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Phytochemical Screening and Gram-Negative Antibacterial Activity of *Carica Papaya* leaves extracts

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ABSTRACT

The use of plants as potential sources of antibiotics needed to reduce Gram-negative antibiotic resistance has been investigated. The extraction of these plants is essential for isolating antibacterial agents with the aim of understanding their role in the treatment of Gram-negative bacterial infections. Therefore, this study was designed to identify the presence of some phytochemicals as well as their antibacterial activities against Gram-negative bacteria *Pseudomonas aeruginosa*. *Carica papaya* leaves were extracted successively with *n*-hexane and dichloromethane at varying pH's using percolation method after which phytochemical screening was carried out on each extract. The qualitative analysis of the phytochemicals was carried out using standard experiment. The in-vitro antibacterial activity of the extracts was carried out against *P. aeruginosa* using disk diffusion assay. The results of phytochemical screening indicated the presence of steroid in all the four extracts. The leave extracts except for the dichloromethane at pH 9.15 inhibited the pathogen with the highest zone of inhibition being 22 mm. The minimum inhibitory concentration (MIC) was analyzed using disk diffusion assay with values ranging between 31.25 $mgmL^{-1}$ to 250 $mgmL^{-1}$ against the pathogen. There were variations in the lowest concentrations of the extracts causing the inhibitions. The dichloromethane at pH 8.3 extract that contained terpenoids was biologically active against *P. aeruginosa* at 250, 125, 62.5 and 31.25 $mgmL^{-1}$ concentrations.

Keywords: Gram-negative, Phytochemicals, *Carica papaya* leaves, Antibiotic resistance.

INTRODUCTION

Cases of multi-drug resistance were being reported soon after antibiotics were first introduced for clinical use [1]. Treatment of patients with antibiotic resistant infectious diseases is often considerably less successful than would be normally anticipated, thus the rise of antibiotic resistant pathogens has a significant impact on healthcare [2], [3], [4]. With the continued rise in antibiotic resistance, it has been estimated that by the year 2050, the mortality rate associated with

antibiotic resistance will rise to approximately 10 million cases [5]. These pathogens; Gram-positive and Gram-negative bacteria have over time become resistant to their treatment, with Gram-negative bacteria being described as pathogens with higher level of resistance and posse more serious threat to the healthcare system by the Center for Disease Control[4], [6]. In 2017, The World Health Organization (WHO) listed 12 families of pathogens already resistant to antibiotics of which the Gram-negative bacteria were highlighted as being multi-drug resistant [7]. However, new treatment i.e. antibiotics for these infections caused mainly by problematic Gram-negative bacteria such as carbapenem-producing Enterobacteriaceae (CRE), carbapenem-resistant *P. aeruginosa* (CRPA) and carbapenem-resistant *Acinetobacter baumannii* (CRAB) infections have recently been launched [8], [9].

The current resistant *P. aeruginosa* treatment available is colistin which is used in combination with imipenem and fosfomycin [10]. Though effective, these medications are quite expensive, have limited accessibility and are only administered as a last resort to terminally ill patients [11]. These medications could control new resistance from occurring but their high cost might render them inaccessible to low income individuals [12], [13]. *C. papaya* leaves have been used as anti-inflammatory, dengue, and malaria, antimicrobial among traditional medicine in Africa [14].

Several metabolites such as cysteine endopeptidases of class-II and III, chitinase and glutaminyl cyclase) were isolated from Carica latex [15]; quercetin and Kaempferol, alkaloids (pseudocarpaine and carpaine) have been isolated from leaves of the plant [16]. Thus, to combat the emerging threat to healthcare by Gram-negative bacteria there is a need to continue to search for new antibiotic natural products, capable of treating current and emerging antibiotic resistant strains. Therefore, the present study was designed to investigate the bioactive phytochemical of *C. papaya* leaves against Gram-negative antibacterial with higher antibiotic resistance.

Methods and Materials

Plant material identification

Leaves from *Carica papaya*, were collected from Shagari Lowcost, Katsina Local Government, Katsina State. The leaves were identified and authenticated as *Carica*

Papaya at the Biology Department of Umaru Musa Yar'adua University (UMYU), Katsina state, Nigeria.

Plant Preparation and Extraction

The leaves of *C. papaya* were washed with tap water and cut into small slices. The slices were pulverized after being air-dried. Dry samples (29 g) were immersed in 500 mL distilled water in a round bottom flask for 46.5 h. The mixture was then filtered to yield 250 mL of aqueous crude extract. Initial liquid-liquid partitioning was carried out using 3 x 250 mL of *n*-hexane both layers were collected, and the pH of the aqueous extract was tested to be pH 8.3. To the aqueous extract at pH 8.3 was extracted 3 x 250 mL with dichloromethane (DCM), the DCM solution was concentrated under reduced pressure to afford a brownish residue. The pH of the resulting aqueous extract was then adjusted to pH 5.3 with a few drops dilute HCl by monitoring the pH change with a pH meter until pH 5.3 was attained. The aqueous extract at pH 5.3 was extracted 3 x 250 mL with DCM, the DCM solution was concentrated under reduced pressure to afford reddish-brown residue of DCM pH 5.3 extract. The pH of the resulting aqueous extract was further adjusted to pH 9.15 with a few drops of NaOH before being extracted 3 x 250 mL with DCM. The DCM solution was concentrated under reduced pressure to afford a greenish residue.

Phytochemical screening of extracts

Preliminary qualitative tests were carried out to determine the phytochemicals present in the leaves of *C. papaya*. The presence of phytochemicals alkaloids, terpenoids, steroids, saponins, and flavonoids were tested in the crude extracts previously obtained. All procedures were developed at room temperature. The extracts were used for the subsequent qualitative analysis of metabolites using methods described by Junaid RS and Patil MK in 2020 [17].

Antimicrobial screening of extracts

The antimicrobial activity of the leaves extracts of *C. papaya* plant was determined using *Pseudomonas aeruginosa*; *P. aeruginosa* was selected because of its increased antibiotic resistance observed in healthcare setting. These resistant strains are easily spread from person to person through contaminated hands, equipment, or surfaces and are very difficult to kill. The bacteriawas obtained from the department of microbiology, Umaru Musa Yar'adua University, Katsina.

Sample preparation and serial dilution

To determine the Gram-negative antibiotic effect that is the minimum inhibitory concentration of *C. papaya* leave crude extracts different concentrations of the different test extracts (250 $mgmL^{-1}$, 125 $mgmL^{-1}$, 62.5 $mgmL^{-1}$ and 31.25 $mgmL^{-1}$) were prepared. For serial dilutions, 250 $mgmL^{-1}$ stock solutions were prepared by autoclaving individual solutions of 0.5 g of each extract obtained in 2 mL of distilled water prior to use. The autoclaved solutions were then transferred into test tubes and dissolved further by stirring vigorously using vortex mixer and water bath sonicator. Three further dilutions of 125 $mgmL^{-1}$, 62.5 $mgmL^{-1}$ and 31.25 $mgmL^{-1}$ concentrations were made using 2-fold serial dilution.

Preparation of nutrient agar (NA)

Nutrient agar growth media was prepared by dissolving whilst stirring 14 g of Himedia M173-500G nutrient agar in 500 mL of distilled water in a sterile 100 mL conical flask, the pH is adjusted to pH 7 at room temperature. The prepared nutrient agar is then autoclaved at 121 °C for 15 min and allowed to cool to 60 °C. Under a lamina air flow, the media was poured into petri-plates to occupy 60-70 % of the petri-plate and allowed to cool to room temperature and solidify.

Disk diffusion assay

The spotted disks are prepared by labeling and spotting each 5 mm disk by immersing (~10 µL) into the different concentrations of 250 $mgmL^{-1}$, 125 $mgmL^{-1}$, 62.5 $mgmL^{-1}$ and 31.25 $mgmL^{-1}$ sets of each extract and allowed to dry for about 15-30 minutes (depends on the solvent used), the positive control (ciprofloxacin) was spotted onto disks, and the negative control (fresh disk).The spotted disk containing different samples are then mounted onto the set impregnated agar plates and incubated in a Healthline IN-G255 Thermostat incubator at 37 °C for 24 h. Diameters of clear zones of inhibition are recorded once the bioassay plate is removed from the incubator.

Results

Phytochemicals of the extracts of *C. papaya* leaves

Phytochemical analysis (Table 1) shows the presence of alkaloids, saponins, flavonoids, terpenoids and steroids in the initial aqueous crude extract, with saponins being present in all the different pH's of DCM extracts but absent in *n*-hexane extract. Flavonoid is present in the *n*-hexane, DCM at pH 8.3 and

pH 9.15 extract but absent in the DCM at pH 5.3 extract. Terpenoids is present in *n*-hexane and DCM at pH 8.3 but absent in DCM at pH 5.3 and pH 9.15 extracts. The presence of alkaloids is observed in *n*-hexane, DCM at pH 5.3 and pH 9.15 extracts but absent in DCM at pH 8.3 extract. The presence of steroids is observed in all the extracts.

Table 1 – Qualitative phytochemical analysis of C. papaya leaf extracts

Phytochemicals	Crude Extracts			
	n-Hexane	DCM 1	DCM 2	DCM 3
Saponins	-	-	+	+
Flavonoids	+	-	+	+
Terpenoids	+	-	+	-
Alkaloids	+	+	-	+
Steroids	+	+	+	+

+ = present, - = absent; DCM 1=DCM at pH 5.3; DCM 2= DCM at pH 8.3; DCM 3= DCM at pH 9.15

Gram-negative antibacterial screening of C. papaya leaf extracts

Antibacterial screening of the extracts against *P. aeruginosa* Gram-negative bacteria was carried out (Table 2). Antibacterial activity i.e. zones of inhibition was observed in *n*-hexane, DCM at pH 5.3 and pH 8.3 extracts but not from DCM at pH 9.15 extracts. The widest zone of inhibition was 22 mm from the DCM at pH 8.3 extract while 0 mm was observed from DCM at pH 9.15 extract. For the minimum inhibitory concentrations experiments of the crude extracts (Table 3), antibacterial activity was observed from the *n*-hexane extracts at high concentrations of 250 and 125 $mgmL^{-1}$ concentrations with the diameter of the zones of inhibition of 9 and 7 mm respectively and none was observed at lower concentrations of 62.5 and 31.25 $mgmL^{-1}$. For DCM extract at pH 5.3 antibacterial activity with a zone of inhibition of 7 mm was observed at the highest concentration of 250 $mgmL^{-1}$ and no zones of inhibition were observed at 125, 62.5 and 31.25 $mgmL^{-1}$ concentrations even after repeats. For DCM extract at pH 8.3 antibacterial activity against *P. aeruginosa* was observed at all the concentrations 250, 125, 62.5 and 31.25

mgmL^{-1} with zones of inhibition of 22, 15, 12 and 11 mm respectively. Finally, no antibacterial activity was observed for DCM at pH 9.15 extracts at all concentrations where zero zones of inhibition were recorded.

Table 2- Antibacterial screening of crude extracts of *C. papaya* leaves

Crude Extracts	Zone of inhibition (mm)
n-hexane	9
DCM at pH 5.3	7
DCM at pH 8.3	22
DCM at pH 9.15	0
Positive control (ciprofloxacin)	28
Negative control (blank disk)	0

Table 3- Minimum Inhibitory Concentrations of crude extracts

Extracts	Concentrations mgmL^{-1}				MIC
	250	125	62.5	31.25	
n-hexane	9	7	0	0	250 mgmL^{-1}
DCM at pH 5.3	7	0	0	0	250 mgmL^{-1}
DCM at pH 8.3	22	15	12	11	31.25 mgmL-^{1}
DCM at pH 9.15	-	-	-	-	NA
Control	28				8 -32 µgmL^{-1}

Discussion

The phytochemical analysis of *C. papaya* leaves extract showed the presence of chemical constituents which are associated with possessing some pharmacological activities [18]. Organic extraction was also carried out based on the physicochemical properties of bioactive natural product(s) of interest by qualitative analysis and by adjusting the pH of the aqueous extract before partitioning with organic solvents. Though not all the five phytochemicals that were tested were present in all extracts screened, the presence of steroids was observed in all extracts (Table 1). From literature *C. papaya* leaves are known to

contain steroids, triterpenoids, and fatty acid methyl esters this supports our findings [19]. Also present in *n*-hexane and DCM at pH 8.3 and pH 9.15 extracts is flavonoid, flavonoids are commonly water-soluble in nature and therefore usually remain in aqueous layer. However, the use of pH change has altered the pH of the aqueous mixture and hence the polarity thereby enabling flavonoids to be salted out into the organic mixture [20]. From literature flavonoids present in plants are bound to sugar and contain aglycone and in this research flavonoid was detected in non-polar organic but not inDCM at pH 5.3 extract. Interestingly is the presence of terpenoids in *n*-hexane and DCM at pH 8.3 extracts but was not detected in DCM at pH 5.3 and pH 9.15 extracts, this suggests that terpenoids are primary in nature comprising of hydrocarbons and oxygen making terpenoids very non-polar in nature. The presence of terpenoids in DCM at pH 8.3 extract could be as a result of terpenoids being within the neutral pH range region [21]. As expected, alkaloids are detected in DCM at pH 9.15 extract because alkaloids are basic in nature with a pH value of 10.5 and mostly appear in the form of neutral molecules possessing high retention factors based on their polarity. In this research alkaloid was detected at a lower pH i.e 5.3 even after the experiment was repeated. From literature, the type of alkaloid such as indole alkaloid present in etractdetermines the extraction method applied. [22]. From literature, the pH range of saponins vary between 3 and 8, saponins was detected in DCM extracts at pH 8.3 and 9.15 the difference could be due to physicochemical differences such as environment, temperature, and climate changes between these plants. Saponins were not detected in *n*-hexane and DCM at pH 5.3 because the saponins present in this *C. papaya* leaves extract are more basic as observed.

Antibacterial activity test using *P. aeruginosa* as the Gram-negative bacteria was carried out on all the extracts collected. Antibacterial activity was observed from most extracts with the exception of DCM at pH 9.15 and their diameters of the zones of inhibition were measured (Table 2). The diameters observed around the disk containing the extracts are an indication of the inhibition of the test organisms therefore the larger the halo size the wider the diameter and the greater the inhibition of the growth of the test organism. There is always a negative control (used to observe that the inhibition is indeed caused by the extracts)

and a positive control (this is a known antibiotic known to inhibit the growth of the test organism). Antibacterial activity was not detected in DCM at pH 9.15 extract as there were no zones of inhibition observed. This finding shows the bioactive compound(s) which can inhibit the growth of *P. aeruginosa* are not present in that extract. Also, observed was the absence of the phytochemical terpenoid in DCM at pH 5.3 and pH 9.15 extracts but its presence in DCM at pH 8.3 extract. This was an interesting finding because DCM extracts at pH 5.3 and pH 9.15 presented little to no antibacterial activity against *P. aeruginosa* whereas DCM at pH 8.3 extract showed antibacterial activity against our test organism. Therefore we postulated that a terpenoid phytochemical was responsible for the biological activity observed.

To determine the minimum inhibitory concentration (MIC), the extracts were diluted serially to yield four different concentrations of 250 $mgmL^{-1}$, 125 $mgmL^{-1}$, 62.5 $mgmL^{-1}$ and 31.25 $mgmL^{-1}$ (Table 3). *n*-hexane extract had an MIC of 125 $mgmL^{-1}$ with a diameter of 9 mm, the diameter is low even at higher concentrations suggesting a weak inhibition of the test organism. For DCM at pH 5.3 the MIC was 250 $mgmL^{-1}$ showing the highest concentration as its MIC with a zone of inhibition of 7 mm. This is another weak inhibition by the metabolite present within the extract which is needed in high concentrations for any biological effect to occur. The DCM at pH 8.3 extract appears to be the most promising extract showing inhibition in all concentrations, the higher the concentration the higher the inhibition with an MIC of 31.25 $mgmL^{-1}$ with a zone of inhibition of 11 mm. Whilst the presence of bioactive compounds in *n*-hexane and DCM at pH 5.3 extracts could arise because of non-polar or acidic bioactive compounds their bioactivity could also be because of residual compounds from extractions. For DCM at pH 8.3 extract however, the biological activity could be as a result of a new or derivative compound of a Gram-negative antibiotic to be isolated from *C. papaya* leaves. This is an interesting discovery because *P. aeruginosa* have been reported by WHO as a priority 1 (critical) since it has acquired resistance to most antibiotics currently within the healthcare systems [10]. With Carbapenem and fluoroquinolones (ciprofloxacin) resistance rates for *P. aeruginosa* between 20 to 40 % when compared to that of *klebsiella pneumonia* which are above 25% the need

for new antibiotics is of high priority[10] [23].

Conclusion

The phytochemical screening of *C. papaya* leaves extracts shows the presence of steroids, alkaloids, terpenoids, saponins and flavonoids, the antibacterial activity of the extracts against *P. aeruginosa* bacteria shows DCM at pH 8.3 extract exhibit the highest bioactivity against *P. aeruginosa*. From the phytochemical screening, the presence of terpenoids phytochemical may be responsible for the Gram-negative antibacterial activity observed. These findings indicate that *C. papaya* leaves extract may be used in the treatment against resistant *P. aeruginosa* Gram-negative bacteria.

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