Exploring the Antimicrobial Properties of Guava leave extract against selected Food Pathogens

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ABSTRACT

Guava (*Psidium guajava L.*), is an important tropical plant with diverse benefits. In this study, the antimicrobial properties of guava leaf extract were explored against some food-borne pathogens (*E. coli, Salmonella,* and *S. aureus*). Three different extracts (water, methanol and ethanol) were obtained from the guava leaves by maceration for 3 days. The phytochemical content and antioxidant activity of the extracts were evaluated. The antimicrobial susceptibility and minimum inhibitory concentration (MIC) of the extracts were also determined. The phytochemical screening revealed constituents such as phenols and tannins, flavonoids, terpenoids, saponins, and glycosides. Phenolic content was highest in the methanolic extract (0.261 mg/ml) while flavonoid content was highest in the ethanolic extract (16.41mg/ml). The aqueous and methanolic extracts recorded the highest zone of inhibition (ZOI) against S. aureus while the aqueous was ineffective against *E. coli* and *Salmonella* and the methanolic extract recorded no ZOI against *Salmonella*. However, the ethanolic extract showed the highest ZOI against E. coli. The MIC of the methanolic extracts was 50% against *S. aureus*, 30% against *E. coli*, and 10% against *S. aureus*. For the ethanolic extract, it was 50% against *E. coli*, 20% against *S. aureus*, and 70% against *Salmonella*. The phytochemicals in the extracts contributed to their antioxidant and antibacterial activities. The ethanolic extract of the guava leaves was most effective against the selected foodborne pathogenic microorganisms. Both gram-positive and negative bacteria were susceptible to the extracts, although some gram-negative bacteria were resistant at certain concentrations of the guava leave extracts.

Keywords: guava, antimicrobial, Salmonella, antioxidant, ethanolic, flavonoid

1.0 INTRODUCTION

Food preservation is instituted on the need to improve food quality and safety by preventing spoilage and reducing the risks of foodborne illnesses (Desrosier & Desrosier, 1977). This dual challenge results from the intrinsic properties of food and the threat of microbial contamination. Foodborne pathogens such as *Escherichia coli, Staphylococcus*

aureus, Salmonella, Listeria monocytogenes, and *Shigella*, present a significant threat as causal agents for a range of foodborne diseases (Bracket, 1999). To address this challenge, various preservation methods have been developed over the years, including the use of food preservatives with some being classified as antimicrobials, antioxidants, and anti-browning agents. Antimicrobials are vital in preserving food quality by inhibiting or preventing microbial growth in food products (Gould, 1996). However, antioxidants have become essential agents in the battle against food deterioration, mostly because of their ability to prevent reactions that can lead to undesirable changes in the food. Recently, there has been a shift towards the use of natural ingredients obtained from plants, given their abundance and role in food preservation. Guava leaves, contain bioactive compounds that hold great potential for preserving food quality and enhancing its safety (Harborne, 2012). Its bioactive constituents which include flavonoids, tannins, saponins, phenolic acids, and terpenoids, offer varied advantages, ranging from anti-inflammatory and antioxidant effects to antimicrobial properties against a range of pathogens including those associated with food (Harborne, 2012). The evaluation of plants or their parts in the form of extracts has the potential to extend the shelf life of various food products. Extraction is fundamental to obtaining valuable natural compounds from raw materials. Such techniques include solvent extraction and distillation (Chemt & Strube, 2015). The solvent extraction technique is mostly used and involves stages such as penetration, dissolution, diffusion, and collection. The efficiency of these extraction processes depends on the choice of solvent, particle size, temperature, duration (time,) and solvent-to-solid ratio (Vermerris & Nicholson, 2017). The extraction of bioactive compounds from guava leaves can be achieved using the maceration method. This involves allowing the solvent to diffuse into the solid plant material (dried guava leaves powder), and facilitates the extraction of compounds (Carson, 2002). The extracts obtained can be applied as antimicrobials. Antimicrobials function by disrupting bacterial cell membranes and interfering with the metabolic processes of microorganisms. Hence, these plant extracts can enhance the safety of food and prevent spoilage as natural antimicrobials (Alberts, 2004).

Foodborne pathogens present a major challenge in the food industry, particularly in the field of food preservation (Hintz *et. al*, 2015). Simultaneously, it stands out as the major cause of foodborne illnesses, especially in developing countries. Most of these foodborne illnesses are caused by gram-negative (such as *Salmonella*, *Escherichia coli*, *Pseudomonas*) and gram-positive bacteria (such as *Staphylococcus aureus* and *Bacillus cereus*) (Al-Bayati, 2008). Chemical preservatives have commonly been used to prevent the growth of food spoilage microbes in the food industry. However, the use of these synthetic preservatives has raised concerns among consumers due to their potential toxicity. In spite of their effectiveness, their frequent

use has become of great concern because of issues related to residues in the food, development of microbial resistance, and its adverse effects on human health (Mostafa, 2018). Some of the chemical preservatives have been found to have carcinogenic and teratogenic traits as showing residual toxicity (Sharifzadeh *et. al*, 2016). As a result of some of these features, the food industry is compelled to reduce or eliminate the use of synthetic preservatives and find natural alternatives that can ensure food safety.

Moreover, emergence of resistance to antibiotics by bacterial species does not only affect public health but also makes it challenging to control food spoilage and treat associated illnesses. Plants have been used as traditional remedies in various cultures and populations around the world. Some plants from which antimicrobials have been obtained include moringa, neem, thyme, pomegranate (Chandra *et. al*, 2017). Plant extracts are not only effective against a particular disease but also provide health benefits owing to their antioxidant properties (Dillard & German, 2000). Additionally, plant extracts are often perceived to trigger less adverse reactions compared to synthetic pharmaceuticals (Holaskova *et. al*, 2015). Plant extracts are now being considered as potent agents against foodborne pathogens. For this study, we explored the potential use of guava leaf extract as an alternative in the preservation of food through the inhibition of the growth of selected food pathogens. Specifically, the study investigated the antimicrobial effectiveness of guava leaf extract in inhibiting the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella*.

2.0 MATERIALS AND METHOD

2.1 Preparation of guava leaves extracts

Guava leaf samples (1.8 kg) were collected from a guava tree (*Psidium guajava)* located at Offei Nkansah at Akropong Akuapem in the Eastern Region of Ghana. Random leaf samples were collected into labeled plastic zip lock bags and stored on ice during transportation to the laboratory for extraction.

The guava leaves were washed and kept under a shade at room temperature to until it was dry and then milled into powder. Three solvents were prepared: ethanol (70%), methanol (70%), and distilled water, and were used for the extraction procedure (maceration). The guava leaves powder was added to each of the solvents in the ratio 1:10 (100g of powder: 1 litre of solvent) in sterile Erlenmeyer flasks and mixed thoroughly. The flasks containing the mixture were wrapped with aluminum foil to limit exposure to light, and placed in the dark for 3 days at room temperature. The mixtures were then filtered, transferred into centrifuge tubes, and centrifuged (SORVALL LEGEND X1R Centrifuge, Thermo SCIENTIFIC) for 10 minutes at 4,000 rpm at 25℃. The supernatant was collected, concentrated using a rotatory shaker incubator (3500I Incubating Orbital Shaker, VWR International,) and stored at 4℃ until further use (Biswas et al. 2013).

2.1 Phytochemical screening

Test for saponins: One milliliter (1 mL) of the extract was placed in a test tube and shaken vigorously. The formation of stable foam was as an indication of the presence of saponins (Trease and Evans, 1989).

Test for phenols and tannins: Three milliliters (3 mL) of the extract was mixed with 2mL of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins (Ngene et al., 2019)

Test for terpenoids (Salkowski's Test): One milliliter (1 mL) of the extract was mixed with 2 mL of chloroform. Then 2 mL of concentrated sulfuric acid (H_2SO_4) was added carefully and shaken gently. A reddish-brown coloration formed at the interphase indicated positive results (Trease and Evans, 1989).

Test for flavonoids: One milliliter (1 mL) of extract was mixed with 4 drops of 10% NaOH solution and heated in a water bath for 10 min. The intense yellow color formed which became colorless upon the addition of 10 drops of 1% Hydrochloric acid (HCl) shows the presence of flavonoids (Ngene et al., 2019)

Test for glycosides: One milliliter (1 mL) of extract was mixed with 2 mL of glacial acetic acid containing 2 drops of 2% $FeCl_3$. The mixture was poured into another tube containing 2 mL of concentrated sulfuric acid (H_2SO_4). The formation of a brown ring at the interphase indicated the presence of glycosides (Trease and Evans, 1989).

2.2 Determination of phenolic content of extract

The Folin–Ciocalteu method with a modification was used to determine the phenolic compound content of the samples (Ainsworth & Gillespie, 2007). About 1 mL of each extract was diluted with 2 mL distilled water and 0.5mL of Folin–Ciocalteu reagent. After 3 minutes, 0.5mL of 10% Na_2CO_3 solution was added to the mixture, and the mixture was allowed to stand for 1 hr at room temperature in the dark. The absorbance of the extract was measured at 760 nm with a UV–visible spectrophotometer (CL laboratory incubator, Pol-Eko-Aparatura). Gallic acid (as a standard) was used for the construction of a calibration curve. Results were expressed as mg gallic acid/g of extract (Sofowora, 1996). The tests were run in triplicate and averaged.

2.3 Determination of flavonoid content of extract

Total flavonoid content was determined by the Aluminium chloride method using catechin as a standard. About 1 mL of the test sample (extract) and 4 mL of water were mixed in a volumetric flask. After 5 minutes, 0.3 ml of 5% Sodium nitrite, and 0.3 ml of 10% Aluminium chloride was added. After 6 minutes of incubation at room temperature, 2 mL of 1M Sodium hydroxide (NaOH) was added to the reaction mixture. Immediately, the final volume was made up to 10 mL with distilled water. The absorbance of the reaction mixture was measured

spectrophotometrically (CL laboratory incubator, Pol-Eko-Aparatura) at 510 nm against a blank. Results were expressed as catechin equivalents (mg catechin/g dried extract) (Sofowora 1996).

2.4 Determination of antioxidant activity using 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay

Varying concentrations of the guava leave extract (1 ml) were added to a DPPH-methanol solution (5mg/100ml, 2ml). The decrease in absorbance at 517nm was measured with a UV-visible spectrophotometer (S- 2150-UV, UNICO). The DPPH scavenging activity was calculated using the equation as follows: DPPH scavenging activity (%) = $[1\text{-}A_{sample} /A_o] \times 100$, where A_{sample} is the absorbance of the sample solution (extract) and A_o is the absorbance of the DPPH solution before the addition of the extract.

2.5 Preparation of microbial culture

The microorganisms namely *E. coli*, *Salmonella*, and *Staphylococcus aureus* were used to test the effectiveness of the antimicrobial properties of the guava leaf extracts by measuring their capacity to inhibit their growth. Each of the bacterial strains was cultured onto nutrient agar plates and incubated for 18 to 24h at 37°C to obtain colonies. After overnight incubation, colonies of each bacterium were selected with sterile inoculating loop, transferred to a glass tube containing sterile physiological saline, and vortexed thoroughly. The turbidity of each bacterial suspension was then adjusted and compared to that of 0.5 McFarland standard solution (containing about 1.5×10^8 CFU/mL).

2.6 Antibacterial activity of the extracts

Antimicrobial susceptibility testing (AST) was done using the disc-diffusion method (Beaur et al., 1959). The leaf extracts were tested on Mueller Hinton Agar (MHA) plates to detect the presence of antibacterial activity. Sterile cotton swab was dipped into the adjusted bacterial suspension and rotated several times by pressing firmly on the inside wall of the tube above the fluid level. The dried surface of the MHA plates were inoculated with the test bacteria which had been previously adjusted to the 0.5 McFarland standard solution by seeding method with the cotton swab. Impregnated paper discs (6 mm filter paper disc soaked with each extract) were placed on the surface of the inoculated agar plates. For each bacterial strain, controls were made with discs impregnated with pure solvents instead of the extract. The plates were placed in an incubator (CL laboratory incubator, Pol-Eko-Aparatura) at 37°C. After 24 hours of incubation, the plates were examined for inhibition zones. A ruler was used to measure the inhibition zones in centimeters (cm) and the average recorded.

2.7 Determination of minimum inhibitory concentration (MIC) of the extracts

A known amount of nutrient agar was prepared, autoclaved, and poured into labeled petri dishes to set. Different concentrations of the various guava leaf extracts were prepared (100%, 90%, 70%, 50%, 30%, 20%, 10%, 5%, 2%, 1%). The previously adjusted bacterial strains were used and sterile cotton swabs were used to streak the surface of the set agar plates. Sterile paper discs were dipped into each concentration of the guava leave extracts and placed on the surface of the agar. The plates were placed in an incubator (CL Laboratory incubator, Pol-Eko-Aparatura) at 37℃. After 24 hours of incubation, each plate was examined for inhibition zones, measured with a ruler and the average recorded. The lowest concentration of the extract which inhibited the growth of the test microorganism (evidenced by the presence of zone of inhibition) was determined as the MIC for each type of the guava leaf extract.

2.8 Data and Statistical Analysis

All analyses were performed in duplicates and the results were presented as mean± standard deviation. The data collected was collated using Microsoft Excel 2023. Data were graphically presented as tables, and bar graphs.

3.0 RESULTS AND DISCUSSION

3.1 Screening of Phytochemicals

The results obtained after the phytochemical screening of the guava leaf extracts are presented in Table 1. Guava leaves have been confirmed to contain bioactive phytochemicals, which include flavonoids, tannins, saponins, phenolic acids, terpenoids, and glycosides (Harborne, 2012). These phytochemicals often require polar solvents such as ethanol, water, and methanol for extraction since they are polar (Fawcett, 2004). The study results revealed the presence and absence of some of the phytochemical compounds in the three different extracts (Table 1). The methanolic extract contained all the phytochemicals except terpenoids, the ethanolic extract contained all the phytochemicals except glycosides, and the distilled water contained all the phytochemicals except flavonoids (Table 1).

Table 1: Phytochemical constituents of guava leaf extracts

Extracts	Saponins	Phenols and Tannins	Terpenoids	Flavonoids	Glycosides
Methanol	+	+	-	+	+
Ethanol	+	+	+	+	-
Distilled water	+	+	+	_	+

+: presence of constituent and **-:** absence of constituent

This observation may be attributed to conditions such as the choice of solvent and its solubility, type of the phytochemical compound, and the presence of interfering substances (Harborne, 2012). However, the absence of a particular phytochemical does not particularly deduce its absence. For example, the absence of flavonoids in the distilled water extract can be attributed to the polar nature of flavonoids and their poor solubility in water (Ginting *et al*, 2022). Flavonoids are usually more soluble in organic solvents (such as methanol and ethanol) due to their hydrophobic properties (Ferreira & Pinho, 2012).

3.2 Phenolic content of extracts

The phenolic concentrations of the various extracts are presented in Figure 1. From Figure 1, the methanolic extract had the highest phenolic concentration (0.261 mg/ml), followed by distilled water extract (0.233 mg/ml), and lastly ethanolic extract (0.217 mg/ml). The choice and polarity of solvent affected the extraction of the phenolic compounds. The methanolic extract had a higher phenolic concentration as compared to the water-based extract due to its ability to dissolve a wide range of phenolic compounds (Higuchi *et al*, 2016). The ethanolic extracts yielded a substantial concentration, although it was slightly lower than the concentration measured for the methanolic extract (Figure 1). It was expected that the ethanolic extracts will yield high phenolic concentration compared to the distilled water extracts (Singh, 2022), but in this study, the opposite was observed. This could be a result of the varying solubilities of the phenolic compounds in ethanol. Furthermore, the selective extraction of certain compounds depends on their polarities, and reaction with ethanol (Singh, 2022). On the other hand, water, being a polar solvent, may have enhanced the overall extraction efficiency for the range of phenolic compounds in the extracts. Water has higher polarity relative to methanol and ethanol and it is expected to extract majority of the phytochemicals.

3.3 Flavonoid content of extracts

The estimated flavonoid concentration of the extracts is presented in Figure 2. The ethanolic extract recorded the highest flavonoid concentration (16.41 mg/ml), followed by distilled water extract (13.87 mg/ml), and lastly, methanolic extract (12.36 mg/ml). The results were inconsistent with other studies which reported that the flavonoid content of water extract is usually higher than the ethanolic and methanolic extracts. As observed in Figure 2, water extract had a slightly higher flavonoid concentration than methanolic extract. This is because water has higher polarity than methanol and therefore has a higher affinity for flavonoids than methanol. Nyirenda *et al*. (2012) from their study, reported that polar compounds such as flavonoids were more soluble in aqueous solvents than in organic solvents. The variation as observed in this study may be due to the concentration of solvent used. In this study, ethanol (70%) and methanol (70%) were used as solvents for the extraction.

Solvents at a concentration of 70% creates a balance between the polarity of water (a polar solvent) and the organic solvent (ethanol or methanol). This intermediate polarity increases the solubility of a range of compounds (Marcus, 2002). Ethanol as an organic solvent is effective at dissolving a varied range of flavonoid compounds than water or methanol and has the ability to pervade the plant matrix and solubilize flavonoids; this enhanced solubility can lead to detection of higher flavonoid concentrations in ethanolic extracts (Higuchi *et al*, 2016).

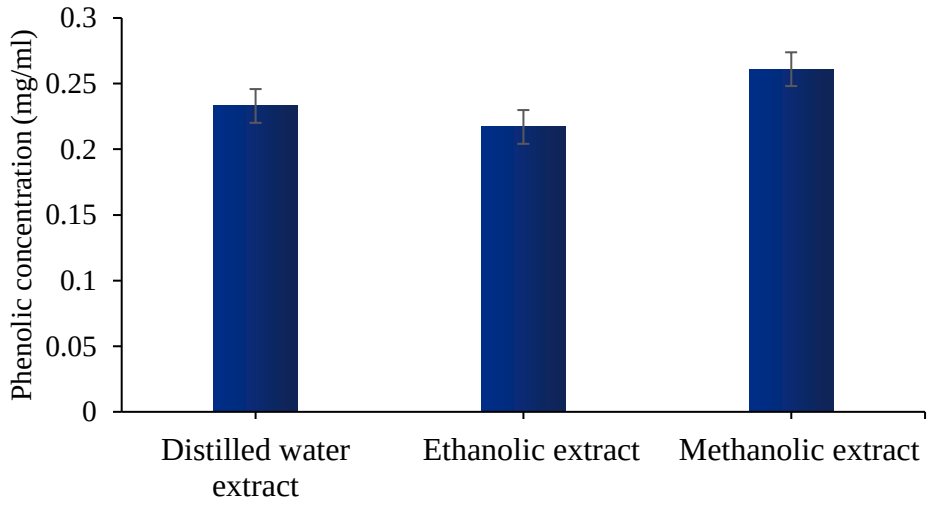

Figure 1: Phenolic concentration of guava leaves extracts

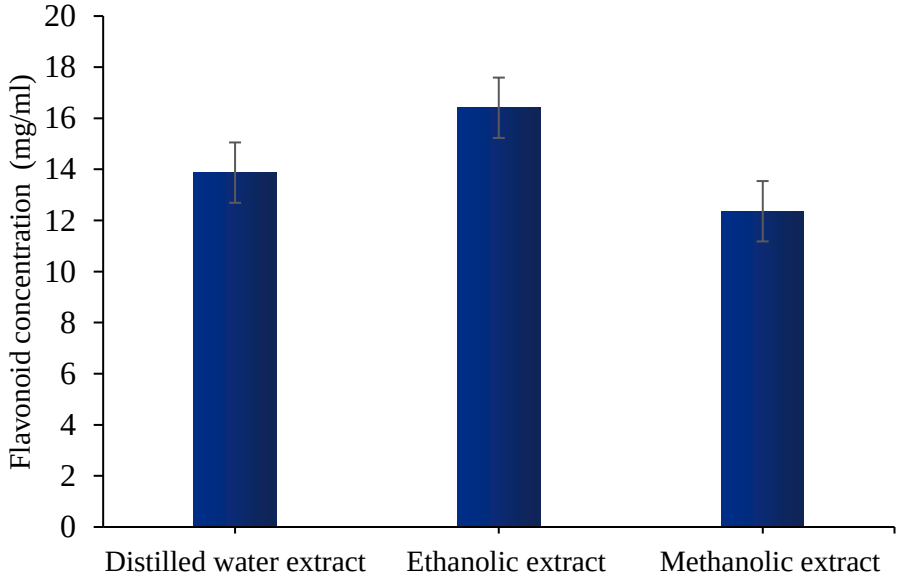

Figure 2: Flavonoid concentration of guava leaves extracts

3.4 Antioxidant activity of extracts

The antioxidant activities of the different guava leaf extracts were evaluated (Figure 3). DPPH (2,2-diphenyl-1-picrylhydrazyl) is a widely used chemical compound for antioxidant assays. It is a stable free radical that accepts electrons or hydrogen atoms (Yeo & Shahidi, 2019). It undergoes reduction via redox reaction when it reacts with antioxidants (Gülcin, 2012). This reduction of DPPH radicals can be used to measure the ability of the antioxidants to scavenge free radicals. Free radicals are harmful molecules that can cause oxidative stress and damage in the body (Gülcin, 2012). From Figure 3, it can be observed that the various extracts showed similar activity. This is because ethanol and methanol solvents have similar polarity towards the flavonoids and phenols in the guava leave and hence are able to dissolve most of these compounds (Hano, 2020). Distilled water, although is highly polar, also dissolves flavonoids and phenols and hence showed antioxidant activity similar to that of the methanolic and ethanolic extracts of the guava leaves (Hano, 2020). Some variabilities in the antioxidant activities of extracts have been attributed to the type of solvent used, incubation time, concentration of the DPPH solution, as well as temperature (Hano, 2020). The stability of plants extracts during storage can impact their antioxidant activity (Hano, 2020). If extracts are not stored appropriately under the right conditions or if they degrade during storage, it can lead to fluctuations in the antioxidant activity.

3.5 Antibacterial activity of extract

The antibacterial activities of the different guava leaf extracts against E. *coli*, *Salmonella*, and *Staphylococcus aureus* were investigated (Table 2). The zone of inhibition (ZOI) is the clear area or zone around the disc impregnated with the antimicrobial where the microorganism did not grow and this indicates its susceptibility (Belkum *et al*, 2019). From Table 2, it can be observed that *E. coli* was resistant to the distilled water extract showing no ZOI, but it was susceptible to the ethanolic and methanolic extracts. From Table 1, Figure 1 and 2, distilled water showed high flavonoid and phenolic content. However, this low antibacterial activity observed may be due to the inability of the distilled water to efficiently extract particular antibacterial phytochemicals from the guava leaves because of its lower affinity for these phytochemicals (Fawcett, 2004).

From this study, E. coli was susceptible to the methanolic and ethanolic extracts and this could be as a result of the strain variability of E. coli (Lin et al, 2015). Some strains may be more susceptible due to genetic differences. Also, the resistance exhibited by gram-negative bacteria such as E. coli could be attributed to its cell wall structure which provides a barrier against penetration by the plant extract (Biswas et al, 2013). From Table 2, S. aureus was observed to be sensitive to all three extracts but the water extract barely showed any activity. This observation was similar to the findings of Nwanneka et al., (2013), who also found that the ethanolic and methanolic extracts of guava leaves were most effective against S. aureus.

Only the ethanolic extract showed efficacy against the generic *Salmonella* (Table 3). *Salmonella* is a gram-negative bacterium and it is having been noted to be more resistant to antimicrobials originating from plant materials and even shows no effect in some instances compared to gram-positive bacteria (Biswas *et al.*, 2013). From Table 2, we can also observe that the ethanolic extract was the most effective since it could inhibit all the microorganisms tested.

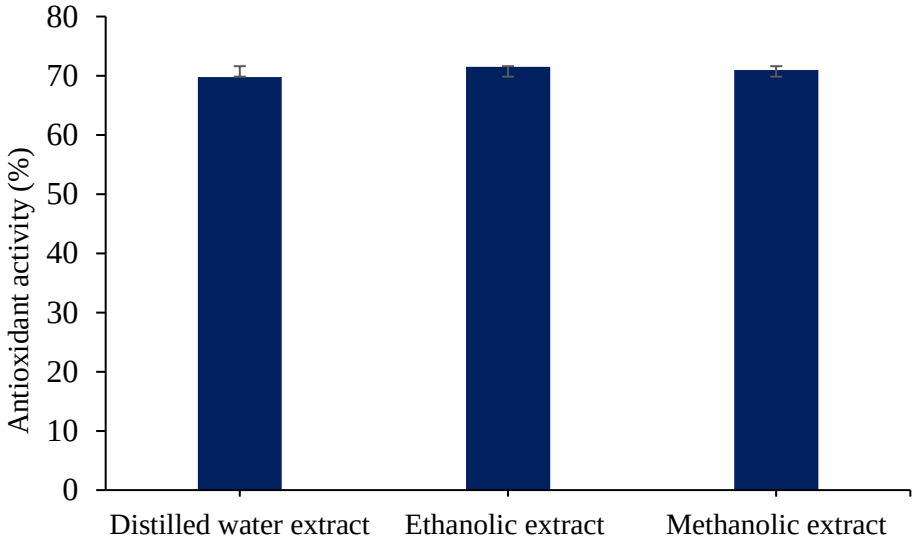

Figure 3: Antioxidant activity (%) of guava leave extracts

Table 2: Zone of inhibition (ZOI) for guava leave extracts

	Methanol Extract	Ethanol Extract	Distilled Water Extract
	M_M (mm)	E_M (mm)	D_M (mm)
E. coli	12.5±1.06 (S)	15.2±0.11(S)	0.00±0.00 (R)
S. aureus	13.0±0.57 (S)	9.5±0.07 (I)	7.00±0.07(I)
Salmonella	0.00±0.00 (R)	12.0±0.14 (S)	0.00±0.00 (R)

M_M: mean ZOI for methanolic extract, E_M: mean ZOI for ethanolic extract, $D_{M:}$ mean ZOI for water extract. Very susceptible (VS) (ZOI: >20mm), Susceptible (S) (ZOI: 10-20mm), Intermediate (I) (ZOI: 5-10mm), Resistant (R) (ZOI: <5mm) (modified ZOI classification by Indriani et al., 2020)

3.6 Minimum inhibitory concentration (MIC) of extracts

The inhibitory activity of different concentrations of the guava leave extracts against E. *coli, Salmonella* and *Staphylococcus aureus* were determined (Table 3). The MIC is described as the lowest concentration of an antimicrobial agent that hinders the noticeable growth of microorganisms (Kowalska & Dudek-Wicher, 2021). The methanolic extract showed moderate inhibitory effect against *E. coli* above 90%. However, no inhibition was observed below 30% (Table 3). The ethanolic extract exhibited a similar pattern to the methanolic extract against *E. coli*. The distilled water extract showed no inhibitory effect against *E. coli* across all the concentrations tested. The methanolic extract showed strong inhibitory effects against *S. aureus* at a minimum concentration of 10% (Table 3). The ethanolic extract exhibited inhibitory effect against *S. aureus* at a minimum concentration of 20%. The distilled water extract showed inhibitory effect against *S. aureus*, at a minimum concentration of 50% (Table 3). Only the ethanolic extract was effective against generic *Salmonella* at a minimum concentration of 70% (Table 3). The presence of lipopolysaccharides (LPS) in the outer membrane of *Salmonella* cells can act as a barrier preventing the penetration of the extract and this may have contributed to the no inhibition observed for the distilled water and methanolic extracts**.**

Table 3: Minimum inhibitory concentration (MIC) of guava leaf extracts

Conc. (%)	*E. coli* (cm)			*S. aureus* (cm)			*Salmonella* (cm)		
	M	E	D	M	E	D	M	E	D
100	1.5	1.7	-	1.5	1.7	1.1	-	1.7	-
90	1.3	1.5	-	1.5	1.7	1.3	-	1.9	-
70	1.1	1.5	-	1.2	1.1	1.1	-	1.5	-
50	0.7	1.2	-	0.9	0.9	0.8	-	-	-
30	0.4	-	-	0.6	0.7	-	-	-	-
20	-	-	-	0.4	0.5	-	-	-	-
10	-	-	-	0.2	-	-	-	-	-

M: methanolic extract, E: ethanolic extract, D: distilled water extract, -: no zone of inhibition

4.0 CONCLUSION

Guava leave extracts contain phytochemicals such as saponins, flavonoids, glycosides, terpenoids, and phenols and tannins. The choice of solvent impacted the extraction of these phytochemicals from the guava leaves. The antioxidant activity correlated with the phenolic content, as methanolic extract displayed the highest antioxidant activity, due to its high phenolic content. The ethanolic extract was the most effective as it showed significant inhibition against *E. coli*, generic *Salmonella* and *S. aureus*. However, the methanolic extract exhibited moderate antibacterial activity, particularly against *E. coli* and *S. aureus.* Distilled water extract showed little to no activity against *S. aureus* at higher concentrations (up to 100%) while the ethanolic extract showed MIC at 50% for *E. coli*, 20% for *S. aureus*, and for *Salmonella* at 70%, making it the most effective extract against the microorganisms tested.

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