Int Journal of Social Sciences Management and Entrepreneurship 7(2): 1031-1040, 2023

ISSN 2411-7323

www.sagepublishers.com

Characterization of Diarrhoeal Bacteria and Their Susceptibility to Guava Leaf (*Psidium Guajava)* Extract in Vihiga County, Kenya

Ilote Bruno Isiaho [1*], Dr. Opande George[2], Dr. Mureithi Loice[3]

[1]School of Science, Kaimosi Friends University;
[2] Dean, School of Science, Kaimosi Friends University;
[3]Lecturer, Department of Agricultural & Biological Sciences, Kaimosi Friends University
*Corresponding author: Ilote Bruno Isiaho. Email address: bilote@kafu.ac.ke (+254721170960)

Abstract

Introduction: Diarrhoea is a disease characterized by loose, watery stool or frequent need to a bowel movement. It may last a few days then disappear without treatment or it can be acute or chronic. It can also be caused by various parasites apart from; fungi, viruses and toxic chemicals This research was carried out within the sub-counties in Vihiga County to characterize the pathogenic intestinal bacteria causing diarrhoea and to determine the susceptibility testing of pure cultures of pathogenic intestinal bacteria identified against *Psidium guajava* leaf extracts.

Methodology: Data on pathogenic intestinal bacteria causing diarrhoea was obtained from all the Sub-County Hospitals Microbiology Laboratory records and the District Health Information System (DHIS) since 2019-2022, the etiological agents of diarrhoea in humans that had the highest frequencies were identified to be *Escherichia coli, Salmonella typhi* and *Shigella dysentriae*, and recorded. The pure cultures of the identified pathogenic bacteria were obtained from Friends Jumuia Hospital Kaimosi Microbiology Laboratory. Susceptibility testing of the obtained pure cultures and *Psidium guajava* leaf extracts was done and their different Zones of Inhibition and Minimum Inhibitory Concentration identified to ascertain if they are of medicinal value.

Results: The results showed both active principles (Carvacrol and thymol) were present in extract A; while extract B, only thymol was identified. The highest concentration in the extracts was Carvacrol with 4.3762 mg/mL in extract A. On the growth of *Salmonella typhi, Escherichia coli* and *Shigella dysentriae* on *Psidium guajava*, the highest zone of inhibition was demonstrated against *Escherichia coli* (9.23mm) by ethanol extracts of leaf. The lowest zone of inhibition was demonstrated against *Shigella dysentriae* (3.49mm) by water extracts of leaf. There was significant difference in growth inhibition among the extracts at $P \leq 0.05$. Acetone extracts had no effect on *Salmonella typhi* and *Shigella dysentriae*. *Escherichia coli* (25mg/ml) with the highest inhibition observed against *Salmonella typhi* (9.13 µM) at 100 mg/ml of acetone extracts.

Conclusion: From the study, it is evident that the Ethanolic extract of *Psidium guajava* leaf extract is of medicinal value but only in lower concentrations on some microorganism. Tests performed in this study, where Tannins, Saponins, Flavonoids and Terpernoids were identified all of which have antibacterial effects, also carvacrol which is a chemical substance with broad antibacterial activity against microorganisms inhibiting bacterial growth was present.

Keywords*: Psidium guajava, Zone of inhibition, Carvacrol and thymol*

Introduction

Acute diarrheal disease is a major cause of morbidity and mortality of children and adults residing in the tropics. The available diagnostic tests include stool microscopy for bacterial and parasitic infections, culture and sensitivity methods for bacterial infections and molecular methods for bacterial, parasitic and viral infections (Brehm, et. al., 2020).

A better understanding of bacterial pathogenesis has grown increasingly important, because of the emergence of new pathogens and increased resistance among enteric pathogens or other enteric flora (such as Vancomycin-resistant enterococci and others). Still other organisms, such as *Shigella* and *Salmonella,* have the capacity to invade epithelial cells or survive intracellularly. Among the mechanisms by which an organism may colonize and disrupt intestinal function to cause malabsorption or diarrhea are microbial attachments, localized effacement of the epithelium, production of secretory enterotoxins, production of cell-destroying cytotoxins, or direct epithelial cell invasion (Kariuki *et, al.,* 2019).

According to Lawley (2019), there is a broad range of physiological conditions in the GIT creating distinct niches for colonization by microorganisms. The indigenous microbiota differs in composition along the length of the alimentary canal. Saliva may contain up to 109 microbial cells/ml but the stomach, with its extremely low pH, is an imposing barrier against microbial entry into the lower intestinal tract.

Methodology

Study area, collection, preparation and preservation of Psidium guajava

The study was conducted in the five sub-counties (Hamisi, Emuhaya, Luanda, Sabatia and Vihiga) of Vihiga county. Plant materials of *Psidium guajava* which included leaves was collected from the farms in Vihiga County and were taken to Friends Jumuia hospital Kaimosi Laboratory for processing and analysis.

Culture medium preparation

The Hektoen Enteric Agar media which is a selective and differential media was used, it was reconstituted and then prepared as per the manufacturer's instructions, the reconstituted broth was then dispensed in conical flasks, then heat to boil until medium dissolves completely, then cool it to 40^0 c. This media is ideal since it helped isolate the preferred bacteria. The samples was inoculated into the Media using streak method on culture plates which was then incubated at 37^0c for 24 hours, then the culture plates were then observed for any growth and morphological characteristics (Akili, *et, al.,* 2019). Columbia Agar by Neogen was used for the isolation and cultivation of fastidious organisms like *Salmonella, Escherichia coli, and Shigella dysenteiae* species. It was prepared by suspending 41g of the medium in 1 litre of purified water, mix and heat to dissolve, autoclave at 121^0c for 15 minutes, add 50 mls of sheep blood to 950 mls of melted medium to make 5% of sheep blood, and cool to 40^0c, then dispense to sterile plates. After 24 hours' incubation, *Salmonella* colonies appears as blue green with black centers, *Shigella dysenteiae* species appears greener with color fading towards colony periphery, and *Escherichia coli* produced orange colonies (Kumar *et, al.,* 2019). Mueller Hinton Agar (Oxoid, UK) medium. They were kept as stock cultures in the refrigerator set at 4^0C.

Ethical consideration

Research protocol was submitted to Masinde University of Science and Technology, Institutional Ethic Review committee for approval before the study commenced. Research permit was also sought from National Commission for Science, Technology and Innovation (NACOSTI). Permission to collect data was sought from management of Vihiga County and Jumuia Hospital- Kaimosi. Further, the study was conducted in accordance with the principles of the Declaration of Helsinki and International ethical

guidelines for biomedical research involving human subjects, published by the Council for International Organisation of Medical Sciences.

Results

Gas chromatography results expressed in mg/mL concentration units

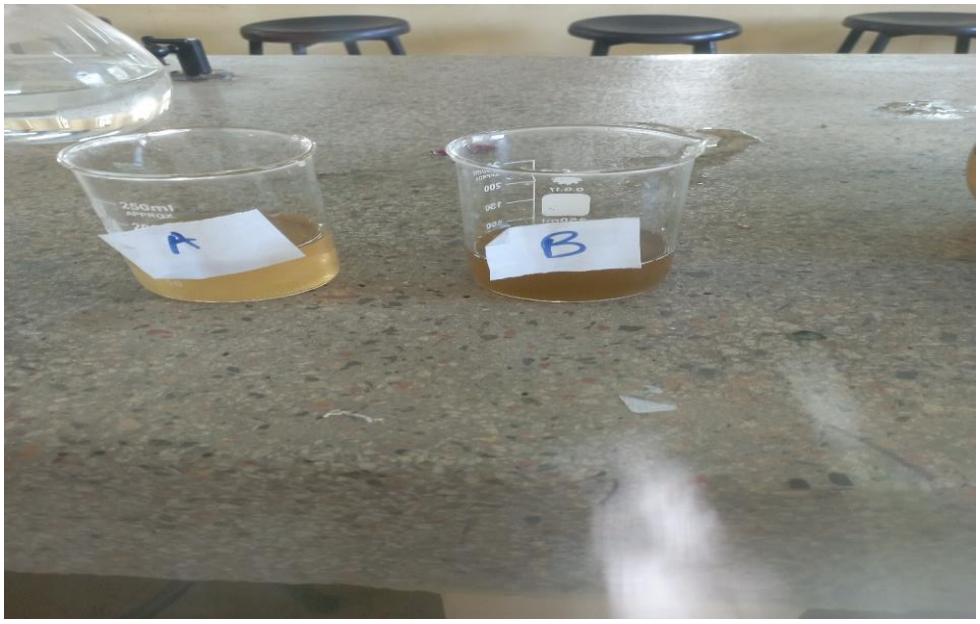

Figure 4.5: An image of *Psidium guajava* extracts A and B, (researcher 2023)

Guava leaf extract	Carvacrol	Thymol
Extract A	4.3762	1.0465
Extract B	0	0.6523

From the results both active principles (Carvacrol and thymol) were present in extract A; while extract B, only thymol was identified. The active principle with the highest concentration in the extracts was Carvacrol with 4.3762 mg/mL in extract A. Medicinal plants are commonly rich in terpenes (carvacrol, citral, linalol and geraniol) and phenolic compounds, effective compounds as food additives (Nile *et al*., 2017).

Chemical composition of guava leaf extracts by gas chromatography

Table 4.2: Secondary metabolites in leaf extracts of *Psidium guajava*

Secondary metabolite	**Presence (+) or absence (-) in guava leaves**
Alkaloids	-
Flavonoids	+
Tannins	+
Phenols	-
Saponins	+
Anthraquinones	-
Glycosides	-
Anthocyanins	-
Terpernoids	+

The chemical composition was determined using a gas chromatography (GC; AgilentTechnologies 6890N series manufactured in the USA), with a DB_WAXetr polar column, at 250^0C and 12.13 psi with a He flow of 36.5 mL min-1 after injection. The conditions for the column were: initial temperature 50^0 C, from zero to two min, increasing from 10 to 100^0C until reaching 250^0C, keeping the temperature constant for 5 min, and then descending to 50^0C for two minutes with a He flow of 1.6 mL min-1 at a pressure of 12.13 psi and an average velocity of 25 cm s-1, using an ionizing flame detector (IFD) at a temperature of 210^0C, with a flow of H_2 of 40 mL min-1 and an air flow of 450 mL min-1.

The phytochemical screening of *Psidium guajava* were carried out and the results recorded. All the tested leaf, indicated the presence and absence of phytochemical shown in table 4.2.

Table 4.3: Total Bacterial mobility

	Extract A	Extract B
+	62	46
-	16	32

(+): Presence of diffuse or total turbidity in the medium. (-): Absence or slight presence of growth

In Table 4.3, the results showed that the Carvacrol standards have broad antibacterial activity against microorganisms inhibiting bacterial growth in relation to the extract A of guava that showed an effect on the inhibition of the mobility of 62 bacteria while extract B inhibits 46 bacteria. Gallegos- Flores *et al.* (2019) reported that Carvacrol at a concentration of 0.3mM decreases the mobility of the strain determined with the Western Blot technique, where they observed a decrease in flagellin synthesis; this protein is found in 8% of the total cellular protein. According to Gallegos- Flores *et al.* (2019), *E. coli* cells grow in the presence of Carvacrol at a concentration of 5 micrometres without flagella synthesis, causing the microorganism to grow without mobility; that is, when the bacterial cell is subjected to stress caused by toxic substances and its survival is at risk; which is capable of suppressing the production of the flagellin protein and conserving energy for other cellular functions, which can therefore be a survival tactic; however, at a concentration greater than 5 micrometres, the bacteria immediately cease mobility and cell death occurs; observing that the concentration of 5 micrometres is the one with the highest number of bacteria that inhibited growth.

The study sought to find out the Carvacrol interaction that is present in extract A and B of guava leaves which shows a greater inhibition in the mobility of bacteria, as long as the concentrations of Carvacrol is concerned. The results are shown in Table 4.4.

Table 4.4: Efficiency of the extracts

Concentration			Extract A				Extract B			
				χ^2	z	p		χ^2	z	p
Carvacrol (µM)	0.3	+	127	81.41	7.84	0.001	111	71.15	5.28	0.001
		-	29				45			
	1	+	122	18.59	7.04	0.001	106	28.85	4.48	0.001
		-	34				50			
	5	+	118	78.21	6.4	0.001	102	67.95	3.84	0.001
		-	38				54			
		-	34				50			

In Table 4.4, the results show that both cases are significant, but a greater magnitude of comparison of extract A with Carvacrol is distinguished, due to the chemical compounds present. Smaller Chi

square (χ^2) magnitude represents greater similarity to the extract; therefore, extract B does not have the inhibiting effect.

Effect of Psidium guajava leaf extracts on growth of Salmonella typhi, Escherichia coli and Shigella dysentriae

The study sought to find out the effect of *Psidium guajava* leaf extracts on growth of *Salmonella typhi, Escherichia coli* and *Shigella dysentriae.* The results are shown in Table 4.5

Table 4.5: Diameter of zone of inhibition (µM) exhibited by *Psidium guajava* leaf extracts against *Salmonella typhi, Escherichia coli* and *Shigella dysentriae*

	Leaf		
	Ethanol	Distilled Water	Acetone
Salmonella typhi	6.13	3.99	0
Escherichia coli	9.23	7.29	6.34
Shigella dysentriae	6.11	3.49	0
LSD $p \leq 0.05$	0.018		

Table 4.5 shows the data presented in the means of three replicates. The results of effect of *Psidium guajava* leaf on growth of *Salmonella typhi, Escherichia coli* and *Shigella dysentriae.* The highest zone of inhibition was demonstrated against *Escherichia coli* (9.23mm) by ethanol extracts of leaf. The lowest zone of inhibition was demonstrated against *Shigella dysentriae* (3.49mm) by water extracts of leaf. There was no inhibition exhibited on *Salmonella typhi, and Shigella dysentriae* by acetone extracts of leaf. There was significant difference in growth inhibition among the extracts at P≤0.05.

Effect of different concentrations of leaf, extracts of water, ethanol and acetone on the growth (µM) of Salmonella typhi, Escherichia coli and Shigella dysentriae.

The disc diffusion method of Mueller Hinton agar was used to determine the antimicrobial activity of *Psidium guajava* leaf extracts with different concentrations as shown in Table 4.6

Table 4.6: Effect of different concentrations of leaf, extracts of water, ethanol and acetone on the growth (µM) of *Salmonella typhi, Escherichia coli* and *Shigella dysentriae.*

Plant part	Extract	Microorganisms	Concentration Mg/ml			
			25	50	75	100
			Zones of inhibition			
Leaf (*Psidium guajava)* extract	Ethanol	*Salmonella typhi*	7.12	4.14	2.16	3.15
		Escherichia coli	11.34	9.25	7.16	7.93
		Shigella dysentriae	0	0	0	0
	Distilled Water	*Salmonella typhi*	8.15	7.11	0	5.78
		Escherichia coli	0	5.13	0	8.13
		Shigella dysentriae	0	0	0	0
	Acetone	*Salmonella typhi*	0	0	0	0
		Escherichia coli	0	8.87	7.94	9.13
		Shigella dysentriae	0	0	0	0

Data presented are the means of three replicates. As shown in table 4.6, increase in the concentration has different effects on the microorganism, plant part and extract used. Leaf extracts did not show any growth inhibition on *Shigella dysentriae* in all the concentrations of the ethanol extract, the highest inhibition was observed in 25 mg/ml ethanol extract on *Escherichia coli* (11.34 µM) and the lowest inhibition was observed in 75 mg/ml ethanol extract on *Salmonella typhi (*2.16 µM). *Shigella dysentriae* didn't show any inhibition in all the concentrations of waters extract, *Salmonella typhi* showed the highest inhibition (7.11 µM) at 50 mg/ml of water extract while the lowest inhibition was against *Escherichia coli* (5.13 µM) at 50 mg/ml of water extract. Acetone extracts had no effect on *Salmonella typhi* and *Shigella dysentriae*, *Escherichia coli* (25mg/ml) with the highest inhibition observed against *Escherichia coli* (9.13 µM) at 100 mg/ml of acetone extracts.

Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration (MIC) of various extracts of leaf, of *Salmonella typhi, Escherichia coli* and *Shigella dysentriae.* The results are shown in Table 4.7.

Table 4.7 Shows Minimum Inhibitory Concentration (MIC) of various extracts of leaf

Plant part	Microorganisms	Minimum Inhibitory Concentration (mg/ml)		
		Water	ethanol	Acetone
Leaf (*Psidium guajava)* extract	*Salmonella typhi* $p \leq 0.05$	0.017	0.025	0
	Escherichia coli $p \leq 0.05$	0.025	0.018	0.1
	Shigella dysentriae $p \leq 0.05$	0	0	0

Table 4.7 shows Minimum Inhibitory Concentration (MIC) of various extracts of leaf, of *Psidium guajava* on the microorganisms, the MIC varies with the microorganism, leaf extract. The MIC values varied from 0.017-0.1 mg/ml for the three extracts. Lowest MIC value 0.025 mg/ml was recorded against *Escherichia coli* and *Salmonella typhi* where against *Salmonella typhi* the lowest MIC observed was 0.017 mg/ml. ($P \leq 0.05$).

Minimum Inhibitory Concentration (MIC) is the lowest concentration able to completely inhibit any visible microorganism growth after overnight incubation with media (Chima *et al*., 2016). The MIC result showed that increasing concentration has an increasing effect in inhibiting the organisms used. Since the MIC values indicated the definite nature of the antimicrobial activities of guava leaf, the inhibition zones values, only indicated the extent of effectiveness of the extract with increasing concentration.

Analysis of Variance of Antimicrobial activity of Psidium guajava, leaf extract on Salmonella typhi, Escherichia coli and Shigella dysentriae.

The statistical analysis examined whether there was a consistent and significant relationship between any of the indicator bacteria and the risk of diarrhoeal disease. Initially, estimates of the concentrations of these bacteria in each water, acetone and ethanol sample were obtained from the number of colonies on the membrane filters. Their density per 100 ml was calculated from the filter colony counts of the three sample dilutions, assuming that the average density was independent of the volume of water filtered and that the number of colonies per plate.

Table 4.8: Analysis of Variance

Model	R Square	Root MSE	Coefficient Variance	Inhibition mean
1	.75	1.673	34.67	6.672

Model		Sum of Squares	Df	Mean Square	F	Sig.
Model	Regression	3040.311	1	23040.311	38.470	.001
	Residual	1169.619	349	31.200		
	Total	4209.931	350			

The study found that the p-value in the analysis is 0.001 which is smaller than the chosen level of significance of 0.05. If the p-value is less than the given significance level, then there is sufficient evidence to conclude that the difference in variances is significant. The overall model explains 75% variation of indicator bacteria and the risk of diarrhoeal disease, and it is not significantly useful in explaining indicator bacteria in *Psidium guajava*, leaf extract, F (1, 1349) = 38.470, $p < .05$. The F-ratio was used to determine the degree of variation contributed by each factor. The factors and their respective interactions were considered in the experimental design as statistically not significant effects at 95% confidence limit. By study of the main effect of each factor the general trends of the influence of the factors toward the process can be characterized.

Discussions

This study dueled on the characterization of diarrhoeal bacteria and their susceptibility to guava leaf (*Psidium guajava)* extract in Vihiga County, Kenya. Phytochemical screening of medicinal plants is very important in identifying the new sources of therapeutically and industrially important compounds. It is imperative to initiate urgent steps for screening of plants for secondary metabolites. The medicinal value of plants lies in some chemical substances that produce a definite physiological action on the human body that varies in different persons (Ekaiko *et al*., 2015a). Phytochemical screening of the leaf extracts of the *Psidium guajava* revealed that the plant contains Flavonoids, Saponins, Tannins, and Terpernoids compounds which was in agreement with Ekaiko *et al*. (2015a).

Flavonoids, Saponins, Tannins, and Terpernoids were isolated and identified from the leaf extracts and are commonly known to have antimicrobial properties (Sikandar *et al.,* 2013). The present study showed that the different parts of *Psidium guajava* possess antimicrobial potential against *Salmonella typhi*, *Escherichia coli* and *Shigella dysentriae,* this may be due to the disruption of the cell wall formation which consequently causes the leakage of cytoplasmic constituents (Chima *et al*., 2016). In line with the present finding, several other studies have reported *Psidium guajava* leaves to have antimicrobial potentials (Chima *et al*., 2016). Results of this study revealed very significant growth inhibition activity with the extracts demonstrating broad spectrum of activity against both bacteria *Escherichia coli* and *Shigella dysentriae.*

In the bacterial test, it was observed that the potency of the activity of *Psidium guajava* leaves extracts against microbes depends on the extraction solvent used. *Psidium guajava* parts in organic extracts such as ethanol and acetone was more effective than *Psidium guajava* in water extracts. This may be due to the better solubility of the active components in organic solvents. The ethanol extracts demonstrated a higher activity than the acetone and water extracts, the better efficacy of the ethanol extract against the acetone and water may be because different solvents have different polarities, hence different degrees of solubility for the various phytoconstituents (Ruhama, 2014). Based on the limited spectrum of activity of the other extracts (acetone and water) compared with the ethanol extracts, it suggested that the active component is more soluble in ethanol than in the other solvents. This is in agreement with Ekaiko *et al*. (2015 b).

Not all the extracts prepared from leaf using acetone, water, and ethanol exhibited antibacterial activity against the tested microorganisms, *Escherichia coli* exhibited higher inhibition with ethanol extract of leaves. There was no inhibition exhibited by acetone, water and ethanol extracts of leaf against *Shigella dysentriae.* This may be due to water, acetone and ethanol not being able to extract the antibacterial chemicals present in the leaf extract (Naomi, 2014). These results are in agreement with results of Yahaya *et al*., 2017. The study showed that increased concentration either increased or decreased the zone of inhibition for the tested microorganisms depending on the extracts used.

Conclusion

From the study, it is evident that the Ethanolic extract of *Psidium guajava* leaf extract is of medicinal value but only in lower concentrations on some microorganism. From the various tests performed in this study, where Tannins, Saponins, Flavonoids and Terpernoids were identified all of which have antibacterial effects, also carvacrol which is a chemical substance with broad antibacterial activity against microorganisms inhibiting bacterial growth was present.

There was a significant correlation between the *Psidium guajava* leaf extracts and the organisms obtained on sensitivity testing where the differences obtained on their various Zones of Inhibition was so evident and significant giving a P-value of ≤ 0.05.

Disclosure

The author reports no conflicts of interest in this work.

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