Psidium guajava,*

Introduction

Today, there is a renewed interest in traditional medicine and increasing demand for more drugs from plant source. This revival of interest in plant derived drug is mainly due to the current widespread belief that "green medicine" is safe and more dependable than the costly synthetic drugs, many of which have adverse side effects (Nair and Chanda, 2007). This situation provided the impetus to the search for new antimicrobial substances from various sources like medicinal

plants (Cordell, 2000). Synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects. Therefore, there is a need to search for new infection fighting strategies to control microbial infections (Dabur *et al*., 2007). The World Health Organization (WHO) estimates that plant extracts or their active constituents are used as folk medicine in traditional therapies of 80 % of the world's population, over 50 % of all modern clinical drugs are of natural product origin.

Psidium guajava L. (Guava) belonging to the Myrtaceae family, is a very unique and traditional plant which is grown due to its diverse medicinal and nutritive properties. The guava tree is an evergreen small tree. The guava leaves are 2 to 6 inches long and 1 to 2 inches wide, aromatic when crushed, and appear dull-green with stiff but coriaceous with pronounced veins (Wilson *et al*., 2001). Guava have been grown to be utilized as an important fruit in tropical areas like Africa and different part of guava i.e. roots leaves, bark, and stem and are widely used for their antispasmodic, cough, fevers etc. concerning human health (Yahaya *et al.,* 2019). There are bioactive components in the guava leaf that can fight against pathogens, regulate blood glucose levels, and can even aid in weight loss. The leaves of guava contain an essential oil rich in cineol, tannins, triterpenes, flavonoids, resin, eugenol, malic acid, fat, cellulose, chlorophyll, mineral salts, and a number of other fixed substances (Ncube *et al*., 2008). *P. guajava* is mainly used as folk medicine many countries. The leaves and decoction prepared from this plant are used for the treatment of vomiting, dysentery, stomach upsets, bleeding gums, and intestinal worms, prevention of hangovers, edema and cough (Abdelrahim *et al*., 2002). A decoction of the leaves or bark of *P. guajava* L. is used for the treatment of ulcer, wounds and eye infections (Lozoya *et al*., 2002). *P. guajava* leaf extracts have improved myocardial function and antioxidant properties (Garcia *et al*., 2003). In various studies, *P. guajava* leaf and bark extracts showed potent antibacterial activity against Staphylococcus *aureus*, *Salmonella typhi, Shigella* spp., *Salmonella paratyphi* A, B, C, *Bacillus* spp., *E. coli*, *Pseudomonas* spp. and *Clostridium* spp. This plant also showed potent antifungal, anti-amebic, anti-yeast and antimalarial properties (Arima and Danno, 2002).

In addition, the global problem of antibiotic resistance impacts negatively on antibiotic administration to treat diseases, hence the prompting researchers to explore traditional knowledge in finding new and innovative antibacterial from plants origin to curb the menace of antibiotic resistance. Also, according to Perez *et al.* (2008), *Psidium guajava* L. has shown to exhibit Gram-positive and Gram-negative antibacterial. Therefore, the study was aimed to determine the antibacterial activity of *P. guajava* leaf extracts against some enteric bacteria associated with diarrhea in children.

Materials and methods

Study Area

The study was conducted at Microbiology Department of Murtala Muhammad Specialist Hospital (MMSH) and Laboratory of Microbiology Department of Kano University of Science and Technology Wudil. Kano State is located in the North-western Nigeria, it is coordinated at latitude 11^0 30 N and longitude 8^0 30 E (Wikipedia, 2021). It share borders with Kaduna State to the South-West, Bauchi State to the South-East, Jigawa State to the East and Katsina State to the North. It has a total area of 20,131km^2 (7,777sqm) and estimated population of 13.4 million (NPC, 2014).

Collection and Identification of Plant Materials

The leaf of the plant (*P. guajava* L.) were collected from Botanical garden of Bayero University Kano at about 06:30 hours. Identification of the plant's leaf was conducted at Herbarium unit in the Department of Plant Science Bayero University Kano with the following voucher identification number BUKHAN 0336. Voucher specimen was deposited in the Herbarium for future reference. The leaves were washed with water to remove dust and rinsed with distilled water. Samples were air dried for two-weeks and pulverized into powder form using sterile mortar and pestle in the laboratory as described by Ali *et al*. (2017). The powdered samples were bagged in a black polythene bag and store in air tight container for further use.

Extraction of Plant Materials

Ethanol and water were used as solvent in the extraction process. One hundred grams (100 g) of the powdered leaves were weighed out and mixed with 500 ml of distilled water and ethanol respectively in a separate sterile conical flask. The ethanol solution was extracted using Soxhlet extractor while the aqueous solution was allowed to stand for three days and extracted by maceration method. The mixtures were filtered using Whatman filter paper and the filtrates were evaporated to dryness using rotary evaporator for ethanol extract and water bath at about 40 ^{0}C for aqueous extracts respectively. The extract yields was weighted, stored in dark air tight container at 4°C (Kumar, 2020).

Preparation of Extracts Concentrations

The stock concentration for the study was 200 mg/ml. The stock concentration was prepared by dissolving 2g of the extract in 10 ml of DMSO. Various working concentration of 100, 50 and 25 mg/ml was prepared from stock concentration by half fold dilution. The concentrations were stored until use (Ali *et al*., 2017).

Phytochemical Screening

Preliminary Phytochemical Screening

Phytochemical screening of the plant materials was conducted using the method adopted by Sofowora (1993) and Tiwari *et al*. (2011). Wagner's test for determination of alkaloid, Ferric chloride test for phenol, gelatin test for tannin, lead acetate test for flavonoid, foam test for saponin, acetic acid test for steroid, Salkowski test for terpenoid detection, Fehling's test for glycoside and Benzene test for determination of anthraquinone.

Quantitative Phytochemical Analysis

Various methods were employed in determining the amount of bioactive components (phytochemicals) present in the plant leaf. Terpenoids and tannins were determined using Spectrophotometric method while phenol was determined using Folin Ciocalteu procedure. The

alkaloids, flavonoids, and the content of saponins were evaluated using analytical method (Harbone, 1998).

Test Isolates

Clinical isolates of *Escherichia coli*, *Salmonella*, *Klebsiella* and *Shigella* isolated from under 5 years children diagnosed with diarrhea attending Murtala Muhammad Specialist Hospital were used for the study. The isolates were screened for Multi drug resistance and extended spectrum beta lactamase production. Reference isolates *E. coli* ATCC 25922 obtained from department of Microbiology, Aminu Kano Teaching Hospital Kano was used in the study as negative control.

Antibacterial Activity of the Extract

The agar well diffusion method was used to determine the antibacterial activity of the leaf extracts as described by Ali *et al*. (2017). A 0.1 ml volume of the standardized organisms (0.5 McFarland) was introduced onto the surface of freshly prepared Mueller Hinton agar in a sterile Petri dish and allowed to set and then labeled. A 6 mm sterile Cork borer was used to punch five (5) wells in the inoculated agar medium. Four of the wells were filled with four different concentrations of the extract which were labeled accordingly; 200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml, while the 5th well contained 25 mg/mL of Ciprofloxacin as control for this research. The plates were left on the bench for one hour to enable proper diffusion of the extracts and then incubated at 37°C for 24 hours. After incubation, the diameter of the zones of inhibition around each well was measured to the nearest millimeters along straight line. The experiment was conducted in triplicate and average value was evaluated.

Determination of Minimum Inhibitory Concentration (MIC)

The MIC was determined using the micro-dilution method as described by Clinical and Laboratory Standards Institute (CLSI, 2010). Serial two-fold dilutions of all the extracts were prepared with sterile broth in a 96-well microtitre plate, obtaining a concentration range from 50 to 3.125 mg/mL (50, 25, 12.5, 6.25 and 3.125 mg/mL). It was followed by addition of 5 µL of the isolates suspension which was added to the wells containing the dilutions. Non-inoculated wells containing sterile broth and extract were used as negative controls. After incubation for 24 hours at 37°C, the samples were observed. MIC was recorded as the lowest concentration of each plant extract that inhibited the bacterial growth as detected by the absence of visual turbidity.

Determination of Minimum Bactericidal Concentration (MBC)

To estimate the MBC, an aliquot of each well that did not show microbial growth in the prior tests were swabbed on the entire surface of Mueller Hinton Agar plates and then incubated for 24 hours at 37°C. The lowest concentration that prevented the bacterial growth was registered as MBC (Ali *et al*., 2017).

Statistical Analysis

The data of average zone of inhibition produced by the isolates against the different extracts of *P. guajava* used was analyzed using One-Way ANOVAs and the statistical program SPSS 21.0 (Statistical Package for the Social Sciences). The results were presented as the means ± standard error. Significance level for the differences was set at $p<0.05$.

Results

Phytochemical Screening of *Psidium guajava* leaves Extracts

The Table below showed that the preliminary phytochemicals screening of aqueous and ethanol extracts of *Psidium guajava* leaves. The result showed that both aqueous and ethanol extracts of *P. guajava* leaves contain alkaloids, flavonoids, phenol, terpenoid, anthraquinone, saponin and tannin except Steroid.

Table 1: Phytochemical Screening of *Psidium guajava* Leaf Extracts

S/N	Phytochemicals	Aqueous extract	Ethanol Extract
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Phenol	+	+
4	Terpenoid	+	+
5	Anthraquinone	+	+
6	Saponin	+	+
7	Steroids	-	-
8	Tannin	+	+

Key: + = Present, - = Absent

Quantitative phytochemical Analysis of *P. guajava* Leaf

The quantitative phytochemical screening of leaf of *P. guajava* is presented in Table 2. The result showed that the leaf has highest percentage of flavonoid (6.75%), followed by alkaloid (5.5%), phenol (3.2%), saponin (3.1), terpenoid (2.25%), tannin and anthraquinone (2.1%) each.

Table 2: Quantitative phytochemical Analysis of leaf *P. guajava*

S/N	Phytochemicals	Leaf (%)
1	Alkaloid	5.50±0.05
2	Flavonoid	6.75±0.02
3	Phenol	3.20±0.01
4	Terpenoid	2.25±0.01
5	Anthraquinone	2.10±0.02
6	Saponin	3.10±0.00
7	Tannin	2.10±0.01

Antibacterial Activity of Aqueous Extract

The antibacterial activity of various concentration of aqueous extract of *P. guajava* leaf is presented in Table 3. The antibacterial activity of the extract depends on its concentration and types of isolates. Highest zone of inhibition is demonstrated by *Klebsiella* (16 mm) at 200 mg/mL. The overall sensitivity of the isolates to the extract indicated that *Klebsiella* spp was the most sensitive with average zone of inhibition of 12.75 mm, followed by *E. coli* with 12.5 mm, *Salmonella* spp with 11.5 mm while least sensitivity was shown by *Shigella* spp with 10.5 mm. The zone of inhibition produced by the control (Ciprofloxacin 25 mg/mL) ranges from to 20 – 23 mm while ATCC 25948 recorded 20 mm at 200mg/mL of the extract

Table 3: Antibacterial activity of *P. guajava* Leaf Aqueous Extract

	Concentration (mg /mL)/zone of inhibition (mm)				
Isolates	**25**	**50**	**100**	**200**	**Control**
Escherichia coli	10.00 ± 0.5^{a}	12.00 ± 0.3^{a}	13.00 ± 0.0^{b}	15.00 ± 0.3^{b}	23.00
Salmonella spp	08.00 ± 0.00^{a}	11.00 ± 0.3^{b}	13.00 ± 0.5^{c}	14.00 ± 0.5^{c}	21.00
Shigella spp	08.00 ± 0.3^{a}	10.00 ± 0.3^{a}	11.00 ± 0.0^{b}	13.00 ± 0.3^{b}	20.00
Klebsiella spp	11.00 ± 0.0^{a}	12.00 ± 0.3^{b}	12.00 ± 0.5^{b}	16.00 ± 0.5^{c}	22.00
ATCC 25948	12.00	15.00	18.00	20.00	25.00

Key: Values having different superscript on the same row are considered significantly different at $p<0.05$

Antibacterial activity of Ethanol Extract

The antibacterial activity of various concentration of ethanol extract of *P. guajava* leaf is presented in Table 4. The antibacterial activity of the extract depends on its concentration and types of isolates. Highest zone of inhibition is demonstrated by *Klebsiella* (17 mm) at 200 mg/mL. The overall sensitivity of the isolates to the extract indicated that *Klebsiella* spp was the most sensitive with average zone of inhibition of 13.75 mm, followed by *E. coli* with 13.5 mm, *Salmonella* spp with 13 mm while least sensitivity was shown by *Shigella* spp with 12.25 mm. The zone of inhibition of the control (Ciprofloxacin 25 mg/mL) ranges from to 20 – 23 mm while ATCC 25948 recorded 22 mm at 200mg/mL of the extract.

Table 4: Antibacterial activity of *P. guajava* leaf Ethanol Extract

	Concentration (mg /mL)/zone of inhibition (mm)				
Isolates	**25**	**50**	**100**	**200**	**Control**
Escherichia coli	10.00 ± 0.0^{a}	13.00 ± 0.5^{b}	15.00 ± 0.3^{b}	16.00 ± 0.5^{c}	23.00
Salmonella spp	10.00 ± 0.00^{a}	12.00 ± 0.3^{b}	15.00 ± 0.5^{c}	15.00 ± 0.5^{c}	21.00
Shigella spp	10.00 ± 0.3^{a}	11.00 ± 0.0^{a}	13.00 ± 0.0^{b}	15.00 ± 0.3^{b}	20.00

Klebsiella spp	11.00±0.00[a]	12.00±0.3[a]	14.00±0.5[b]	17.00±0.3[c]	22.00
ATCC 25948	13.00	16.00	18.00	22.00	25.00

Key: Values having different superscript on the same row are considered significantly different at $p<0.05$

MIC and MBC of the Extract

The MIC and MBC of *P. guajava* leaf extracts are represented in Table 5. The result showed dilutions of various concentrations of aqueous and ethanol extracts can inhibit and/or kill the isolates. Lower MIC (12.5 mg/mL) was shown by ethanol extract against *Klebsiella*, *E. coli* and *Salmonella* than aqueous extract (25 mg/mL) except Klebsiella with 12.5 mg/mL. MBC of the extracts ranges between 25 - 50mg/mL. However, the MBC of Shigella was not found in aqueous extract.

Table 5: Minimum Inhibitory Concentration (MIC) and MBC of the *P. guajava* Leaf Extracts

Isolates	Aqueous extract		Ethanol extract	
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)
Escherichia coli	25	25	12.5	25
Salmonella spp	25	50	12.5	25
Shigella spp	25	NA	25	50
Klebsiella spp	12.5	50	12.5	50

Key: NA = Not available

Discussion

The presence of these secondary metabolites in plants, produce some biological activity in man and animals and it is responsible for their use as herbs. These compounds also serve to protect the plant against infection by microorganisms, predation by insects and herbivores, while some give plants their some cases, the activity has been associated with specific compounds or classes of compounds. These active constituents can be used to search for bioactive lead compounds that could be used in the partial synthesis of more useful drugs (Yahaya *et al*., 2019). Preliminary phytochemical analysis of *Psidium Guajava* in the present study was carried out to identify the bioactive components such as alkaloids, flavonoids, steroids, terpenoid, anthraquinone, phenol, saponin and tannin present in the leaf of the plant.

Preliminary phytochemical analysis of aqueous and ethanol leaf extract *P. guajava* showed the presence of alkaloids, flavonoids, phenol, terpenoid, anthraquinone, saponin and tannin except Steroid. From the result, there is highest percentage of flavonoid (6.75%), followed by alkaloid (5.5%), phenol (3.2%), saponin (3.1), terpenoid (2.25%), tannin and anthraquinone (2.1%) each. Earlier researches have revealed the presence of alkaloids, flavonoids, glycosides, phenols,

reducing compounds, saponin and tannins in the aqueous extract of *P. guajava* leaves (Uboh *et al.*, 2010; Offo, 2015).

The phytochemical analysis of *P. guajava* leaves showed the presence of more than 20 isolated compounds, including alkaloids, carotenoids, essential oils, fatty acids, lectins, phenols, saponins, tannins, triterpenes and vitamin C. Further studies on the quantitative analysis of the various bioactive compounds present in *P. guajava* could contribute significantly to the health management of man and could be recommended in our daily need of nutrition. This finding can be attested to the work of Offo, (2015) who also reported similar finding on the phytochemical of guava leaf extract, which contain alkaloid, saponin, flavonoids, phenol, steroid, tannin, protein and glycoside. Pandey and Shweta, (2012) reported the phytochemicals mainly present in *P. guajava* were reducing sugar, tannin, saponin, phlobatannin, terpenoid, alkaloid and phenols.

The finding of the present study was also in conformity with that of Joseph and Priya, (2012) who reported the presence of glycoside, saponin, Anthraquinones, flavonoids, tannin and alkaloids in the leaf of *P. guajava*. Reports on the antimicrobial activity of *P. guajava* leaf extracts were attributed to the presence of tannins, terpenoid and flavonoids in the leaves (Q'adan *et al*. 2005). This finding supported the findings of Elekwa *et al*. (2008) which state that the phytochemical of *P. guajava* leaf revealed the presence of alkaloids, saponin, steroidal rings, reducing sugar, carbohydrate and glycosides. However, the finding of the present study contradict that of Nwanneka *et al*. (2003) where the preliminary phytochemical analysis of *P. guajava* leaf reveals absent of Alkaloid.

The alkaloids comprising a large group of nitrogenous compounds are widely used as cancer chemotherapeutic agents, anaesthetics and Central Nervous Stimulants (Madziga *et al*., 2012). It also interferes with cell division, hence the presence of alkaloids in *P. guajava* leaf could account for its use as antimicrobial agents. Terpenoid have been found to be useful in the prevention and therapy of several diseases, including cancer. Terpenoid are also known to possess antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, antispasmodic, antihyperglycemic, antiinflammatory and immune modulatory properties (Abaoba *et al*., 2011). Flavonoids are also present in the extracts as a potent water-soluble antioxidant and free radical scavenger, which prevent oxidative cell damage and also have strong anticancer activity (Ali *et al*., 2018). It also helps in managing diabetes induced oxidative stress. Saponin and tannin were also present in the extracts. Saponin protect against hypercholesterolemia and antibiotics properties (Ali *et al*., 2018). In addition, it has been documented that saponin have antitumor, antioxidant and anti-mutagenic activities and can lower the risk of human cancers by inhibiting the growth of cancer cells (Prohp and Onoagbe, 2012). The growth of many fungi, yeast, bacteria and viruses was inhibited by tannins (Chung *et al*., 1998). Tannins are used as antiseptic and this activity is due to presence of the phenolic group. Tannin rich medicinal plants are used as healing agents in a number of diseases (Ali *et al*., 2018).

The antibacterial activity of aqueous and ethanol extract of *Psidium guajava* leaf in this study was evaluated by well agar diffusion method. *Psidium guajava* at concentrations of 25, 50, 100, 200mg/ml was used. The antimicrobial activity of the extract was dose dependent with 200 mg/ml having higher activity. The ethanol extract showed higher antibacterial activity against the test isolates when compared to aqueous extract. This shows similarities to the findings of

Nwanneka *et al*. (2013) which investigated the antimicrobial activity of *Psidium guajava* leaf extract, the results showed that both aqueous and ethanol extracts of guava leaf inhibited the growth of the bacteria and fungi tested but the ethanol extract showed stronger inhibition than the aqueous extract against the organisms. The finding of this study was also inconformity with that of Pandey and Shweta (2012) where the results of antibacterial activity of *Psidium guajava* leaf and stem reveals that ethanol extract showed stronger anti-bacterial activity than aqueous extract. However, this was contrary to the findings of Emmanuel (2010) he stated that the antimicrobial activity of aqueous extract of *Psidium guajava* is higher than that of ethanol extract. The result of antimicrobial effects of *Psidium guajava* leaf extract by Elekwa *et al*. (2009) showed that aqueous extract of guava leaf had higher inhibitory effects on some organisms than ethanol extract. This is contrary to present findings. Higher activity by the ethanol extracts in this study is attributed to better solubility of the active component of the leaf by the ethanol than water.

Based on the sensitivity of the isolates to the extracts, the results of antibacterial activity of the extracts was more pronounced in *Klebsiella*, followed by *E. coli*, *Salmonella* and less active on *Shigella* spp. The antimicrobial activity of *P. guajava* leaf extracts on the bacteria isolates was attributed to the presence of some phytochemicals such as tannins, terpenoid, alkaloid, saponin and flavonoids in the leaf. From the results of MIC determination, the minimum inhibitory concentration, it showed dilutions of various concentrations of aqueous and ethanol extracts can inhibit and/or kill the isolates. Lower MIC was shown by ethanol extract against *Klebsiella*, *E. coli* and *Salmonella* than aqueous extract. MBC of the extracts ranges between 25 – 50 mg/mL. However, the MBC of Shigella was not found in aqueous extract. Minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation and minimum bactericidal concentration (MBC) is the lowest of antimicrobial agent that will prevent the growth of an organism after subculture on to antibiotic free media (Abdallah *et al*., 2019).

Conclusion

Preliminary phytochemical screening of leaf extract of *P. guajava* showed the presence of alkaloids, flavonoids, phenol, terpenoid, anthraquinone, saponin and tannin except Steroid. The leaf has the highest percentage of flavonoid (6.75%), followed by alkaloid (5.5%), phenol (3.2%), saponin (3.1), terpenoid (2.25%), tannin and anthraquinone (2.1%) each. The antimicrobial activity of the extracts showed that the ethanol extract showed higher antibacterial activity against the test isolates when compared to aqueous extract. However, the antibacterial activity of the extracts was more pronounced in *Klebsiella*, followed by *E. coli*, *Salmonella* and less active on *Shigella* spp. The antibacterial activity of the extracts is attributed to the presence of phytochemicals in it. It is recommended that there is need to exploit the potentials of the plants especially in areas of traditional medicine and pharmaceutical industries.

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