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Efficacy of leaf extracts of *Psidium guajava* (L.) on enteric bacterial isolates from faecally impacted groundwater

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ABSTRACT

The efficacy of leaf extracts of guava *Psidium guajava* (L.) were assessed on enteric bacteria isolated from two wells (W1 and W2) prone to faecal contamination in Akure, Nigeria. The load of enteric bacteria in the water samples was determined using a standard microbiological method. Leaf extracts of *P. guajava* (L.) were prepared using ethanol and hot distilled water and the antibacterial effects of the extracts on bacterial isolates were determined using the agar well diffusion method. Phytochemical components of the leaf extracts were carried out using standard methods. Results revealed the following mean concentrations of enteric bacteria in W1 and W2: *Salmonella* (3.8 and 3.9 $\log_{10}$ CFU/100 ml) and *Shigella* (3.6 and 3.7 $\log_{10}$ CFU/100 ml). The ethanol leaf extracts of *P. guajava* (L.) exhibited a zone of inhibition of 22 mm at 200 mg/ml on *Shigella* and no zone of inhibition was observed on *Salmonella*. The phytochemical components of ethanol and hot distilled water leaf extracts of *P. guajava* (L.) revealed the presence of saponin as (13.64 mg/g) and (59.82 mg/g). The findings of this study revealed that ethanol leaf extracts of *P. guajava* (L.) may be useful as an antibacterial agent against *Shigella*.

Key words: diarrhea, enteric bacteria, groundwater, inhibition, leaf extracts, phytochemical components

HIGHLIGHTS

- The level of faecal pollution in the wells was 'critical' based on *E. coli* concentration.
- Hot distilled water leaf extracts of guava had no effect on *Salmonella* and *Shigella*.
- Resistance of *Salmonella* was plasmid-based.
- Bioactive components of ethanol leaf extracts of *P. guajava* contributed to the antibacterial effect on *Shigella*.
- Antibacterial potential of *P. guajava* leaves may be enhanced by extracting with ethanol.

INTRODUCTION

Waterborne diseases are the major cause of morbidity and mortality in low- and middle- income countries and antimicrobial agents are essentially important in reducing the global burden of waterborne infectious diseases (Mandal *et al.* 2011). Most waterborne diseases are often transmitted via the faecal-oral route, and this occurs when human faecal material is ingested through drinking contaminated water or eating contaminated food as a result of inadequate water, sanitation and hygiene.

Plants have been a veritable source of drugs and the scientific exploration of medicinal plants for the benefit of man is intensified because these plants may likely reduce the dependence on orthodox chemotherapeutic agents (Ayodele *et al.* 2019). Medicinal plants are important with respect to new drug and pharmacological research development and are widely used and accepted as home remedies and raw materials for the pharmaceutical industry (Mule *et al.* 2013). Development of antibiotics resistance by enteric bacterial pathogens including *Salmonella* and *Shigella*, against easily accessible and commonly prescribed drugs has become a major concern throughout the world, particularly in developing countries (Oncho *et al.* 2021). There is a need to search for new antimicrobial agents among plant extracts because plant-based antimicrobials represent a vast untapped source of medicine.

Guava (*Psidium guajava* L.) belongs to the family *Myrtaceae* and is considered to have originated from tropical South America. Guava trees grow in tropical and sub-tropical regions of the world such as Asia, Africa and Hawaii (Biswas *et al.* 2013). The leaves have long been recognized for their antimicrobial activity. *P. guajava*

Linn is a phytotherapeutic plant used in folk medicine for the management of various disease conditions including enteric bacterial infections and it is also a good source of natural antioxidants (Oncho *et al.* 2021). Studies on phytochemical components of different guava extracts has revealed numerous bioactive compounds such as tannin, flavonoid, terpenoid, steroids, glycoside, cardiac glycoside, alkaloid, phlobatannin, polyphenol, saponin anthraquinones and phytosteroid (Oncho *et al.* 2021). Plant-derived bioactive compounds are promising sources of antimicrobials. These compounds act by the inhibition of microbial cell wall development, disruption, and lysis, hampering biofilm formation, repression of DNA replication and transcription, impeding adenosine triphosphate (ATP) production, suppression of bacterial toxins, and the generation of reactive oxygen species (ROS) (Kumar *et al.* 2021). Guava leaves, owing to the presence of different organic and inorganic antioxidants and anti-inflammatory compounds, have been shown to demonstrate antimicrobial activities against *Pseudomonas aeruginosa*, *Escherichia coli*, *Streptococcus faecalis*, *Staphylococcus aureus* and *Bacillus subtilis* (Kumar *et al.* 2021).

Several studies have reported the antidiarrheal potential of guava leaves. For instance, Mazumdar *et al.* (2015) observed that a dosage level of extracts of guava leaves at a concentration of 500 and 750 mg/kg had antidiarrheal effect in rats. Similarly, Kumar *et al.* (2021) reported that guava leaf extracts at doses of 52–410 mg/kg when administrated orally were found to combat diarrhea, and also resulted in reduced intestinal transit and dilatory removal of unwanted gastric products in rodents. In addition, symptoms, such as secretion of interstitial fluid and wetness of faecal droppings in a dose-dependent manner were reduced significantly. In another related study, Dewi *et al.* (2013) combined aqueous leaf extracts of guava and leaf extracts of green tea at different ratios and reported that all combinations had potent antidiarrheal activities, depicted by enhanced stool weight, stool onset, stool consistency and diarrhea period.

This study set out to assess the antibacterial effects of leaf extracts of guava *P. guajava* (L.) on enteric bacteria isolated from selected groundwater sources in Akure, Nigeria. The objectives of this study were to determine the level of faecal contamination of the wells; examine the phytochemical components of the leaf extracts of guava *P. guajava* (L.); and assess the antibacterial effects of the leaf extracts on enteric bacteria from the wells.

MATERIALS AND METHODS

Collection, preparation and extraction of plant leaves

Fresh leaves of *P. guajava* (L.) were collected from guava trees growing at a residential house in Owo, Ondo State, Nigeria. The leaves were collected into plastic bags, stored in a cool box with ice packs and transported to the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, Nigeria for identification. Thereafter, the leaves were washed thoroughly with running tap water, dried, homogenized to fine powder, stored in an air tight bottle and kept at room temperature until required. One hundred grams (100 g) each of the dried powdered plant leaves was weighed and sequentially extracted with 150 ml of ethanol and hot distilled water by maceration method. The extracts were filtered using Whatman No. 1 filter paper and filtrates were concentrated appropriately, weighed, labelled and stored at 4 °C. Two grams (2 g) of the filtrate were dissolved in 10 ml Dimethyl sulfoxide (DMSO) containing 70% distilled water and 30% DMSO to achieve a 100% concentration.

Sampling site and collection of water samples

The study area was New Castle Hostel along Deeper Life Camp Ground, FUTA North gate, Akure, Ondo State, Nigeria. Both wells (W1 and W2) were situated at approximately 6 m and 5 m from a septic tank, respectively, and were also in close proximity to a landfill (approximately 10 m from the wells). W1 and W2 were within coordinates 5° 8° 30°E and 7° 18° 30°N as shown in Figure 1. Water samples were collected in duplicates using 500 ml sterile bottles labelled A and B over a period of 16 weeks (March to June, 2019), totaling 32 water samples from each well. The samples were transported in a cool box with ice packs to the laboratory for analyses within one hour.

Enumeration of enteric bacteria in water samples

Membrane faecal coliform agar (*m*-FC), membrane lauryl sulphate agar (MLSA), *Salmonella Shigella* agar (SSA) and Eosin Methylene Blue (EMB) agar were used as the selective media. The concentrations of faecal coliforms, *E. coli*, *Salmonella*, and *Shigella* in the water samples were determined using the membrane filtration technique. Membrane filters (0.45 μm) were placed on prepared media aseptically and agar plates were incubated at 44 °C

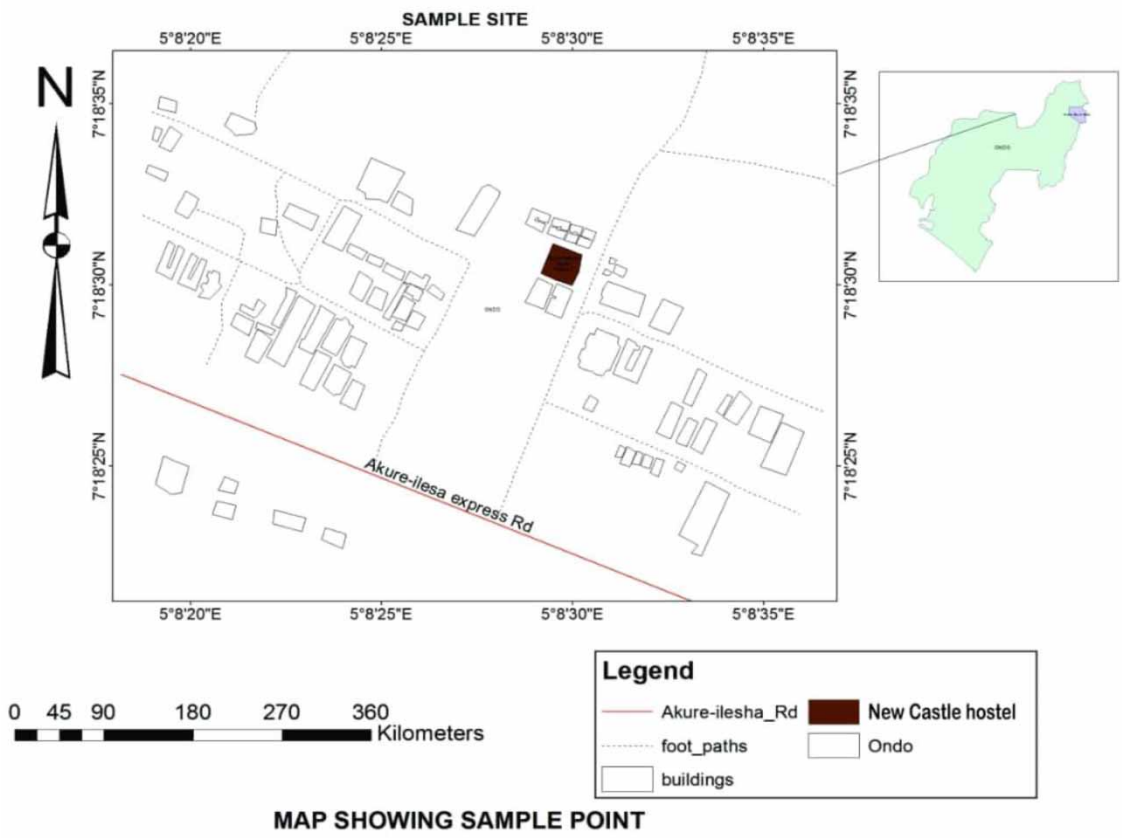

Figure 1 | Map showing the location of New Castle Hostel as the sample point.

for 24 hours (*m*-FC), 37 °C for 24 hours (MLSA, EMB, SSA). Colonies were counted, calculated and expressed as colony forming units (CFU) per 100 ml.

Assessment of antibacterial activity of leaf extracts of *P. guajava* (L.)

Bacterial colonies from 24 hours overnight culture were used to make a suspension. Four or five isolated colonies of the organism to be tested were picked with a sterile inoculating loop and suspended in 2 ml of sterile saline. The saline tube was mixed thoroughly using a vortex and the turbidity of the suspension was adjusted to a 0.5 McFarland standard. More organism was added to the suspension when it was too light and diluted with sterile saline when the suspension was too heavy to achieve a 0.5 McFarland standard. The suspension was used within 15 minutes of preparation.

The agar well diffusion method was used for the assessment of antibacterial activity of leaf extracts of *P. guajava* (L.). Mueller Hilton agar was prepared according to manufacturer's specification and a standardized inoculum was swabbed on the agar. Four wells were made on each plate using a sterile cork borer of 6 mm diameter. The wells were filled with 0.1 ml of different diluted concentrations (200 mg/ml, 100 mg/ml, 50 mg/ml and 25 mg/ml respectively) of the extract with the aid of a filter syringe. The plates were allowed to stand for 15 minutes to allow pre-diffusion of the extracts, and thereafter incubated at 37 °C for 24 hours. Diameters of zones of inhibition were measured using a transparent plastic ruler and recorded appropriately.

Determination of phytochemical components of leaf extracts of *P. guajava* (L.)

The leaf extracts of *P. guajava* (L.) were tested qualitatively for the presence of saponin, tannin, phlobatannin, flavonoid, steroids, terpenoids and alkaloids using standard methods (Poongothai *et al.* 2011). In addition, the leaf extracts of *P. guajava* (L.) were tested quantitatively for the presence of saponin, tannin, flavonoid, steroids and terpenoids using standard methods (Sofowora 2008).

Plasmid profiling and curing of antibiotics resistant isolates

About 600 μl of bacterial culture was added to a 1.5 ml microcentrifuge tube, and centrifuged for 30 seconds at 14,000 rpm. The supernatant was discarded and 100 μl of 7X lysis buffer (blue) 1 was added and mixed. About 350 μl of cold neutralization buffer (yellow) was added to the solution, mixed thoroughly and centrifuged at 11,000–16,000 rpm for 2–4 minutes. The supernatant was transferred into Zymo-Spin™ IIN column and placed into a collection tube, then centrifuged for 15 seconds. Other buffers (Endo-wash, Zyppy™ wash, Zyppy™ elution) were added appropriately to obtain the plasmid DNA. Plasmid curing was performed by inoculating 100 ml of culture grown in broth containing 10% of Sodium Dodecyl Sulfate (SDS). After inoculation, the cultures were incubated at 37 °C for 24 h. After 24 h incubations, the above procedure was used to purify the plasmid. Antibiotics sensitivity testing of isolates was carried out. Briefly, a sterile swab was dipped into the broth suspension containing the isolate and excess moisture was removed. The surface of a plate containing Mueller-Hinton agar was inoculated with the isolate by streaking and was allowed to dry for about 5 minutes before placing the antibiotic disks on the agar. Thereafter, the plates were inverted and placed in an incubator at 37 °C for 24 h. Diameters of zones of inhibition were measured using a transparent plastic ruler (Kirby *et al.* 1996).

Statistical data analysis

Data obtained were transformed to $\log_{10}$ using Microsoft Excel and examined using Statistical Package for Social Sciences (SPSS) Version 23.0. All experiments were performed in triplicates and data derived from the study were subjected to 2-way analysis of variance (ANOVA) and level of significance was documented at $P < 0.05$.

RESULTS

Detection of enteric bacteria in water samples from the wells

The mean concentrations of the faecal coliforms, *E. coli*, *Salmonella* and *Shigella* in water samples from the wells (W1 and W2) over the study period are shown in Table 1. The mean concentrations of faecal coliforms in the water samples from W1 and W2 were 4.1 and 4.4 $\log_{10}$ CFU/100 ml, respectively, whereas those of *E. coli* in the water samples from W1 and W2 were 3.2 and 3.1 $\log_{10}$ CFU/100 ml, respectively. Similarly, the mean concentrations of *Salmonella* in the water samples from W1 and W2 were 3.8 and 3.9 $\log_{10}$ CFU/100 ml, respectively, whereas those of *Shigella* in in the water samples from W1 and W2 were 3.6 and 3.7 $\log_{10}$ CFU/100 ml, respectively.

Table 1 | Mean concentrations of enteric bacteria in water samples from the wells

Enteric bacteria	W1 ($\log_{10}$ CFU/100 ml) mean ± SD (min- max)	W2 ($\log_{10}$ CFU/100 ml) mean ± SD (min- max)
Faecal coliforms	4.1 ± 0.5 (2.7–4.5)	4.3 ± 0.4 (3.0–4.5)
E. coli	3.2 ± 0.4 (2.3–4.1)	3.1 ± 0.2 (2.6–3.5)
Salmonella	3.8 ± 0.5 (2.5–4.1)	3.9 ± 0.5 (2.5–4.2)
Shigella	3.6 ± 0.5 (2.3–4.1)	3.7 ± 0.5 (2.2–4.0)

Key: W1 – Well 1; W2 – Well 2; values are presented as mean ± standard deviation (minimum- maximum); $n = 32$.

Antibacterial activity of leaf extracts of *P. guajava* (L.) on the isolates

The antibacterial activity of ethanol and hot distilled water leaf extracts of *P. guajava* (L.) measured at varying concentrations (200, 100, 150 and 25 mg/ml) against *Salmonella* and *Shigella* revealed that ethanol leaf extracts of *P. guajava* (L.) had higher antibacterial effect compared to hot distilled water leaf extracts of *P. guajava* (L.). *Salmonella* was observed to be resistant to both ethanol and hot distilled water leaf extracts of *P. guajava* (L.), whereas *Shigella* was susceptible to ethanol leaf extracts of *P. guajava* (L.) with the highest zone of inhibition (22.0 mm) at 200 mg/ml and the least zone of inhibition (6.7 mm) at 25 mg/ml (Table 2).

Phytochemical components of leaf extracts of *P. guajava* (L.)

The qualitative phytochemical components of ethanol leaf extracts of *P. guajava* (L.) revealed the presence of saponin, tannin, flavonoid, steroid, terpenoid, whereas those present in hot distilled water leaf extracts of

Table 2 | Antibacterial activity of ethanol and hot distilled water leaf extracts of *P. guajava* (L.)

Isolates/zone of inhibition (mm)			
Leaf extracts	**Concentration (mg/ml)**	***Salmonella***	***Shigella***
Ethanol	200	0.0 ± 0.0^{a}	22.0 ± 0.0^{c}
	100	0.0 ± 0.0^{a}	17.0 ± 0^{c}
	50	0.0 ± 0.0^{a}	10.3 ± 0.3^{b}
	25	0.0 ± 0.0^{a}	6.7 ± 0.3^{a}
Hot distilled water	200	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	100	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	50	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}
	25	0.0 ± 0.0^{a}	0.0 ± 0.0^{a}

Values are means ± standard error. Means carrying the same alphabet in the same column are not significantly different ($P < 0.05$).

P. guajava (L.) include saponin, tannin, flavonoid, terpenoid. However, phlobatannin and alkaloid were absent in both ethanol and hot distilled water leaf extracts of *P. guajava* (L.) (Table 3).

Table 3 | Qualitative phytochemical components of leaf extracts of *P. guajava* (L.)

Components and tests	**Ethanol extracts (mg/g)**	**Hot water extracts (mg/g)**
Saponin	+	+
Tannin	+	+
Phlobatannin	−	−
Flavonoid	+	+
Steroid	+	−
Terpenoid	+	+
Alkaloid	−	−
Keller kiliani test	+	+
Salkwoski test	+	+
Lieberman test	+	−
Legal test	+	+

Key = + present; − absent.

The quantitative phytochemical components of ethanol leaf extracts of *P. guajava* (L.) showed that terpenoid (25.80 mg/g) had the highest quantity, while steroid (3.71 mg/g) had the least. On the other hand, the quantitative phytochemical components of hot distilled water leaf extracts of *P. guajava* (L.) showed that saponin (59.82 ± 0.21 mg/g) had the highest quantity while steroid (0.00 mg/g) had the least (Table 4).

Table 4 | Quantitative phytochemical components of leaf extracts of *P. guajava* (L.)

Components	**Ethanol extracts (mg/g)**	**Hot water extracts (mg/g)**
Saponin	13.64 ± 0.10^{ab}	59.82 ± 0.21^{b}
Tannin	9.64 ± 0.00^{b}	3.51 ± 0.02^{a}
Flavonoid	15.11 ± 0.01^{ab}	2.20 ± 0.00^{a}
Steroid	3.71 ± 0.01^{a}	0.00 ± 0.00^{a}
Terpenoid	25.80 ± 0.01^{ab}	11.05 ± 0.04^{ab}

Key: Values expressed in Mean ± Standard error ($n = 3$) with the same superscript down the column are not significantly different ($P < 0.05$).

Plasmid profiling and curing of resistant isolates

The gel image of profiling and curing of *Salmonella* revealed that the resistance of the isolates was plasmid based and the DNA plasmid molecules were lost after curing, as shown in Figure 2. Septrin and ciprofloxacin exhibited

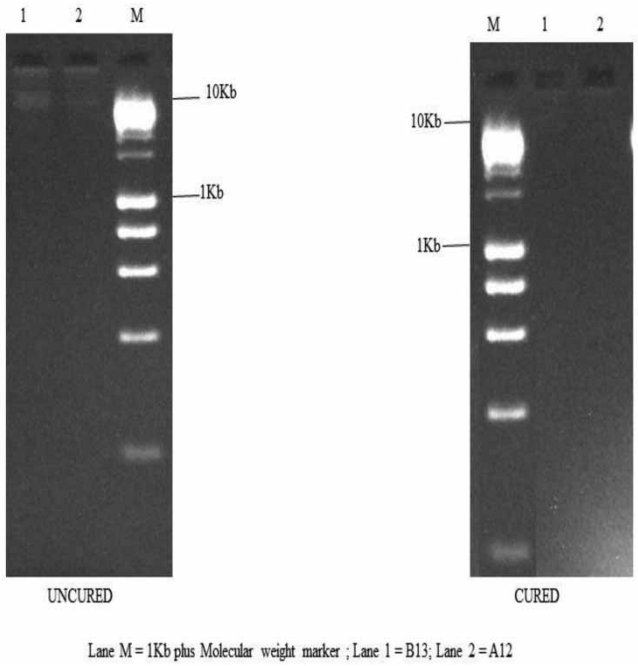

Figure 2 | Gel image of profiling and curing of resistant isolates of *Salmonella*.

the highest zone of inhibition (25.67 mm), while gentamycin exhibited the least zone of inhibition (20.67 mm) on *Salmonella* (Table 5).

Table 5 | Antibiotics sensitivity pattern of *Salmonella* after plasmid curing

Salmonella	SEP	CHL	CIP	AUG	GEN	OFL	STRE
1	25.67 ± 0.33^{f}	24.67 ± 0.33^{d}	25.67 ± 0.33^{g}	23.33 ± 0.33^{b}	22.33 ± 0.33^{a}	23.33 ± 0.67^{c}	25.00 ± 0.00^{e}
2	23.67 ± 0.33^{c}	23.00 ± 0.58^{b}	23.67 ± 0.88^{d}	24.33 ± 0.33^{e}	20.67 ± 0.33^{a}	25.00 ± 0.00^{f}	25.00 ± 0.58^{g}

Key: SEP – Septrin; CHL – Chloramphenicol; CIP – Ciprofloxacin; AUG – Augmentin; GEN – Gentamycin; OFL – Ofloxacin; STRE – Streptomycin; Superscripts with the same alphabet are significantly different ($P < 0.05$).

DISCUSSION

The antibacterial effects of leaf extracts of guava *P. guajava* (L.) on enteric bacteria isolated from selected groundwater sources in Akure, Nigeria were assessed. The occurrence of faecal coliforms in the water samples from the wells suggest faecal pollution and degraded groundwater quality. Studies have shown that consumption of faecally contaminated water may lead to the occurrence of gastrointestinal diseases (Folorunso *et al.* 2014; Onyango *et al.* 2018). In this study, the concentration of *E. coli* in water samples from well 1 and well 2 were higher than those observed by Olalemi *et al.* (2021) in water from a well situated in Apatapiti, Akure, Nigeria and this may likely be as a result of the landfill and septic tanks sited around the wells. Onyango *et al.* (2018) had reported that the presence of *E. coli* in higher counts in groundwater indicate contamination with faecal matter and other pathogens that may compromise the safety of the water source. The level of faecal pollution in the wells (W1 and W2) were 'critical' based on *E. coli* concentration. The results also revealed the presence of *Salmonella* and *Shigella* in the water samples from the wells. This observation is similar to the findings of Folorunso *et al.* (2014) where the authors reported the presence of the pathogens in the water samples obtained from plastic containers used in storing well water and suggested that the bacteria thrive well in polluted and untreated water source. It also in agreement with Mahagamage *et al.* (2020), where the authors observed high counts of *Salmonella* and *Shigella* in groundwater sources in Kelani River Basin in Sri Lanka.

Ethanol leaf extracts of *P. guajava* (L.), which showed higher antibacterial activity on *Shigella* compared to hot distilled water leaf extracts of *P. guajava* (L.), may likely be attributed to better solubility of the bioactive

components of leaf extracts of *P. guajava* (L.) in ethanol than hot distilled water. This is in agreement with the findings of Nwanneka *et al.* (2013) where the authors investigated the antimicrobial activity of leaf extracts of *P. guajava* and observed that ethanol leaf extracts of guava exhibited higher inhibition on tested bacteria than aqueous leaf extracts of guava. The resistance of *Salmonella* to ethanol leaf extracts of *P. guajava* (L.) may be due to the presence of resistance genes present in the Gram negative organism. This is in contrast to the findings of Chanda & Kaneria (2011) and Oncho *et al.* (2021), where the authors reported an inhibitory effect of guava extracts on *Salmonella*.

Studies have shown that phenolic compounds, particularly flavonoids, are responsible for antibacterial properties of guava (Farhana 2017). Quercetin is one of the most predominant flavonoids of guava leaves with high pharmacological activity (Hirudkar *et al.* 2020). The presence of other components such as terpenoids, glycosides and saponins in guava extracts has been demonstrated to correlate positively with antibacterial activity (Johnson *et al.* 2017; Kumar *et al.* 2021). Similarly, water-soluble tannins present in guava leaves may act as bacteriostatic agents, with mechanism of actions such as withholding substratum, hampering oxidative phosphorylation, and extracellular enzyme inhibition (Das & Goswami 2019; Kumar *et al.* 2021). The quantitative phytochemical components of ethanol and hot distilled water leaf extracts of *P. guajava* (L.) in this study revealed differences for the same plant species. Studies have shown that the results of phytochemical analysis may differ because of various factors such as biochemical reaction within species, plant genotypes, developmental stages and geographical locations. Furthermore, variations in extraction methods are usually found in the length of the extraction period, pH, temperature, particle size, and the solvent-to-sample ratio (Taura *et al.* 2014; Oncho *et al.* 2021).

A key factor that has led to the rise and global dissemination of multidrug-resistant (MDR) bacteria is mobile antimicrobial resistance genes (ARGs). These are frequently located on plasmids, which are pieces of usually circular, self-replicating DNA that can code for a variety of different functional gene groups (Michelle *et al.* 2018). Antimicrobial resistance genes that pose the most significant threat to human medicine are typically found in Gram-negative bacteria (Michelle *et al.* 2018). Previous reports have shown that drug resistance of *Salmonella* is an emerging global challenge, especially in low- and middle-income countries where misuse and abuse of antimicrobial agents is on the increase (Ocean *et al.* 2015). Furthermore, the emergence and spread of plasmid-borne resistance genes that undergo horizontal gene transfer are threatening many last-line antibiotic therapies (Crofts *et al.* 2017). In this present study, *Salmonella* was observed to be resistant to leaf extracts of *P. guajava* (L.) and this resistance may likely be plasmid-mediated because the antibiotics sensitivity pattern of *Salmonella* after curing suggest that the plasmids responsible for resistance had been removed. This was evident in the marked sensitivity of *Salmonella* to antibiotics such as septrin, chloramphenicol, augmentin, gentamycin and streptomycin.

CONCLUSION

Antibacterial activity of ethanol and hot distilled water extract of leaf of *P. guajava* (L.) against *Shigella* and *Salmonella* isolated from water samples from the selected wells revealed that the ethanol leaf extracts of *P. guajava* (L.) had inhibitory effect on *Shigella* but not on *Salmonella.* The hot distilled water leaf extracts of *P. guajava* (L.) had no inhibitory effect on the isolates. Qualitative and quantitative phytochemical components of leaf extracts of *P. guajava* (L.) showed the presence of saponin, flavonoids, glycoside, terpenoid, steroid and tannin that contributed to the antibacterial effects of the leaf extracts of *P. guajava* (L.) on *Shigella*. This study further provides more evidence supporting the hypothesis for the use of leaf extracts of *P. guajava* (L.) in the treatment of gastrointestinal infections caused by *Shigella*. Ethanol leaf extracts of *P. guajava* (L.) may be used for treatment in combination with commercially available antibiotics against gastrointestinal infections. The potential antibacterial effects of *P. guajava* could be enhanced by extracting with ethanol instead of water as applied in the traditional practice.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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