ORIGINAL RESEARCH

ASSESSMENT OF ANTIMICROBIAL POTENTIALS AND MINIMUM INHIBITORY CONCENTRATION (MIC) OF EXTRACTS OF DRY *carica papaya* SEEDS ON MULTIDRUG RESISTANT (MDR) CLINICAL MICROBIAL ISOLATES

Authors:
OKEKE-NWOLISA, Benedictta C[1*], BENEDICT, Joshua Chinonso[1],

Author Affiliations:
[1]Department of Medical Microbiology and Public Health, Faculty of Medical Laboratory Sciences, Nnamdi Azikiwe University, Nnewi Campus, Nnewi, Nigeria.

***Correspondence:**
Benedictta C Okeke-Nwolisa
bc.okeke-nwolisa@unizik.edu.ng
+234 8064312596
Orcid ID: https://orcid.org/0000-0003-1285-0961

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ABSTRACT

Background: Infections caused by drug-resistant microorganisms are usually more difficult to treat, require longer hospital stays, and are associated with higher mortality rates. Researchers and scientists have begun using medicinal plants as alternatives to synthetic drugs. Various parts of medicinal plants including *carica papaya* are being examined.

Aim: This study aimed to investigate the antimicrobial activities of aqueous and 40% ethanolic extracts of dry c*arica papaya* seeds against some clinical isolates, the Minimum Inhibitory Concentration (MIC) and the median lethal dose (LD_{50}) of the extracts.

Methods: Both extracts were tested at 10mg/ml (5%), 50mg/ml (25%), 100mg/ml (50%) and 200mg/ml (100%) on the bacterial and fungal isolates. The in-vitro antibacterial and antifungal activities of the aqueous and 40% ethanolic extracts were determined using Disc diffusion technique. Test organisms were obtained from the laboratory, resuscitated in peptone water,

sub-cultured into nutrient agar for the bacteria test organisms and Sabouraud dextrose agar (SDA) for the fungi test organisms and incubated at 37^0C for 24hours. The test organisms were standardized by matching with the appropriate McFarland's standard. LD_{50} was ascertained using modified Lorke's method (Lorke 1983) using 26 Wistar rats (*Rattus norvegicus*).

Results: Both extracts had antimicrobial activities of varying degrees based on Clinical Laboratory Standards Institute (CLSI) interpretive criteria (CLSI, 2012). The 40% ethanolic extract gave a higher antibacterial activity on the test organisms than the aqueous extract. MIC of 12.5mg/ml was observed from the 40% ethanolic extract. LD_{50} for both extracts was found to be greater than 5000mg/kg.

Conclusion: It can be inferred that extracts of dry Carica papaya seeds have antibacterial and antifungal potential.

Keywords: *Antimicrobial, Carica papaya, seeds, aqueous extract, ethanolic extract, Wistar rats.*

INTRODUCTION:

Multidrug resistance (MDR) is indeed a serious problem caused by the abuse and misuse of antibiotics and other antimicrobials. Infections caused by drug-resistant microorganisms are usually more difficult to treat, require longer hospital stays, and are associated with higher mortality rates[1].Antibiotic resistance has been on a steady rise because of the abuse of antimicrobial agents and the development of resistant strains of microbes, researchers and scientists have begun using medicinal plants as alternatives to synthetic drugs[2] . Various parts of medicinal plants including Carica papaya are being examined[2,3]. *Carica papaya*, commonly known as papaya or pawpaw belongs to the family *caricaceae* in the order *brassicales*. It is a tropical fruit tree native to Central America and Mexico. It is widely cultivated for its edible fruit and medicinal properties[4]. Papaya fruit is highly regarded for its nutritional and economic benefits. Notably, various parts of the plant, including the roots, bark, leaves, peels, seeds and pulp have medicinal properties[5,6] .

Of these, the seeds hold the greatest therapeutic value despite being discarded and accounting for just 7% of the fruit's weight.

Multidrug resistance (MDR) is a critical problem arising from the abuse and misuse of antibiotics and other antimicrobials. Infections caused by drug-resistant microorganisms are often more difficult to treat, require longer hospital stays, and are associated with higher mortality rates[1] .This problem has caused scientists and researchers to explore other medicinal sources such as plants, as alternatives to the commercially available antibiotics.

Though *carica papaya* has been used in traditional medicine for its medicinal properties, there is paucity of data on the antimicrobial activity of seeds of *carica papaya* on multidrug resistant microbes. Hence, this study is intended to investigate the antimicrobial properties of the aqueous and 40% ethanolic extracts of c*arica papaya* seeds and how they can be used as a better alternative to chemotherapeutic drugs in treating antibiotic resistant microbes.

Figure 1: Diagram of *Carica papaya* tree with fruits (A) and longitudinally cut sections of the ripe fruits, exposing the seeds (B).

MATERIALS AND METHODS

Study design:

The *Carica papaya* seeds were sourced from randomly collected ripe *Carica papaya* fruit samples from fruit sellers within Nnewi metropolis. The seeds from the fruits were air-dried, ground and extracted using two extraction solvents (40% ethanol and water). Eight microorganisms were used for the antimicrobial assays, three Multidrug Resistant (MDR) bacteria isolates and three non-MDR bacteria isolates, one MDR fungal isolate and one non-MDR fungal isolate. The results obtained were compared with the results from similar study work and that of the control.

Study area:

The study area that was used for this study is within Nnewi North metropolis. It is a commercial city in Anambra State, South Eastern Nigeria. Nnewi as a metropolitan city has two Local Government Areas, which are Nnewi North and Nnewi South. Nnewi North comprises four autonomous Communities: Otolo, Uruagu, Umudim and Nnewichi[7]. The 2016 population estimation showed that Nnewi had a population of over 900,000. The city spans over 200 square miles (520 km^2) in Anambra State. Nnewi Metroprolitan Area and its Statellite towns are home to nearly 2.5 million residents as of 2005[8].

Sample extraction of the pawpaw seeds and determination of the median lethal dose was carried out in Department of Physiology Laboratory, Faculty of Basic Medical Sciences, Nnamdi Azikiwe University, Nnewi Campus, Nnewi; while the antimicrobial activity testing of the extracts analysis was done in Microbiology Laboratory at Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi.

Materials: This research used seeds of ripe fruits of *carica papaya*, 40% ethanol, sterile water, Dimethylsulfoxide (DMSO), Wistar rats (*rattus norvegicus*), Whatman filter paper, rotary evaporator, refrigerator, autoclave, electronic weighing balance,

incubator, measuring cylinder, beaker, conical flask, Petri dishes, forceps, paper puncher, laboratory hot air oven, electronic blender, bunsen burner, wire loop, micro-titer plate, 2,3,5-triphenyltetrazolium chloride (TTC), swab sticks, Nutrient agar (ACCUMEDIA/Swemed Diagnostics LTD. Bangalore, India), Sabouraud dextrose agar (SDA) (ACCUMEDIA/Swemed Diagnostics LTD. Bangalore, India), clinical isolates of bacteria and fungi, personal protective equipment (PPE).

Identification of the fruits:
Before sample preparation, the ripe pawpaw fruits were identified by a Taxonomist at the Department of Botany Sciences, Faculty of Natural Sciences, Nnamdi Azikiwe University Awka. The herbaruim number of the plant is **NAUTH-190^{A}**.

Sample preparation
The ripe fruits were washed in running tap water and air-dried under room temperature. The seeds were aseptically removed from the fruit samples, and weighed on a weighing balance. Approximately 1kg was obtained. The seeds were washed under running tap water, air dried at room temperature on the clean table for a week and weighed. The dried seeds were grounded to fine powder using a sterile electronic blender and stored in a sterile bottle at 4^0C for analysis.
This preparation method was done with modifications according to the method by Dagne *et al*., (2021)[9].

Preparation of aqueous extracts of dry *carica papaya* seeds:
The *Carica papaya* aqueous seed extract was prepared by dissolving 20g of the fine seed powder in 200ml of distilled water in a 250ml capacity conical flask. The extract was obtained by macerating 20g of the fine seed powder in 200ml of distilled water for 48 hours until the soluble materials dissolves. The extract was then filtered using a clean handkerchief and further filtration was done using Whatman no.1 filter paper into a clean glass jar.
The residue of the extract was weighed and the volume of the filtrate was measured.
The filtrate was concentrated by evaporation to dryness in a hot air oven at 40^0C until the solvent from extract evaporates. The resulting extract was put in a vial and stored at 20^0C. This extraction method was adopted and done with modifications according to the method by Dagne *et al*., (2021)[9].

Preparation of 40% ethanolic extracts of dry *carica papaya* seeds:
The *Carica papaya* 40% ethanolic seed extract was prepared by dissolving 20g of the fine seed powder in 200ml of 40% ethanol in a 250ml capacity conical flask. The extract was obtained by macerating 20g of the fine seed powder in 200ml of 40% ethanol for 48 hours until the soluble materials dissolves. The extract was then filtered using a clean handkerchief and further filtration was done using Whatman no.1 filter paper into a clean glass jar. The residue of the extract was weighed and the volume of the filtrate was measured. The filtrate was concentrated by evaporation to dryness in a hot air oven at 40^0C until the solvent from extract evaporates.
The resulting extract was put in a vial and stored at 20^oC. This extraction method was adopted and done with modifications according to the method by Dagne *et al*., (2021)[9].

Preparation of stock solution of the extracts:
0.5gram each of the aqueous and 40% ethanolic extract was taken and dissolved in 2.5ml of DMSO respectively. Thus

200mg/ml of stock was obtained as a standard concentration of the extracts (Was prepared using standard procedures). Different concentrations of 5% (10mg/ml) [0.2 in 10ml]; 25% (50mg/ml) [0.5g in 10ml]; 50% (100mg/ml) [0.5g in 5ml], and 100% (200mg/ml) aqueous and ethanolic extracts were prepared by diluting the appropriate grams of the extract in the required volume of DMSO.

Laboratory tests: isolation, purification and identification of bacterial and fungal microorganisms

The test microorganisms used were Gram positive bacterium (*Staphylococcus aureus*), Gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and fungus (*Candida albicans*) both MDR strains and non MDR strains. Pure cultures of the test organisms were gotten to avoid mixed growth by sub-culturing an already isolated and identified test microorganism in the laboratory's incubator into freshly prepared nutrient agar for bacteria isolates and into Sabouraud Dextrose Agar (SDA) for the fungal isolate. At the end of incubation period, pure bacterial and fungal isolates were selected based on morphological and biochemical characteristics [10]. The antimicrobial activity of the aqueous and ethanolic seeds extracts of *Carica papaya* was done by agar disc diffusion method for the MDR isolates and agar dilution method for the non MDR isolates while the MIC was done by broth micro-dilution method.

Sensitivity testing:

preparation of Macfarland standards:

0.5 and 1.5 McFarland standards were prepared by mixing 0.05ml of 1.175% barium chloride hydrate ($BaCl_2.2H_2O$) with 9.95ml of 1% sulfuric acid (H_2SO_4) with constant stirring for the 0.5 McFarland while 0.15ml of 1.175% barium chloride hydrate ($BaCl_2.2H_2O$) with 9.85ml of 1% sulfuric acid (H_2SO_4) were mixed to prepare 1.5 McFarland standard.

The 0.5 McFarland standard is expected to give an approximate cell density (1.5×10^8 CFU/ml) giving optical density (absorbance) of 0.1 at 600nm wavelength while the 1.5 McFarland standard was expected to give an approximate cell density (4.5×10^8 CFU/ml) giving optical density (absorbance) of 0.357 at 600nm wavelength.

The prepared McFarland standards were put into tightly screwed tubes and stored at room temperature away from sunlight[11].

Preparation of test organisms:

Two colonies of the already isolated bacterial test microorganisms were emulsified in 4-5ml of sterile nutrient broth and matched with 0.5 McFarland's standard. Same was done for the fungal test organism but it was matched with 1.5 McFarland's standard. The broths containing the microorganisms were allowed to stand for 2 -3 hours before it was used[12].

Antimicrobial susceptibility testing:

This was done by filter paper disc diffusion technique as described in[13] with modifications for the MDR isolates and agar dilution method as described in[14] Kenneth *et al*., (1973) with modifications.

Preparation of discs

Diffusion discs of 6mm diameter prepared from Whatman filter paper by using a sterile paper puncher and sterilized at 120^0C for 1hour in hot air oven. Subsequently, the sterilized discs were impregnated aseptically by applying 0.02ml (20µl) of the aqueous and ethanolic extracts of the plant at

concentrations of 10mg/ml (5%), 50mg/ml (25%), 100mg/ml (50%) and 200mg/ml (100%) using a sterile micropipette and then allowed to dry in an hot air oven at 40^0C.
The discs were then placed in sterile container and stored at 4^0C.

Disc diffusion technique:
Sterile nutrient agar medium (14g nutrient agar in 500ml distilled water, autoclaved at 115^0C and 15psi for 30 minutes) was prepared, cooled to 50^0C, poured into the sterile Petri dishes and allowed to solidify. Pure isolates were collected from the broth culture of the test organisms and streaked on the agar with a sterile swab. The discs were placed on the surface of the medium using a flame sterilized forceps. The discs were also placed on fungal isolate inoculated in SDA.
The culture plates were then incubated at 37^0C for 24 hours to obtain zones of inhibition.
The diameter of these zones of inhibition was measured using a meter rule, vertically, horizontally and diagonally.
The mean was calculated and presented in millimeter[15,16] and read as resistant, intermittent or sensitive based on the length of the inhibition zone. The zones of inhibition were compared with that of the conventional antibiotic discs.

Agar dilution technique:
Using a sterile micro pipette, 1ml of the different concentrations of the 40% ethanolic and aqueous extracts were put in about 20ml each of already prepared molten nutrient agar in a sterile universal container for the bacteria isolates and SDA for the fungi isolate when the agar media were still in molten form (50^0C). The mixture was mixed properly by gently turning the corked tube it was in up and down three times. The mixture was then poured into sterile Petri dishes and allowed to cool and solidify. Each Petri dish/culture plate contained a specific concentration of the extracts.
The Petri dishes containing the mixture were then swab inoculated aseptically with the different test organisms from the standardized broth culture of the organisms and allowed to incubate at 37^0C for 24 hours to check for the presence or absence of growth which was read as resistant[-] or sensitive[+][14].

Determination of minimum inhibitory concentration (mic):
Minimum inhibitory concentration is the lowest concentration of antimicrobial agent that will completely inhibit visible growth of the test-organism.
The most preferred method for determination of MIC using extracts is the broth micro-dilution method. This method as described by [17], was performed using a new sterile 96-well micro-titer plates. The 96-well plates made up of 8 rows (A - H) and 12 columns (1 -12) were prepared by dispensing 50µl of Nutrient broth into the specific row to be used. Each row is made up of 12 wells labelled 1-12. 50µl from the specific concentration of the test extract to be used was added into well 1(positive control) and 3 of the specific row of the plate to be used.
Then two fold serial dilutions were performed by using a sterile micro pipette from the 3rd well to the 12th well and the last dilution discarded.
Determination of mic cont.:
The resulting dilutions were as follows: 1 in 2(1/2) for well 3, 1 in 4(1/4) for well 4, 1 in 8(1/8) for well 5, 1 in 16(1/16) for well 6, 1 in 32(1/32) for well 7, 1 in 64(1/64) for well 8, 1 in 128(1/128) for well 9, 1 in 256(1/256) for well 10, 1 in 512(1/152) for well 11 and 1 in 1024(1/1024) for well 12. 10µl of the test inocula was added to the 2nd well (negative control) till the 12th well

except the 1st well. The specific concentration of extract to be used with broth (1st well) was used as a positive control and inoculum with broth (2nd well) was used as a negative control. The test plates were then incubated at 37ºC for 18hours.

After 18hours, 50µl of 0.01% solution of 2, 3, 5- triphenyltetrazolium chloride (TTC) was added to the wells with a sterile micropipette and the plate incubated for another hour. Since the colorless tetrazolium salt will be reduced to red/pink colored product by biological active bacteria, thus the inhibition of growth can be detected when the solution in the well doesn't change to pink/red after incubation with TTC.

The **MIC** is the lowest sample concentration showing no colour change (clear) and exhibited complete inhibition of growth.

Determination of 50% lethal dose ld_{50} (acute toxicity):

The acute toxicity or the median lethal dose (LD_{50}) of the aqueous and 40% ethanolic extracts of dry seeds of *Carica papaya* were evaluated in 26 rats of the Wistar strain(13 rats for each extract) using a modified Lorke's method[18]. Prior to the test, the rats were acclimatized to standard animal cage conditions for a week and fasted overnight. 2.04g of the ethanolic extract was dissolved in 20mls of distilled water and administered using an oral cannula. The process was done in two phases for both the aqueous and 40% ethanolic extract.

In the first phase, a total of three groups were formed, and within each group, three rats were randomly placed. The rats were given the 40% ethanolic extracts, orally at doses of 10, 100, and 1000 mg/kg body weight (b.w). The rats were observed for 24 hours for signs of toxicity (morbidity and mortality).

During the second phase, four groups with one rat in each group were given the 40% ethanolic extracts orally. The doses administered were 1200, 1600, 2900, and 5000 mg/kg b.w in a similar manner as above. The rats again were observed for 24 hours for any signs of toxicity.

After the last phase, the LD_{50} was calculated as the geometric mean of the highest non-lethal dose (a) and the least toxic dose (b).

$LD_{50} = \sqrt{a \times b}$

Ethical approval: The study was approved by Nnamdi Azikiwe University Animal Research Ethics Committee (NAU-AREC), Awka (NAU/AREC/2024/0076).

Data analysis: The data obtained in this study was presented in tables and figures.

RESULTS

Antimicrobial Susceptibility Testing (AST)

In the results according to this study it was observed that the aqueous and 40% ethanolic extracts showed no signs of antimicrobial activity against the MDR isolates in which disc diffusion method was used as seen in Table 1, Figures 1a, 1b, 1c, 1d, 2a, 2b, 2c, 2d but showed varying degrees of activity against non-MDR isolates in which agar dilution was used as seen in Table 2, Figure 4c-7d

Table 1: Zone of inhibition of MDR-microorganisms in 40% ethanolic and aqueous extracts of *Carica papaya* seeds using disc diffusion method.

MDR Microorganisms	Zone of Inhibition diameter (mm) for *Carica papaya* seed extracts							
	Aqueous extract (AE)				Ethanolic extract (EE)			
	5%	25%	50%	100%	5%	25%	50%	100%
Escherichia coli	0	0	0	0	0	0	0	0
Pseudomonas aeruginosa	0	0	0	0	0	0	0	
Staphylococcus aureus	0	0	0	0	0	0	0	0
Candida albicans	0	0	0	0 0	0 0	0		

KEY: MDR-Multidrug Resistant.
0-Resistant (Okeke-Nwolisa *et al*., 2023a)

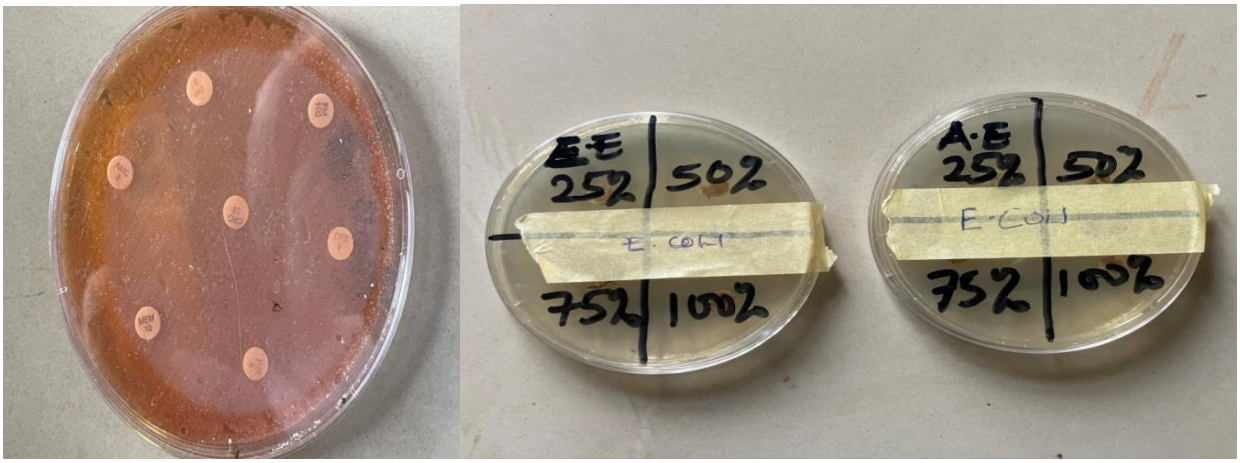

Figure 1a: Culture plate showing MDR *Escherichia coli*
Figure 1b: Aqueous and ethanolic *Carica papaya* seed extract on MDR *E.coli* showing no zone of inhibition after incubation at 37^0C for 24 hours

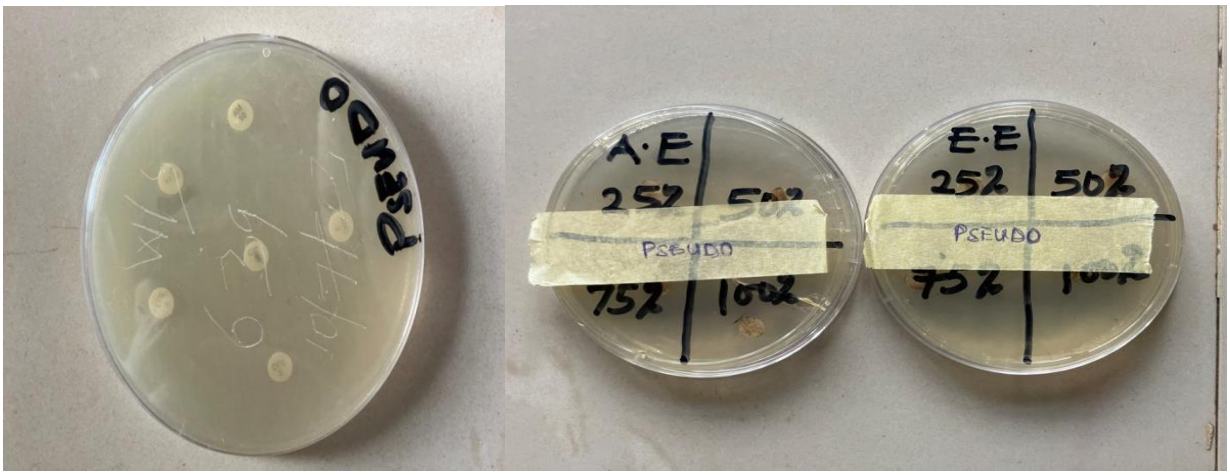

Figure 1c: Culture plate showing MDR *Pseudomonas aeruginosa*
Figure 1d: Aqueous and ethanolic *Carica papaya* seed extract on MDR *P.aeruginosa* showing no zone of inhibition after incubation at 37^0C for 24 hours

Figure 2a:Culture plate showing MDR *Staphylococcus aureus*
Figure 2b: Aqueous and ethanolic *Carica papaya* seed extract on MDR *S. aureus* showing no zone of inhibition after incubation at 37^0C for 24 hours

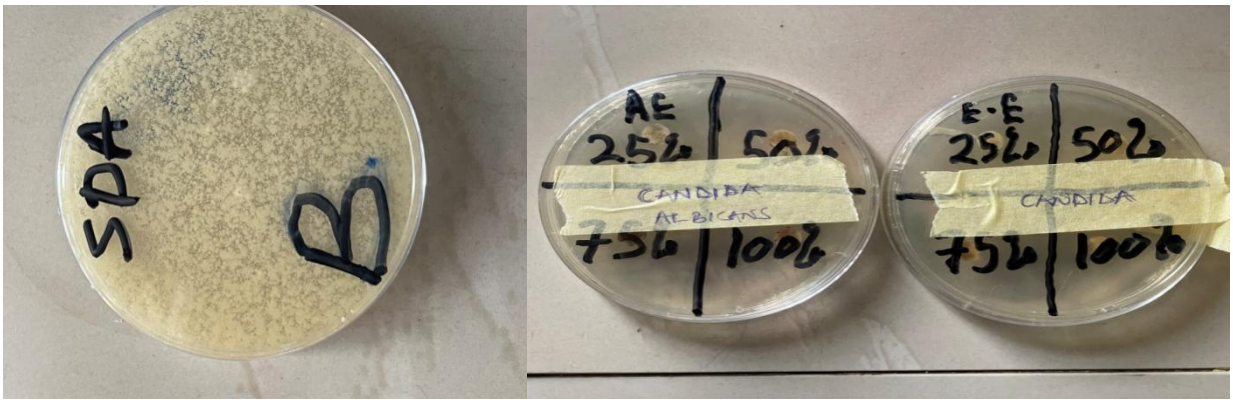

Figure 2c: SDA Culture plate showing MDR *Candida albicans*
Figure 2d: Aqueous and ethanolic *Carica papaya* seed extract on MDR *C.albicans* showing no zone of inhibition after incubation at 37^{0}C for 24 hours
KEY: MDR (Multidrug Resistant).
AE- Aqueous extract
EE- Ethanolic extract

Table 2: Presence or absence of inhibition in 40% ethanolic and aqueous extracts of *Carica papaya* seeds on Non-MDR microorganisms.

Non-MDR Microorganisms	Aqueous extract				Ethanolic extract			
	5%	25%	50%	100%	5%	25%	50%	100%
Escherichia coli	-	-	-	-	-	+	+	+
Pseudomonas aeruginosa	-	-	+	+	-	-	-	-
Staphylococcus aureus	-	-	-	-	-	-	-	-
Candida albicans	-	-	-	+	-	+	+	+

KEY: SENSITIVE (+)
RESISTANT (-) (Kenneth *et al*., 1973).
MDR (Multidrug Resistant).

It was also observed that the 40% ethanolic extract showed higher antimicrobial activity than the aqueous extract. The lowest concentration of the 40% ethanolic extract that showed inhibition was the 25% concentration (50mg/ml), having inhibitory effect against *Escherichia coli* and *Candida albicans* as seen in Table 2, Figure 6c and 6d while that of the aqueous extract was the 50% concentration (100mg/ml) having slight inhibitory effect against *Pseudomonas aeruginosa* as seen in Table 2, Figure 5a. Both extracts showed increased inhibitory effect on increased concentration against the susceptible microorganisms as seen in Table 4.2 and Figure 4c-7d

The 5% and 25% concentrations of the aqueous extract and 5% concentration of the 40% ethanolic extracts showed little to no activity against the non-MDR test organisms as seen in Table 2 and Figure 4c, 4d, 4e, 4f, 6a and 6b. The 50% and 100% concentrations of the aqueous extract, 25%, 50% and 100% concentrations of the ethanolic extract inhibited the growth of some the non-MDR test organisms as seen in Table 2 and Figure 5a, 5b, 5c, 5d, 6c, 6d, 7a, 7b, 7c and 7d.

Candida albicans showed the highest susceptibility as it was inhibited by both the

aqueous extract (100% concentration) and the 40% ethanolic extract (25%, 50% and 100% concentrations) as seen in Table 2 and Figure 5c, 6c, 7a and 7c. It is followed by *Escherichia coli* which was inhibited by 25%, 50% and 100% concentrations of the ethanolic extract as seen in Table 2 and Figure 6d, 7b, 7d. *Staphylococcus aureus* showed the least susceptibility as it was resistant to all concentrations of both aqueous and 40% ethanolic extracts as seen in Table 2 and Figure 4d, 4f, 5b, 5d, 6b, 6d, 7b and 7d. It was followed by *Pseudomonas aeruginosa* which was only susceptible to 50% and 100% concentrations of the aqueous extract as seen in Table 2 and Figure 5a and 5c.

Antimicrobial susceptibility testing revealed that the Non-MDR test organisms were resistant to some of the control antibiotics used as seen in Table 3 and Figures 3a, 3b and 3c below.

Table 3: Antibiotic Susceptibility Test results of Non-MDR test organisms on control drugs.

Non-MDR Microorganisms	Zone of Inhibition diameter (mm) ANTIBIOTICS							
	GEN	**CRX**	**CXM**	**OFL**	**AUG**	**NIT**	**CPR**	**CAZ**
Escherichia coli	20	-	16	23	-	18	25	-
Pseudomonas aeruginosa	13	-	-	20	-	-	26	-
Staphylococcus aureus	6	-	-	-	-	26	-	-

KEY: MDR- Multidrug Resistant
GEN - Gentamicin
CRX – Cefuroxime
CXM – Cefixime
OFL – Ofloxacine
NIT - Nitrofurantoin
CPR - Ciprofloxacine
CAZ - Ceftazidine
(**-**) - Resistance.

Figure 3a: Antibiotic sensitivity discs on *Staphylococcus aureus* showing zones of inhibition after incubation at 37^{0}C for 24 hours

Figure 3b: Antibiotic sensitivity discs on *Escherichia coli* showing zones of inhibition after incubation at 37^{0}C for 24 hours

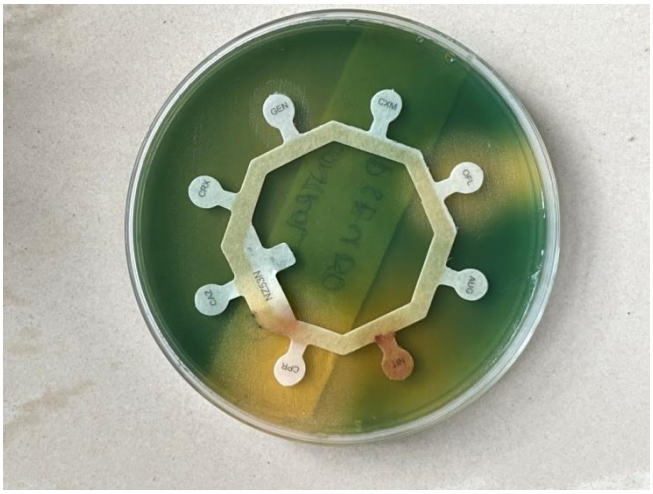

Figure 3c: Antibiotic sensitivity discs on *Pseudomonas aeruginosa* showing zones of inhibition after incubation at 37^0C for 24 hours

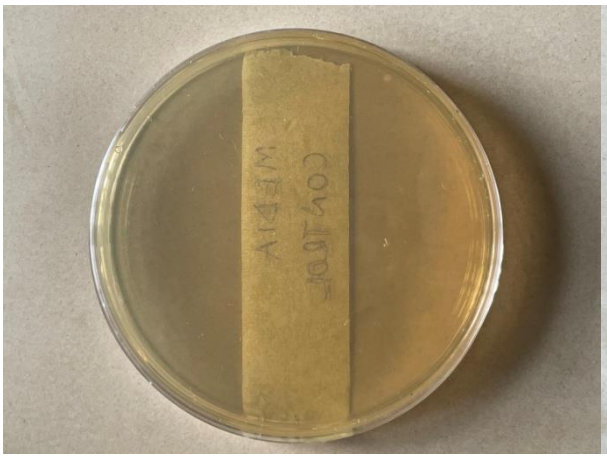

Figure 4a: Media control culture plate plate

Figure 4b: 40% Extract control culture

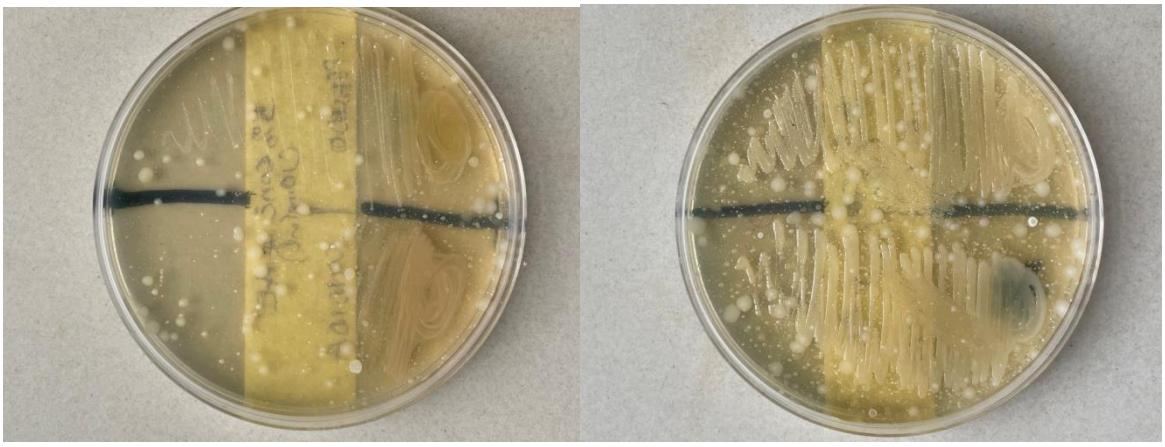

Figure 4c: 5% AE on *Pseudomonas aeruginosa* and *Candida albicans* after incubation at 37^{0}C for 24 hours

Figure 4d: 5% AE on *Escherichia coli* and *Staphylococcus aureus* after incubation at 37^{0}C for 24 hours

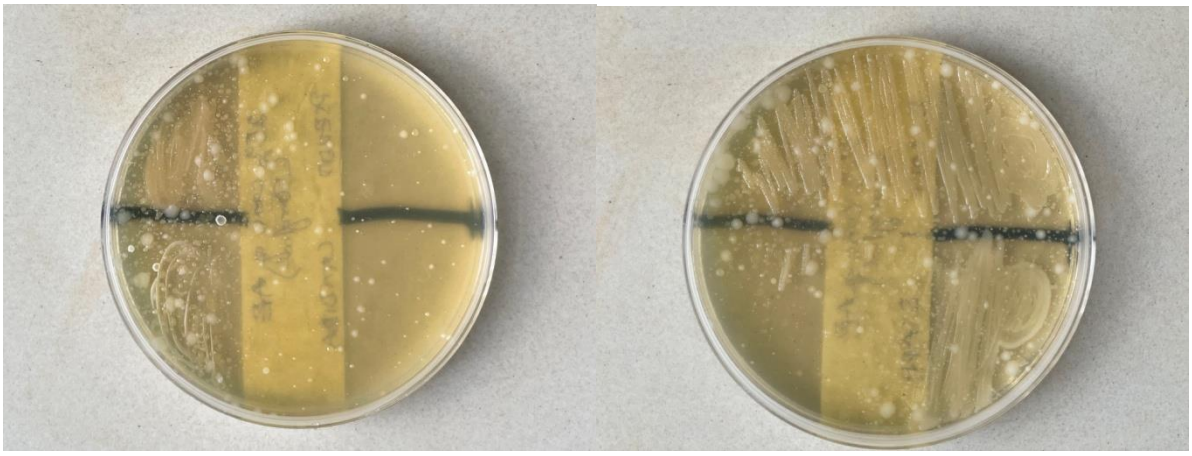

Figure 4e: 25% AE on *Pseudomonas aeruginosa* and *Candida albicans* after incubation at 37^{0}C for 24 hours

Figure 4f: 25% AE on *Escherichia coli* and *Staphylococcus aureus* after incubation at 37^{0}C for 24 hours

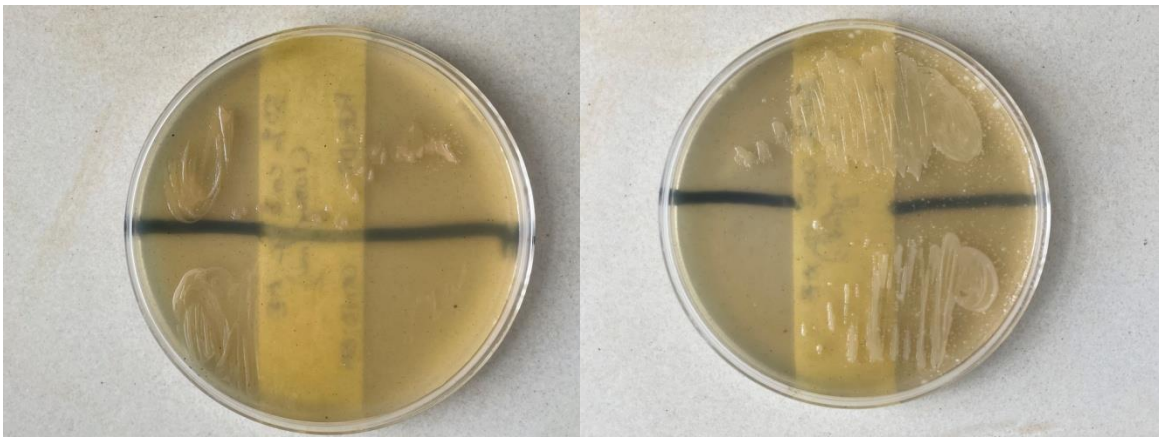

Figure 5a: 50% AE on *Pseudomonas aeruginosa* and *Candida albicans* after incubation at 37^0C for 24 hours

Figure 5b: 50% AE on *Escherichia coli* and *Staphylococcus aureus* after incubation at 37^0C for 24 hours

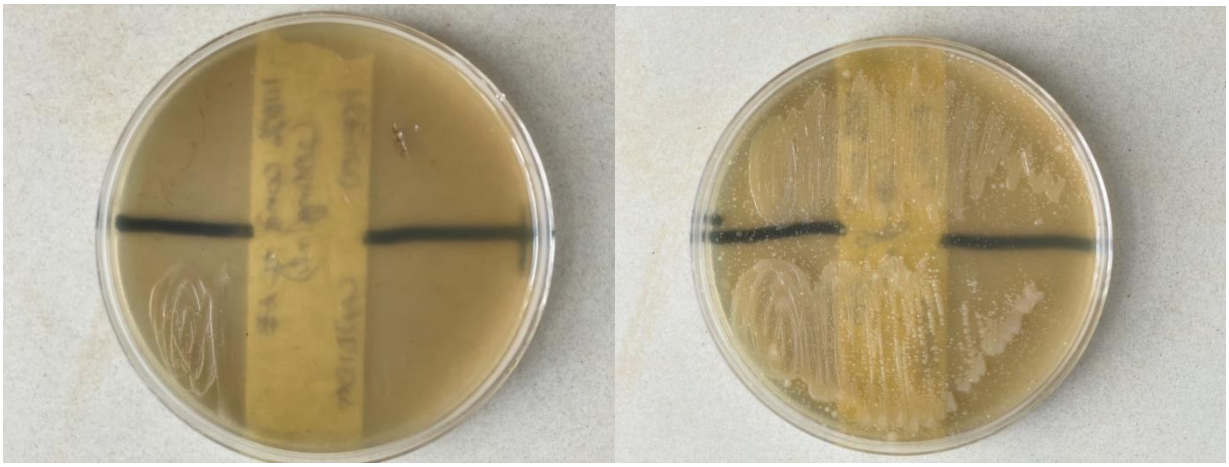

Figure 5c: 100% AE on *Pseudomonas. aeruginosa* and *Candida albicans* after incubation at 37^0C for 24 hours

Figure 5d: 100% AE on *Escherichia. coli* and *Staphylococcus. aureus* after incubation at 37^0C for 24 hours

KEY: AE-Aqueous Extract

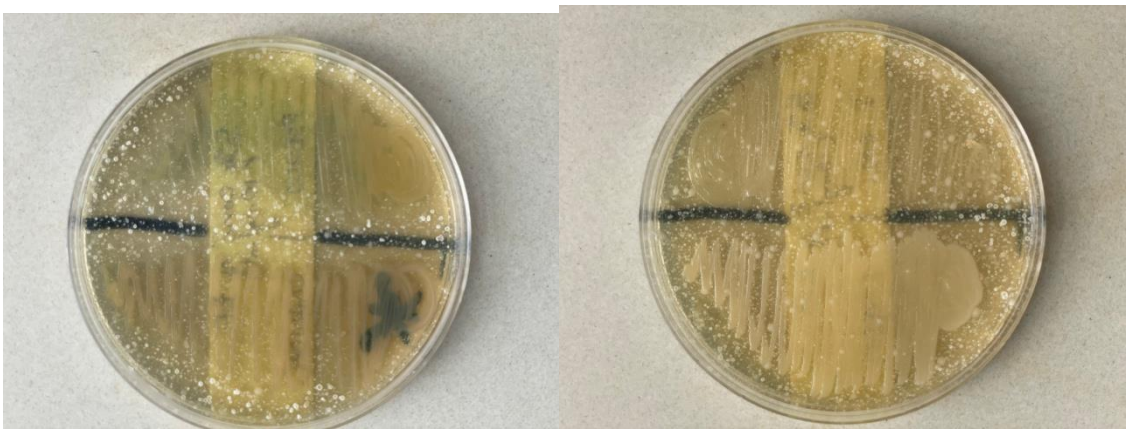

Figure 6a: 5% EE on *Pseudomonas. aeruginosa* and *Candida. albicans* after incubation at 37ºC for 24 hours

Figure 6b: 5% EE on *Escherichia. coli* and *Staphylococcus. aureus* after incubation at 37^0C for 24 hours

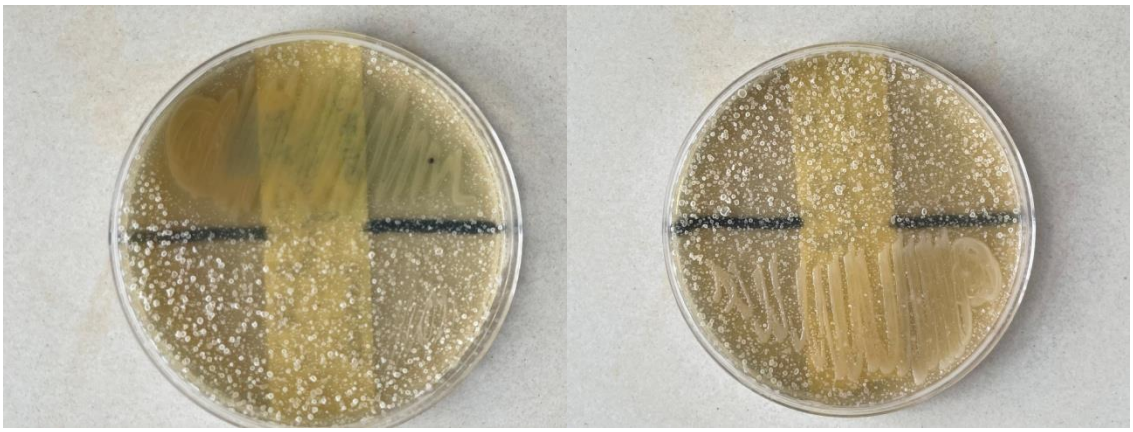

Figure 6c: 25% EE on *Pseudomonas. aeruginosa* and *Candida. albicans* after incubation at 37^0C for 24 hours

Figure 6d: 25% EE on *Escherichia. coli* and *Staphylococcus. aureus* after incubation at 37^0C for 24hours

KEY: EE-Ethanolic Extract.

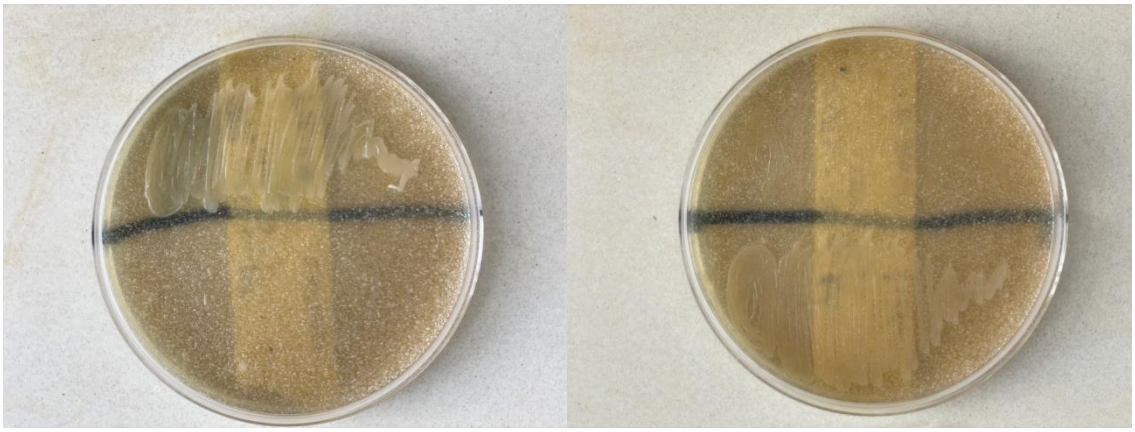

Figure 7a: 50% EE on *Pseudomonas. aeruginosa* and *Candida. albicans* after incubation at 37^0C for 24 hours

Figure 7b: 50% EE on *E. coli* and *S. aureus* after incubation at 37^0C for 24hours

Figure 7c: 100% EE on *Pseudomonas. aeruginosa* and *Candida. albicans* after incubation at 37^0C for 24 hours

Figure 7d: 100% EE on *Escherichia. coli* and *Staphylococcus. aureus* after incubation at 37^0C for 24hours

KEY: EE-Ethanolic Extract.

Minimum Inhibitory Concentration (MIC)

This was carried out to determine the effectiveness of the various extract concentrations that were able to inhibit the growth of the test organism in the agar dilution method.

Table 4 and Figure 8a, 8b and 8c below shows decreasing antimicrobial activity with decreasing concentration of papaya seeds extracts

Table 4: Determination of MIC using Broth micro-dilution method

	MIC for *Carica papaya* seed extracts in mg/ml						
Non-MDR	**Aqueous extract**				**Ethanolic extract**		
Microorganisms	**50%**	**100%**			**25%**	**50%**	**100%**
Escherichia coli	-	-			6.25	12.5	12.5
Pseudomonas aeruginosa	25	25	-	-	-		
Candida albicans	-	50	6.25	12.5	12.5		

KEY: MDR- Multidrug Resistant

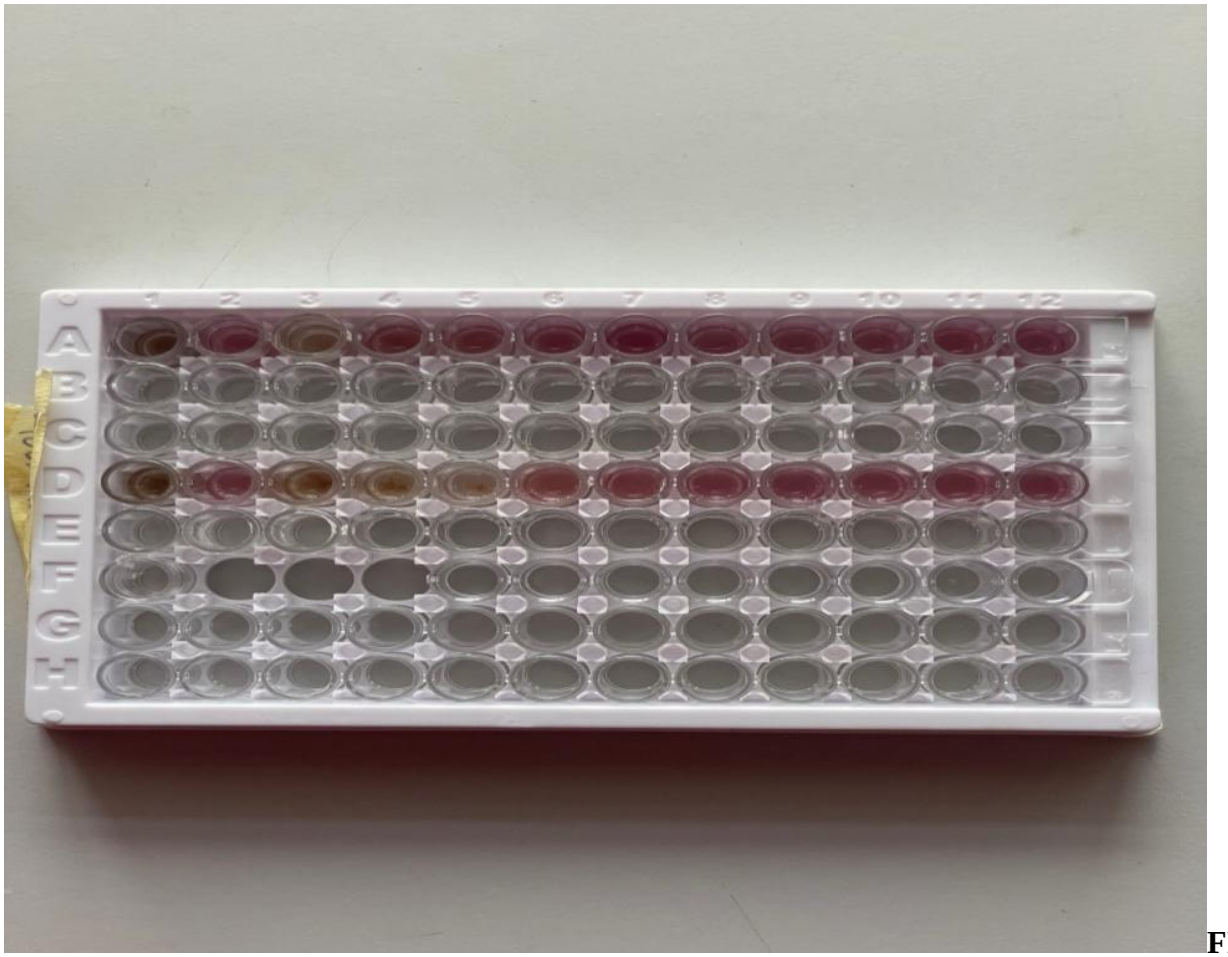

FIGURE 8a: MIC of 50% and 100% AE on *Pseudomonas aeruginosa*
KEY: AE- Aqueous Extract
Row A: 50% Aqueous extract, **Row D**: 100% Aqueous extract.
Column 1: Positive control, **Column 2**: Negative control, **Column 3**:1/2 diltuion, **Column 4**: 1/4 diltuion, **Column 5**: 1/8 diltuion, **Column 6**: 1/16 diltuion, **Column 7**: 1/32 diltuion, **Column 8**: 1/64 diltuion, **Column 9**: 1/128 diltuion, **Column 10**: 1/256 diltuion, **Column 11**: 1/512 diltuion, **Column 12**: 1/1024 diltuion.

Table 5: Showing the different dilutions in each column of the microtitre plate

Column	1	2	3	4	5	6	7	8	9	10	11	12
Dilutions	**+ve**	**-ve**	½	¼	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024

KEY: +ve -Positive control (Nutrient broth plus extract)
-ve -Negative control (Nutrient broth plus organism)

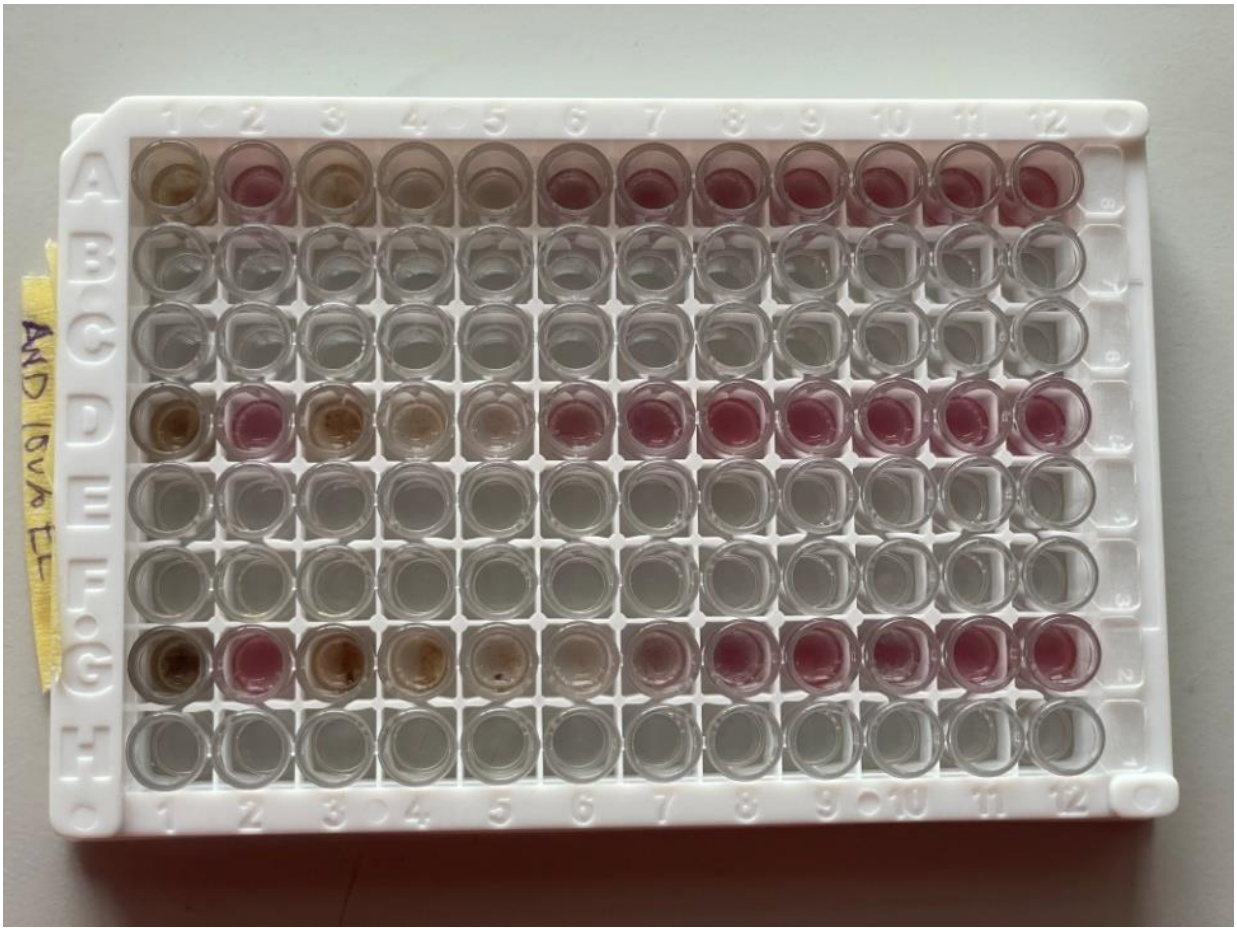

Figure 8b: MIC of 25%, 50% and 100% EE on *Escherichia coli*
KEY: EE- Ethanolic Extract
Row A: 25% Ethanolic extract, **Row D**: 50% Ethanolic extract, **Row G**: 100% Ethanolic extract.

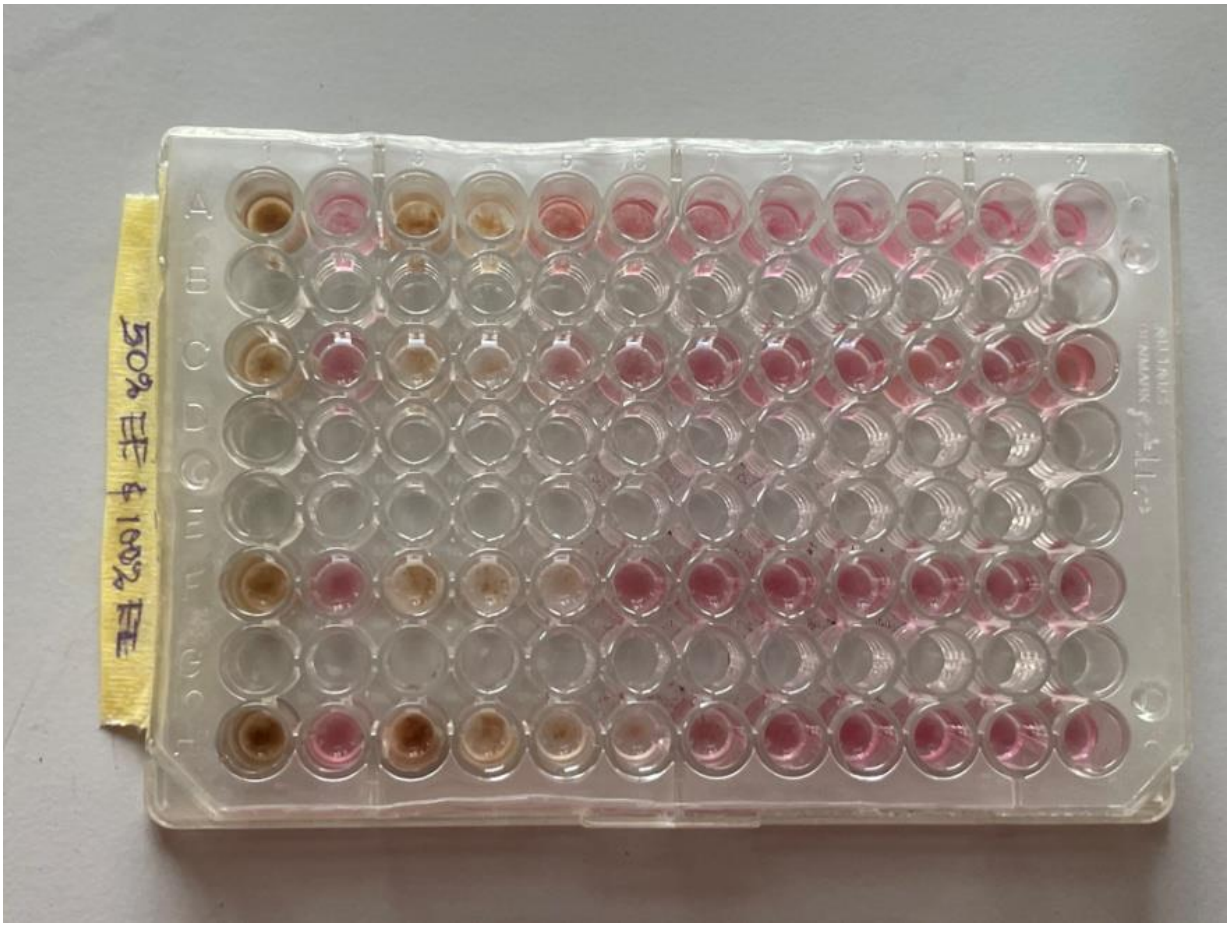

Figure 8c: MIC of 100% AE, 25%, 50% and 100% EE on C*andida albicans*
KEY: AE- Aqueous Extract
EE- Ethanolic Extract
Row A: 100% Aqueous extract, **Row C**: 25% Ethanolic extract, **Row F**: 50% Ethanolic extract, **Row H**: 100% Ethanolic extract.

For *Pseudomonas aeruginosa*, 50% (Row A in Figure 8a) and 100% (Row D in Figure 8a) concentrations aqueous extract were used as seen in Figure 8a. For *Escherichia coli*, 25% (Row A in Figure 8b), 50% (Row D in Figure 8b) and 100% (Row G in Figure 8b) concentrations of 40% ethanolic extract were used as seen in Figure 8b. For *Candida albicans*, 100% concentration of the aqueous extract (Row A in Figure 8c), 25% (Row C in Figure 8c), 50% (Row F in Figure 8c) and 100% (Row H in Figure 8c) concentrations of the 40% ethanolic extract were used as seen in Figure 8c.

Among the 100% concentration (200mg/ml) of the aqueous seed extract, the MIC was 25mg/ml (1/8 dilution as seen in Row D, column 5 of Figure 8a) for *Pseudomonas aeruginosa* and 50mg/ml (1/4 dilution as seen in Row A, column 4 of Figure 8c) for

Candida albicans. Among the 100% concentration (200mg/ml) of the 40% ethanolic seed extract, the MIC was 12.5mg/ml (1/16 dilution as seen in Row G, column 6 of Figure 8b) for *Escherichia coli* and 12.5mg/ml (1/16 dilution as seen in Row H, column 6 of Figure 8c) for *Candida albicans.*

Acute Toxicity Testing

In Table 5, which is an acute toxicity study of the 40% ethanolic seed extract of *Carica papaya*, no animal death was recorded both in phase 1 and phase 2 with a maximum dose of 1000mg/kg and 5000mg/kg per phase. A total of 13 Wistar rats were used to carry out this study.

Table5: Acute Toxicity study of ethanolic seed extract of *Carica papaya* for phase 1 and 2

	Phase	Dose(mg/kg/bw)	Death (13)	Observation
40% ethanolic seed extract of *Carica papaya*	1	10	0/3	The rats remained normal
		100	0/3	The rats remained normal
		1000	0/3	The rats remained normal
	2	1200	0/1	The rats remained normal
		1600	0/1	The rats remained normal
		2900	0/1	The rats remained normal
		5000	0/1	No death occurred

KEY: **mg** - milligram; **kg** - kilogram **bw** - body weight

In Table 6, which is an acute toxicity study of aqueous seed extract of *Carica papaya*, no animal death was recorded both in phase 1 and phase 2 with a maximum dose of 1000mg/kg and 5000mg/kg. A total of 13 Wistar rats were used to carry out this study.

Table 6: Acute Toxicity study of aqueous seed extract of *Carica papaya* for phase 1 and 2

	Phase	Dose(mg/kg/bw)	Death (13)	Observation
Aqueous seed extract of *Carica papaya*	1	10	0/3	The rats remained normal
		100	0/3	The rats remained normal
		1000	0/3	The rats remained normal
	2	1200	0/1	The rats remained normal
		1600	0/1	The rats remained normal
		2900	0/1	The rats remained normal
		5000	0/1	No death occurred

KEY: **mg** - milligram; **kg** - kilogram; **bw** - body weight

DISCUSSION

This study showed differences in antimicrobial activities between the two extracts used which is dependent on the extraction solvent used. This implied that the release of bioactive compounds of plant parts depend on the extraction solvent[9]. The result of this study showed that the aqueous extract was less effective than the ethanolic extract and this is in agreement with the works of Wemambu *et al*., (2018)[19], and Dagne *et al*., (2021)[9], all recorded higher antimicrobial activity of ethanolic extract of *Carica papaya* seeds. This may be due to the better solubility of the active components of the seeds in organic solvents [20].

It was also observed that the extracts showed higher antibacterial activity against Gram negative bacteria organisms than the Gram positive bacteria organism tested. This coincides with the findings of previous works of Mangalanayaki and Nirosha (2013)[21] and Peter *et al*., (2014)[22]. This result however contradicts the results of Jigna and Sumitra, (2006)[23], that recorded higher antibacterial activity against Gram positive bacteria than Gram negative bacteria. This might be due to slight differences in the method used for antimicrobial testing.

The MIC was carried out to determine the effectiveness of the various dry *Carica papaya* seeds extract concentrations that were able to inhibit the growth of the test microorganisms in the agar dilution method. The dry *Carica papaya* seeds which were extracted with different solvents showed different MIC. The 100% concentration of the 40% ethanolic extract of dry *Carica papaya* seeds inhibited *Escherichia coli* and *Candida albicans* at 12.5mg/ml. So, it has a potential to treat infections caused by *Candida albicans* and *Escherichia coli*.

In this study, the fact that the extracts showed activity against fungi, Gram negative bacteria and in other works stated above, against Gram positive bacteria may indicate broad spectrum of activity. Several other reports have shown that *Carica papaya* have significant antibacterial activity in various extracts from different tree parts [24;25 and 26]. Zakira *et al*., 2006 analyzed the antimicrobial activity of *Carica papaya* flowers against bacterial pathogens[27]. Romasi *et al*., 2011 reported that the extracts of papaya leaves could inhibit the growth of *Rhizopus stolonifer*[28].

The antimicrobial effectiveness of plant extracts have been attributed to the presence of secondary metabolites in the investigated plant part which probably plays a vital role in its usefulness as a medicinal plant [29]. Sukadana *et al*., (2008)[30] in Martiasih (2014)[31] had reported that papaya seeds contained a potential antibacterial, triterpenoid aldehyde compound [32]. Triterpenoid has ability to disrupt cell pores so the membrane permeability was disrupted as well.

Another reported antibacterial compound in papaya seeds was carpaine alkaloid. Carpaine digests proteins from microorganisms and changes it into peptone. According to Sabir (2005)[33], flavonoid contents in *Trigona sp. propolis* inhibited the growth of *Streptococcus mutans* in vitro.

Flavonoid denatures proteins and disrupts the cell membrane leading to the death of bacterial cell. Mustikasari and Ariyani (2010)[34] stated that alkaloids had antimicrobial activity by disrupting the microbial cell wall [32]. All these active components play a role in the antimicrobial activities of *Carica papaya.*

The investigation also revealed that the median lethal dose (LD_{50}) of *Carica papaya* seeds extract were found to be over 5000mg/kg for both the 40% ethanolic and the aqueous seeds extracts as seen in Table 4.5 and 4.6. This outcome is due to the absence of any instances of subject mortality at this dosage level administered to the wistar rats. This discovery aligns with the work of Kanadi

et al., 2019[35] and Chinoy *et al*., 1994[36], which indicated that the aqueous extract exhibited an LD_{50} greater than 5000mg/kg. This finding also agrees with the guideline of OECD which states that any substance having LD_{50} above 5000mg/kg ingested orally is safe or essentially non-toxic[37].

CONLUSION

According to this study, dry *Carica papaya* seeds extracts possess antimicrobial activities and therefore is a potential source of therapeutic agent against certain bacteria and fungi. MIC of 12.5mg/ml was observed from the 40% ethanolic extract of dry *Carica papaya* seeds extracts. The aqueous and 40% ethanolic decoction prepared from the dry seeds of *Carica papaya* is tolerable at a dose of up to 5000mg/kg/bw.

RECOMMENDATIONS

Further pharmacological studies are necessary in order to give better insight on the therapeutic potential of the plant so as to properly extract and utilize the appropriate bioactive components which can be used in the treatment of diseases and to ascertain its effectiveness against multidrug resistant organisms so as to reduce the increasing rise of antimicrobial resistance. In addition, further studies are required to eliminate the possibility of toxicity in conditions of more chronic exposure or use of the extract.Researchers should explore other medicinal indigenous African plants for antimicrobial activities to help tackle the problem of multidrug resistance among pathogenic microbes.

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REFERENCES

1.European Centre for Disease Prevention and Control (ECDC). (2018). Annual epidemiological report for 2018: Antimicrobial consumption.

2.Jyotsna, K.P.,Yashab,K., Priyanka, P. and Harison, M.(2014).Antibacterial activity of seed and leaf extract of Carica papaya var. Pusa dwarf Linn. IOSR Journal of Pharmacy and Biological Sciences 9(2):29 - 37.Doi:10.9790/3008-09272937

3.Kadiri,O.,Olawoye, B.,Samson, O. and Adalumo, O.A.(2016). Nutraceutical and Antioxidant Properties of the Seeds,Leaves and Fruits of *Carica papaya*: Potential Relevance to Human Diet, the Food Industry and the Pharmaceutical Industry - A Review.Turkish Journal of Agriculture-Food Science and Technology/4(12).1039-1052. Doi:10.24925/turaf.v4i12.1039-1052.569

4.Cárdenas-Conejo, Y., Aguiñiga-Sánchez, I., Ruiz-Velasco-Santana, D., Hernández-Pérez, T., Soto-Hernández, M., and Jiménez-Estrada, M. (2020). Flavonoids from *Carica papaya*: a comprehensive review on its phytochemistry, bioactivities and mechanisms of action. *Food and Chemical Toxicology, 145*, 111702.

5.Mwangi, J. W., Thoithi, G. N., and Kibiti, C. M. (2020). Phytochemical, pharmacological and traditional uses of *Carica papaya* Linn.: A mini review.

Journal of Medicinal Plants Research, *14*(14), 838-846.

6.Dwivedi, G., and Chauhan, A. K. (2021). Nutritional and Medicinal Potential of *Carica papaya* Linn.: A Review. *Journal of Pharmacognosy and Phytochemistry,10*(1), 1579-1582.

7. Latitude and Longitude Finder; Where is Nnewi, Anambra, Nigeria on Map Lat Long Coordinates (© 2012-2023). Retrieved from https://www.latlong.net/place/nnewi-anambra-nigeria-24864.html

8.Cityfacts (© 2019-2023). Retrieved from https://www.city-facts.com/nnewi

9.Dagne E., Dobo B. and Bedewi Z. (2021). Antibacterial Activity of Papaya (*Carica papaya*) Leaf and Seed Extracts Against Some Selected Gram-Positive and Gram-Negative Bacteria. *Pharmacogn J*, *13*(6): 1727-1733.

10.Cowan and Steel (2013).Manual of Identification of Medical Bacteria. Ed Cowan Cambridge University Press, pp.317.

11.McFarland Nephelometer: an instrument for media used for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *J Am Med Assoc 14*:1176-1178.

12.Bauer, A. W., Kirby, W. M., Sherris, J. C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology, 45*(4), 493-496.

13.Okeke-Nwolisa, B. C., Egeonu, C. O., Enweani-Nwokelo, I. B., Anukam, K. C., and Eyeghre, O.A.(2023a). Antimicrobial activities of aqueous extract of ripe Annona muricata linn (soursop) fruit pulp on clinical isolates. *Journal of Biomedical Investigation*, *11*(1), 1-8.

14.Kenneth C.H., Anne H.M. and Edythe W. (1973). Agar Figure dilution method for routine antibiotic susceptibility testing in a hospital laboratory. *American Journal of Clinical Pathology, 60*(3), 384-394, https://doi.org/10.1093/ajcp/60.3.384

15.Pratiwi S.I. (2008). Antibacterial Activity of Poison Nut (Jatropha curcas L.) Leaf Flour against Bacteria Consortium of Chicken Digestive Tract in vitro. Bog

16.CLSI. (2012). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard, 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

17. Basri, D.F. and Fan, S.H. (2005). The potential of aqueous and acetone extracts of galls of *Quercusinfectoria* as antibacterial agents. *Indian J Pharmacol* ;*37*(1), 26-29.

18.Lorke D. (1983). A new approach to practical acute toxicity testing. *Archemy Toxicology*, 54,275-287.

19.Wemambu I.I., Ajose D.J. and Eni C.C. (2018) "Antibacterial Effect of *Carica papaya* Root Extract on Some Selected Pathogens from Clinical Isolates". *Acta Scientific Microbiology1*(7): 06-10.

20.de Boer H.J., Kool A., Broberg A., Mziray W.R., Hedberg I. and Levenfors J.J. (2005). Antifungal and antibacterial activity of some herbal remedies from Tanzania. *Journal of Ethnopharmacology. 96*:461-469.

21.Mangalanayaki, R. and Nirosha, N. (2013). Antibacterial Activity of Leaves

and Stem Extract of *Carica papaya* L. *International Journal of Advances in Pharmacy, Biology and Chemistry. 2*(3): 473-476.

22.Peter J.K., Kumar Y., Pandey P., Masih H. (2014). Antibacterial Activity of Seed and Leaf Extract of *Carica papaya* var. Pusa dwarf Linn. *IOSR Journal of Pharmacy and Biological Sciences, 9*(2): 29-37.

23.Jigna P and Sumitra C. (2006). In vitro antimicrobial activity of medicinal plants. Afr. *J. Biomed, 9*(2):89-93.

24.Ifesan B.O.T., Fashakin J.F., Ebosele F. and Oyerinde S.A. (2013). Antioxidant and antimicrobial properties of selected plant leaves. *European Journal of Medicinal Plants.3*(3): 465-473.

25.Nirosha N. and Mangalanayaki R. (2013). Antibacterial activity of leaves and stem extract of *Carica papaya*. *International Journal of Advanced Pharmacy and Biochemistry. 2(*3): 473-476.

26.Doughari,JH, Human,IS, Bennade, S, et al. Phytochemicals as chemotherapeutic agents and antioxidants: Possible solution to the control of antibiotic resistant verocytotoxin producing bacteria. Journal of Medicinal Plants Research. 2009;15(2)173 - 177.

27.Zakaria, Z. A., Jais, A.M.M., Sulaiman, M.R., Isa, M.S.P.P. and Riffin, S. (2006). The in vitro antibacterial activity of *Carica papaya* flowers and *Mangifera indica* leaves. *J. of Pharmacology and Toxicology*. *1*(3): 278-283

28.Romasi ,EF, Karina, J, Parhusip, AJN.Antibacterial activity of papaya leaf extracts against pathogenic bacteria. Makara Technologi.2011;15(2):173-177.

29.Oboh F.O.J. and Masodje H.I. (2009) "Nutritional and antimicrobial properties of *Ocimum gratissimum* leaves". *Journal of Biological Sciences 9*(4): 3

30.Sukadana, I.M., Santi, S. R. and Juliarti, N. K.(2008). Antibacterial activity of triterpenoid group compounds from papaya seed (*Carica papaya* L.) *J. Kim. 2*(1) pp 15–8

31.Martiasih M., Sidharta B.B.R. and Atmodjo P.K. (2014). Antibacterial activity of extract papaya seeds (*Carica papaya* L.) againts*Escherichia coli* and Streptococcus pyogenes *J. Penelit.* Pp 5–7

32.Masfufatun, Yani N.P.W. and Putri N.P.Y.K. (2019). Antimicrobial assay of papaya seed ethanol extract (*Carica papaya* Linn) and phytochemical analysis of its active compounds. IOP Conf. Series: *Journal of Physics: Conf. Series 1277*; 012018. doi:10.1088/1742-6596/1277/1/012018

33.Sabir A. (2005). Antibacterial activity of Trigona sp. propolis flavonoid against Streptococcus mutans (in vitro*) J. Kedokt Gigi 38*(3) pp 135–4

34.Mustikasari K. and Ariyani D. (2010). Phytochemical screening of Kalangkala (Litseaangulata) seed methanol extract *SainsdanTerap Kim.4*(2) pp 131

35.Kanadi, M.A., Alhassan, A.J., Ngwen, A.L., Yaradua, A. I., Nasir, A. and Wudil, A. M. (2019). Acute Toxicity Studies and Phytochemical Constituents of Different Solvents Extracts of *Carica papaya* Seeds. *Asian Journal of Research in Botany; 2*(3): 1-9.

36.Chinoy, N. J., D'Souza, J. M. and Padman, P. (1994). Effects of crude aqueous extract of *Carica papaya* seeds in male albino mice. Reproductive toxicology (*Elmsford, N.Y.*), *8*(1), 75–79.

https://doi.org/10.1016/0890-6238(94)90070-1

37.OECD (2001). Harmonized integrated classification system human health and environmental hazards of chemical 9substances and mixtures. *OECD Series on Testing and Assessments 33* (6). pp. 21-24.