Synergistic antibacterial effect of ethanolic extract of Carica papaya and Psidium guajava leave extract on Salmonella typhi, Escherichia coli and Klebsiella pneumoniae

Article · September 2022

DOI: 10.56781/ijsrls.2022.1.1.0025

CITATIONS
0

READS
258

3 authors, including:

Alexander Philip
Bingham University
18 PUBLICATIONS **66** CITATIONS
SEE PROFILE

Lois Zakari
Bingham University
1 PUBLICATION **0** CITATIONS
SEE PROFILE

International Journal of Scholarly Research in
Life Sciences
Journal homepage: https://srrjournals.com/ijsrls/
ISSN: 2961-3264 (Online)

(RESEARCH ARTICLE)

Check for updates

Synergistic antibacterial effect of ethanolic extract of *Carica papaya* and *Psidium guajava* leave extract on *Salmonella typhi, Escherichia coli* and *Klebsiella pneumoniae*

Alexander Philip [1,*], Aisha E Ibrahim [2] and Zakari Lois [3]

Faculty of Science and technology, Department of Biological science Bingham University Karu, Nigeria.

International Journal of Scholarly Research in Life Sciences, 2022, 01(01), 039–045

Publication history: Received on 09 June 2022; revised on 16 August 2022; accepted on 18 August 2022

Article DOI: https://doi.org/10.56781/ijsrls.2022.1.1.0025

Abstract

This study assessed the synergistic antibacterial effect of ethanol extract of *Carica papaya* and *Psidium guajava* on *Salmonella typhi, Escherichia coli* and *Klebsiella pneumoniae* using agar well diffusion method and microdilution with Microtitre plates. Qualitative phytochemical analysis of the extract reveals the presence of alkaloids, saponins, glycosides, steroids, soluble carbohydrates and phenols. Five (5) grams of the freeze-dried extract was dissolved in 10ml of 10% Dimethyl Sulphoxide (DMSO) and used for sensitivity against selected standard isolates. Upon sensitivity testing, this study observes a significant inhibition zone diameter at the highest concentration of the extract (500 mg/ml) against *Klebsiella pneumoniae* with an inhibition zone diameter of 31.0mm relative to the inhibition zone diameters of *Escherichia coli* and *Salmonella typhi* with (19.5 mm) and (20.5 mm) respectively. The inhibition zone diameters of the positive control (ciprofloxacin solution) at 500 mg/mL for *Klebsiella pneumoniae, Escherichia coli* and *Salmonella typhi* was 36.0 mm, 37.5 mm and 34.5 mm respectively. *Escherichia coli* has minimal inhibitory concentration (MIC) of 0.977 mg/ml while *Klebsiella pneumoniae* and *Salmonella typhi* have their minimal inhibitory concentration (MIC) at 31.25 mg/ml and 62.5 mg/ml respectively.

Keywords: Synergy; Antibacterial; *Carica papaya; Psidium guajava*; Extract

1. Introduction

Antibacterial resistance is posing a great challenge to the effective management and treatment of infectious diseases globally, thus have necessitated the need to seek for alternative antibacterial materials to complement existing ones in order to curtail the scourge of infectious disease spread and attend to effective treatment and management of infectious diseases' caused by pathogenic bacteria. The antibiotics used to treat infectious diseases caused by these bacteria have side effects that can prove harmful to the host body. Some of these side effects can range from digestive issues to bone damage, diarrhea and in women, vaginal yeast infections (1). For people in rural communities, especially in third world countries like Nigeria, these antibiotics are relatively expensive and are not easily accessible to them since most people don't have access to primary health care but they are however familiar with the use of herbs for treatment.

This study aims to assess the synergic antibacterial efficacy of *Psidium guajava* and *Carica Papaya* leave extract as potent antibacterial agent against *E. coli, K. pneumonia* and *S. typhi*. It also seeks to find the phytochemicals present in the mixture of the leaves and ascertain the relative minimum inhibitory concentration.

1.1 Study area

The laboratory work was carried out in the Microbiology Laboratory, Department of Biological Sciences, Bingham University located at Auta-Balefi, Karu, Nasarawa state, Nigeria.

* Corresponding author: Alexander Philip
Faculty of Science and technology, Department of Biological science Bingham University Karu, Nigeria.

1.2 Study samples

Pure clinical isolates of the test organisms i.e *Salmonella typhi, Escherichia coli* and *Klebsiella pneumonia* were obtained from the National Veterinary Research Institute (NVRI), Vom, Jos, Plateau state. The microorganisms were sub-cultured on nutrient agar and pure isolates of the resulting growth were obtained to be used for the laboratory practical work.

1.3 Source of plant material

Fresh leaves of the *Carica papaya* and *Psidium guajava* tree were obtained from around Bingham University campus, Karu, Nasarawa state, Nigeria.

1.4 Processing of the plant samples

The fresh leaves of *Carica papaya* and *Psidium guajava* were rinsed with distilled water to remove dirt and soil from the leaves. It was then allowed to dry at room temperature for fourteen (14) days by airdrying. The dried samples were then pounded using a mortar and pestle and further crushed into fine powdery form with an electric blender and stored in a labelled polythene bag for analysis.

2. Extraction of plant materials

2.1 Preparation of ethanol extract of the plant material

- Dried leaves were ground to powder using the mortar and pestle and further ground into fine powder using the electric blender.
- 200 gram of *Carica papaya* plant material and 200 grams of *Psidium guajava* plant powder was weighed on a weighing balance and transferred into a conical flask
- 1500ml of Ethanol solution was measured using a glass calibrated cylinder and gradually poured into the conical flask containing the *Carica papaya* and *Psidium guajava*, it was then mixed vigorously using a stirring rod, shaken intermittently every 30 minutes and stored in a refrigerator for 48 hours
- The mixture was then filtered out using a sieve and then further filtered out using a Whatmann no. 1 filter paper.
- Furthermore, the filtrate was poured into a plastic container in order to separate the Ethanol from the extract. A rotary evaporator was used so that a solid residue is obtained.
- The extract was scrapped using a clean spatula into a sterilized Amber bottle and then stored in the refrigerator.

2.2 Determination of extraction yield

$$\text{Extraction yield (\%)} = \frac{\text{weight of extract after freeze} - \text{drying}}{\text{Dry weight of sample}} \times 100\%$$

3. Qualitative phytochemical analysis

3.1 Test for alkaloids

A known quantity of the extract, 0.1 mg was added to 6 ml of 1% dilute hydrochloric acid (HCl) and boiled, cooled and filtered. The filtrate was divided into three portions and subjected to the following tests. To the first portion, 2 drops of Dragendroff's reagent was added. The formation of a red precipitate indicates the presence of alkaloids. To the second portion, 2 drops of Meyers reagent was added. A creamy white precipitate indicates the presence of alkaloids. To the third portion, 2 drops of Wagner's reagent was added. A reddish-brown precipitate indicates the presence of alkaloids.

3.2 Test for flavonoids

A quantity (1g) of the extract was boiled in ethylacetate (10 ml) at 70^{o}C or 65^{o}C for 3 minutes, filtered and cooled. Then, the filtrate (4ml) was shaken with 1 ml of dilute ammonia (NH_3) solution. An intense yellow colouration indicates the presence of flavonoids.

3.3 Test for tannins

1ml of the extract was added to 10 ml of deionized water and it was then treated with 3 drops of 1% ferric chloride. A greenish-brown precipitate indicates the presence of tannin.

3.4 Test for saponins

A quantity of 5 ml aliquot of the extract was diluted with 20 ml of deionized water, shaken vigorously and observed. The persistent foaming indicates the presence of saponin.

3.5 Test for glycoside

3.5.1 Keller-Kiliani Test

The sample was filtered and 2 ml of the sample was added into a test tube, 1 ml of glacial acetic acid was added followed by 1 ml of $FeCl_3$ and 1 ml of concentrated sulphuric acid (H_2SO_4) was added. Green-bluish colouration indicates the presence of glycosides

3.6 TEST FOR PHENOL

1 ml of extract solution was added to 1 ml of water into which few drops of 5% ferric chloride solution was added, dark green/bluish black colour indicates the presence of phenols with slight modification.

3.7 Preparations of the extract solutions

- A weighing balance was used to weigh 5 grams of the freeze-dried ethanolic extract material of *Carica papaya* and *Psidium guajava*.
- This was then transferred into a sterilized bijou bottle containing 10ml of Dimethyl sulfoxide (DMSO) which was used to prepare the extract solution. It was then stirred using a glass rod to help it dissolve. This gave a concentration of 500mg/ml for the ethanolic extract
- Serial dilution was made by withdrawing 5ml of the concentration from the bijou bottle to another bijou bottle containing 5 ml of dimethyl sulfoxide (DMSO) for ethanolic extract and mixed to obtain 250mg/ml solution. This procedure was used to prepare concentrations of 125 mg/ml, 62.5 mg/ml and 31.25 mg/ml and 15.625 mg/ml for the ethanolic extract.

3.8 Preparation of resazurin dye

The resazurin working solution was prepared by 1:100 dilution of prepared stock solution of Phosphate buffered saline (1 x PBS). This was done by dissolving 0.5 g resazurin sodium salt into 100 ml of 1 x PBS. The resazurin solution was prepared in the dark and was stored in an amber bottle. 1 liter of 1 x PBS was prepared by placing 800 ml of sterile distilled water in a suitable container. 8 g of NaCl (sodium chloride), 200 mg of KCl (potassium chloride), 1.44 g of Na_2HPO_4 (sodium dihydrogen phosphate) was added sequentially. The solution was mixed well and the p^H was adjusted to 7.4 and stored at 4^oC until further analysis. Distilled water was added to make the volume 1 liter.

3.9 PREPARATION OF mcfarland's STANDARD

This preparation involves the mixture of sulphuric acid (1%) and Barium Chloride (1.175%). 9.95 ml of sulphuric acid (H_2SO_4) was added to 0.05ml of barium chloride ($BaCl_2$) to form a precipitate suspension. This served as the 0.5 McFarland standard which served as the turbidity standard for the test organism.

3.10 Preparation of the cell suspension (inocular)

The McFarland standard was mixed well on a vortex mixer before use and then 10 ml was dispensed into a sterile test tube that is the same size and diameter as the tube used to prepare the solution. The test organism was sub-cultured on nutrient agar medium and incubated at 37^oC for 15-24 hours. In the presence of good lighting, the growth from each plate was transferred into a test tube containing 5ml of 0.9% Normal saline and the volume was adjusted to attain a turbidity which matched that of 0.5 McFarland standard's standard. The clarity of the lines was compared on a Wickerham card. This meant that the cell suspension contained appropriately $1.5x10^8$ colony forming unit per ml (CFU/ml).

3.11 Preparation of culture media

The agar used for culturing the microorganisms includes nutrient agar, Mueller-Hinton Broth and Mueller-Hinton Agar.

3.12 Nutrient agar preparation

- 9.8 grams of nutrient agar powder was suspended in 350 ml of distilled water (this may vary according to manufacturer's instruction given on the nutrient agar container).

- It was then mixed and dissolved completely using a vortex mixer.
- The liquid was sterilized by autoclaving at 121°C for 15 minutes
- The liquid was poured into the petri dish and was allowed to solidify. This preparation was done under strict aseptic conditions to prevent any form of contamination.

3.13 Mueller-hinton broth preparation

- 10.5 grams of Mueller-Hinton broth powder (CM0405B) was added in 500 ml of distilled water, was mixed thoroughly and dissolved completely.
- It was then poured into the final containers (e.g conical flask) and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes

4. Result

Positive if there is growth(turbidity) and negative if there isn't any growth

NOTE: Once prepared and not used immediately, it was stored at a temperature below 25°C

4.1 Mueller-hinton agar preparation

- 19 grams of the Mueller-Hinton powder (CM0337B) was added in 500 ml of distilled water
- It was mixed and dissolved completely
- Sterilized by autoclaving at 121°C for 15 minutes
- 4.The liquid was poured into petri dishes, allowed to cool, solidify and the plates were inverted.

NOTE: Once prepared, if not used immediately it should be stored at temperatures between 2-8°C

4.2 Sensitivity test by agar well diffusion method (kirby-bauer method)

- Wells were made aseptically near a Bunsen burner using an 8 mm cork borer to make the agar wells.
- The wells were then impregnated with extracts (30µl) after the Mueller-Hinton Agar plates have been inoculated with the test organisms using a sterile swab stick, which was well labelled.
- The plates were then allowed to stand at room temperature to allow proper diffusion and solidification of the extract and it was then incubated at 37°C for 24 hours.
- Ciprofloxacin tablet (500 mg) (one tablet was dissolved in 20 ml of distilled water) was used as positive control and pure DMSO (Dimethyl Sulphoxide) was used as a negative control.

The antibacterial activities of the plants were determined by measuring the clear zones of inhibition to the nearest millimeter in diameter using a digital Vernier caliper. This was done in duplicates and the mean of the two results taken. The inhibition zone diameters (IZDs) were interpreted based on the CLSI standards.

4.3 Determination of minimal inhibitory concentration (mic) using broth dilution method

The susceptibility of the test organisms to the combined extract of *Carica papaya* and *Psidium guajava* leaves was tested for ethanolic extracts using three Microtiter plates for the three clinical isolates to be tested i.e. *Salmonella typhi, Klebsiella pneumonia* and *Escherichia coli*. Sterile 96 well round bottom Microtiter plates with lid well labelled was used for this method. Each solvent extract was tested in duplicates on the Microtiter plate while Ciprofloxacin and Dimethyl Sulphoxide (DMSO) without the addition of the leaves extract was used as both positive and negative control respectively. The tests were carried out in the titer wells and the procedures includes.

4.4 Procedure

- 100 µl of Mueller-Hinton broth was dispensed to all the wells using a multichannel pipette
- 100µl of each of the extract (which contains 500 mg/mL for ethanolic extract) was pipette out and dispensed into the first column and mixed thoroughly by sucking up and down 6-8 times.
- 100 µl was withdrawn from column 1 and added to column 2, then mixed thoroughly; this made column 2 a twofold dilution of column 1. 100 µl was withdrawn from column 2 to column 3, followed by thorough mixing. This procedure was repeated down to column 6 and 100 µl was withdrawn from the 6th column and discarded. The micropipette tips were changed or discarded after each dilution. Each plate had a set of controls: a column

with broad-spectrum antibiotic (ciprofloxacin) serially diluted as a positive control and a column with pure DMSO is used as a negative control.

- 30 µl of the bacterial suspension that had been standardized was added to all the wells up to the 6th column aseptically, except the column with the negative control. The dilutions to be obtained were 250 mg/ml, 125 mg/ml, 62.5 mg/ml, 31.25 mg/ml and 15.625 mg/ml for the extracts.
- The plates were covered with the lid wrapped in foil paper and incubated at 37°C for 30 minutes.
- 30 µl of the resazurin blue solution was added to all the wells until the 6th column and it was then stored in the incubator for 1 hour and observed for any colour change.

Table 1 Qualitative phytochemicals present in the combined ethanolic leave extract of *Carica papaya* and *Psidium guajava*

Parameters	Ethanolic extract of *Carica papaya* and *Psidium guajava*
Alkaloids	+
Flavonoids	-
Tannins	-
Saponins	+
Glycosides	+
Terpenes	-
Steroids	+
Soluble carbohydrates	+
Phenols	+

Keys: +=Present; -= Negative

$$\text{Extraction yield (\%)} = \frac{\text{weight of extract after freeze} - \text{drying}}{\text{Dry weight of sample}} \times \frac{100\% = 35}{400} \times \frac{100 = 3500}{400} = 8.75\%$$

weight of extract after freeze-drying = 35 grams

Dry weight of sample = 400 grams

Extraction yield = 8.75%

Table 2 Microorganisms and their inhibition zone diameters for different concentrations of the Ethanolic extract of *Carica papaya* and *Psidium guajava* and the positive control

Concentrations of the Extracts (mg/mL)	Inhibition zone diameters (IZD) in mm		
	Salmonella typhi	***Escherichia coli***	***Klebsiella pneumonia***
500mg/mL	20.5 mm	19.5 mm	31.0 mm
250mg/mL	16.5 mm	18.0 mm	27.5 mm
125mg/mL	14.0 mm	13.0 mm	26.5 mm
62.5mg/mL	11.0 mm	10.5 mm	22.0 mm
31.25mg/mL	9.5 mm	8.0 mm	19.0 mm
15.625mg/mL	8.5 mm	7.5 mm	16.0 mm
Positive control	34.5 mm	37.5 mm	36.0 mm

Once inoculated onto Mueller-Hinton agar media containing agar wells with the concentrations of the extract, the isolates were inhibited and showed inhibition zones. This result showed that the antibiotic used (fluconazole solution) as the positive control for the isolates at 500 mg/ml was more effective against those isolates than even the highest concentration of the extract used (500 mg/ml) with inhibition zone diameters (IZD) of 34.5 mm, 37.5 mm and 36.0 mm for *Salmonella typhi, Escherichia coli* and *Klebsiella pneumoniae* respectively. The result also showed that the extract was very effective against *K. pneumoniae* with all values for the IZD being from susceptible to intermediate for all concentrations of the extract from 500 mg/ml to 15.625 mg/ml since according to CLSI standards ≤14 mm means the organism is resistant to the extract or antibiotic with the lowest for *K. pneumoniae* being at 16 mm. The zones of inhibition of *S. typhi* and *E. coli* showed resistance at 125 mg/ml with zone diameters of 14.0 mm and 13.0 mm respectively.

Table 3 Minimal inhibitory concentrations (MIC) of the ethanolic extract and positive control for the different microorganisms

Ethanolic extract solvents and positive control	**Minimum Inhibitory concentrations (MIC) in mg/mL**		
	Salmonella typhi	***Escherichia coli***	***K. pneumonia***
Ethanolic	62.5 mg/mL	0.977 mg/mL	31.25 mg/mL
Positive control	0.244 mg/mL	0.244 mg/mL	0.244 mg/mL

Standardized suspensions of the bacterial isolates (30 µl) were inoculated onto Microtiter plates that had been added with Mueller-Hinton broth (100 µl) and different concentrations of the combined extract material (100 µl), incubated for 30 minutes, and the resazurin working solution added to all the wells and incubated for 24 hours. Minimal inhibitory concentration (MIC) for the isolates was taken by observing for lowest concentrations in the titer wells that still retained the dark-bluish colour of the resazurin dye. The result of the MIC showed that the ethanolic extract was more inhibitory against *E. coli* than the other two isolates used. The result for Minimal inhibitory concentration (MIC) also showed that the antibiotic used as positive control was more effective against the isolates than the extract solutions.

5. Discussion

The presence of bioactive substances has been reported to confer resistance to plants against bacteria, fungi and pests and therefore explains the demonstration of antibacterial activity by the plant extracts used in the study (2). Based on Clinical and laboratory standards institute (CLSI) standards for zones of inhibition ≥20 mm means the organism is susceptible to the extract while between 15-19 mm means the organism is intermediate while ≤14 mm means the organism is resistant to the extract. From the work done for zones of inhibition, for *Klebsiella pneumoniae,* it is highly susceptible to the extract with inhibition zone diameter of 31.0 mm at the highest concentration of the extract (500 mg/mL) respectively followed by *Salmonella typhi* with an inhibition zone of 20.5 mm while *Escherichia coli* is intermediate to susceptible with an inhibition zone of 19.5 mm. compared to work done by (3) who worked on *Carica papaya* leaves only, and reported antibacterial activity against *Salmonella typhi* and *Klebsiella pneumoniae* with zones of inhibition of 8mm and 10mm respectively. Similarly, (4) working on *Psidium guajava* leaves, it had zones of inhibition against *K. pneumoniae* with zone diameter of 17.1 mm. This shows that the combined extract material of *C. papaya* and *P. guajava* had more antibacterial activity against *S. typhi* and *K. pneumoniae*, thus having synergy effect against those organisms. For *Escherichia coli,* the zone of inhibition was 19.5 mm at the highest concentration, compared to work done by (2) who worked on pawpaw leaves extract only, the zone of inhibition for *E. coli* was 18.5 mm and work done by (5) using guava leaves extract, the inhibition zone diameter was 22 mm. This shows that the combined effect was mostly due to the *P. guajava* leaves and thus there was a synergic effect between the two plants.

The MIC values for *Escherichia coli* is at an extremely low concentration (0.977 mg/mL) and this is of great importance since this organism is associated with enteric infections and this goes to show that this organism can be inhibited at low doses when ingested into the body taking into account the physiological processes that can affect the action of this combined extract. The extract is more inhibitory against *E. coli* with an inhibitory concentration of 0.977 mg/mL compared to *K. pneumoniae* and *S. typhi* with inhibitory concentrations of 31.25 mg/mL and 62.5 mg/mL, compared to work done by (6) who worked on only *C. papaya* leaves against *Escherichia coli*, there was no MIC values for the concentrations which they used, and (5) who worked on *P. guajava* leaves, whose MIC was 40 mg/ml for *Escherichia coli*. This shows that *P. guajava* leaves had more effect on the organism than *C. papaya* leaves and thus there was a

combined synergic effect of both plants against the organism since the MIC for the combined plant material is at a lower concentration.

The low MIC values of the ethanolic extract on *Klebsiella pneumoniae* (31.25 mg/mL) is also of special interest since this bacterium is mostly associated with nosocomial infections and is highly resistant to many antibiotics compared to work done by (4) using *P. guajava* leaves, the MIC value was 6.25 mg/ml And this is also evident in the zones of inhibition gotten from the agar well diffusion test in which the values for the zones of inhibition of the ethanolic extract against *Klebsiella pneumoniae* are large compared to *Escherichia coli* and *Salmonella typhi*. It can also be deduced that the ethanolic extract of the combined plant material of *Carica papaya* and *Psidium guajava* can be effective against *Salmonella typhi* due to the low MIC values for it.

6. Conclusion

In conclusion, the inhibition zone diameters (IZD) of the three isolates shows that the extract solution was very effective against *Klebsiella pneumoniae* with even the lowest concentration being intermediate, while for *Salmonella typhi* and *Escherichia coli* the isolates were resistant to it at concentrations of 125 mg/ml with zone diameters of 14.0mm and 13.0 mm respectively. The MIC values of the extract for the three different isolates were 62.5 mg/ml, 0.977 mg/ml and 31.25 mg/ml for *Salmonella typhi, Escherichia coli* and *Klebsiella pneumoniae* respectively, with the extract being extremely effective against *E. coli* and thus this low values shows that this extract contains drug active compounds that can inhibit the growth of such organisms and expressed significant antibacterial effect as can be seen from the inhibition zone diameters in the result tables above. Thus, it could be used as readily available cheap alternative to currently used antibiotics.

Compliance with ethical standards

Acknowledgments

This manuscript is the authors original work, all authors have read, understood and agreed to submit this manuscript in accordance to ethical standards and policy of this journal.

Disclosure of conflict of interest

All the authors hereby declares that there is no conflict of interest.

References

[1] Webster RK, Rubin GJ. Predicting expectations of side-effects for those which are warned versus not warned about in patient information leaflets. Annals of Behavioral Medicine. 2021 Dec;55(12):1253-61.

[2] Srinivasan D, Nathan S, Suresh T, Perumalsamy PL. Antimicrobial activity of certain Indian medicinal plants used in folkloric medicine. Journal of ethnopharmacology. 2001,1;74(3):217-20.

[3] Fasoyinu BT, Oyetayo OV, Ajayi AT. Antibacterial activity of fermenting unripe pawpaw parts (Carica papaya) against some enteric bacteria. International journal of pathogen research. 2019;3(2):1-0.

[4] Hackman KH, Arhin RE, Azumahi KB, Boateng D, Nwosu B, Apenteng M. in vitro antibacterial activity of Psidium guajava (guava) leaves extract on carbapenem-resistant Klebsiella pneumoniae causing multi-drug resistant systemic infections. Journal of Medicinal Plants Research. 2020; 14(9): 475-479.

[5] Ngene CA, Aguiyi CJ, Chibuike JC, Ifeanyi VO, Ukeagbu-Obi MK, Kim GE, Onyemegbulem OB. Anitbacterial activity of Psidium guajava leaf extract against selected pathogenic bacteria. Advances in microbiology. 2019; 9: 1012-1022

[6] Anibijuwon LL, Udeze AO. Antimicrobial activity of Carica papaya (pawpaw leaf) on some pathogenic organisms of clinical origin from South-Western Nigeria. Ethnobotanical leaflets. 2009; 13: 850-864.

[7] Fasoyinu BT, Oyetayo OV, Ajayi AT. Antibacterial activity of fermenting unripe pawpaw parts (Carica papaya) against some enteric bacteria. International Journal of Pathogen Research. 2019; 3(2): 1-10.