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Bioguided isolation, structure elucidation and evaluation of new antimicrobial flavonoid from *Carica papaya* leaves

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Abstract
The emergence of antimicrobial resistance is a global concern, particularly in developing countries. The aims of this study were to isolate and identify antimicrobial fractions of *C. papaya* based on their antimicrobial activity and evaluate the antimicrobial effects of flavonoid to provide natural resources against *S. enteretidis*, *S. typhi*, *E. coli*, *S. flexneri* and *K. pneumonia* using the broth microdilution method. The aqueous fraction showed high inhibition potentials against all the tested bacteria with a Minimum Inhibitory Concentration value of 500 µg/mL. Subsequent phytochemical investigation of aqueous fraction by bioactive-guided column chromatography identified by ^{1}H NMR, ^{13}C NMR and ESI-MS experiments, one new flavonoid named 6''-*O*-acetyl-3'-*O*-methyl orientin. The antimicrobial effects of flavonoid against the same pathogens were close to those of the aqueous fraction. This result showed that 6''-*O*-acetyl-3'-*O*- methyl orientin is responsible for the antimicrobial activity of *Carica papaya* leaves and may be considered as antimicrobial lead compound.

Keywords: Bioguided isolation, fractions, antibacterial, flavonoids, *C. papaya*

1. Introduction
Bacteria are widely distributed in the environment and live in terrestrial and aquatic environments. Water, food and fishery products are the main potential sources of transmission of bacterial infection [1, 2, 3]. Nowadays, the therapeutic control is mostly based on the use of antibiotic drugs. However, excessive and uncontrolled use of antibiotics led to the emergence of resistant and multi-resistant bacteria strains [4, 5]. Therefore, new antibiotic drugs are needed for efficient management of infectious diseases.

In recent years, there have been an increase interest on investigating natural resources including medicinal plants for new therapeutic molecules [6]. The wealthy biodiversity of the Cameroonian flora has been over the years a source of great relief for local populations. Among them, *Carica papaya* (Caricacea) is generally known through its fruits which is a rich source of nutrients (vitamins, minerals and fibers). The other parts, including, leaves, roots, and seeds are used in traditional medicine for the treatment of several ailments. For instance, in West Africa (Nigeria, Ghana, Zimbabwe, and Uganda), the decoction of leaves are administrated orally to fight malaria and fever [7, 8]. In association with the roots, the mixture is efficient against diarrhea and dysentery [9, 10]. In some parts of Asia, the young leaves of the papaya are steamed and eaten like spinach. They contain carpain which facilitate the digestion and kills microorganisms that often interfere with the digestive function [11, 12].

Several phytochemical investigations revealed the presence of several classes of metabolites including alkaloids, terpenoids and phenolic compounds in *C. papaya* [13, 14]. In fact, polyphenols are subdivided into phenolic acid, flavonoids, tannins, quinones, coumarins and stilbenes and they are considered among the major and relevant secondary metabolites in plants. Phenolic compounds are bioactive molecules that inhibit a broad spectrum of the microorganisms [15, 16, 17, 18, 19].

However, according to literature surveys, there have been few studies of *Carica papaya* focusing on its bioassay-guided isolation, and almost all describe investigation using crude extracts or fractions.

The aims of the current study were to isolate and identify antibacterial fractions of *C. papaya* based on their antimicrobial activity, and evaluate the antimicrobial effects of the new isolated flavonoid 6''-*O*-acetyl-3'-*O*-methyl orientin *in vitro* to provide natural resources against bacterial infections. This is the first report of the bioguided isolation of antimicrobial flavonoid from *C. papaya*.

2. Materials and methods

2.1 Plant Material

The leaves of *C. papaya* were collected in the period of January 2020 from Banwa located at West region of Cameroon. They were identified at National Herbarium by comparison of the previous sample under collection number 18647/SRF Cameroon and were deposited in the Laboratory for Phytobiochemistry and Medicinal Plants Studies, Antimicrobial and Biocontrol Agents Unit, University of Yaounde I. The fresh leaves were washed with water, cut into small pieces and kept for shade drying at room temperature (20ºC-25ºC). Thereafter, the complete dried sample was crushed in powder by using blender and was submitted for extraction and isolation of phenolic compounds.

2.2 Extraction and isolation

C. papaya leaves were subjected to cold maceration [20]. The solvents were used in order of increasing polarity: hexane< methanol< water. To obtain used fractions, 560 g of the powdered leaves were soaked separately in 600 mL of pure hexane to remove mostly fats and oils. After 72 h of maceration, the mixture was filtered. The filtrate was concentrated using a rotavapor. To obtain the hexane fraction (3 g), the maceration was repeated twice in order to maximize the yield. The residue obtained after the hexane maceration was dried for 24h at room temperature. The same procedure was repeated to obtain the methanol fraction (13.8g). However, the resulting residue obtained after the methanol fraction was extracted 3 times in 500 mL of water using the Biobase lyophilize to yield a water fraction (20.2g).

The aqueous fraction (20g) was subjected to column chromatography over silica gel eluting with gradients of CH_2Cl_2- MeOH to afford 48 fractions of 200mL each. These fractions were combined on the basis of their TLC profiles into one major fraction:

Fraction A (14g, 1-48) (CH_2Cl_2/MeOH 20:1) was purified by silica gel column chromatography with a gradient of CH_2Cl_2/MeOH (30/1) and on sephadex LH20 column chromatography with acetone to afford compound 1 (17 mg).

2.3 Evaluation of antimicrobial activity

2.3.1 Bacterial strains

In this study, five bacterial strains were used: *Salmonella enteretidis* (CPC), *Salmonella typhi* (CPC) obtained from Pasteur Center of Cameroon; *Escherichia coli* (ATCC 25922), *Shigella flexneri* (NR-518) and *Klebsiella pneumonia* (ATCC 700603) from BEI Resources (USA) Laboratory.

2.3.2 Preparation of stock solution of fractions, isolated compound and reference antibacterial (ciprofloxacin).

Stock solutions were prepared at 100 mg/mL by dissolving 100 mg of extract in 1 mL of absolute dimethyl sulfoxide (DMSO).

2.3.3 Determination of the antibacterial activity

The Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) were determined by broth microdilution method according to the Clinical and Laboratory Standards Institute guidelines CLSI 2012 (protocol M07-A9) [23]. Cell viability was evaluated by colorimetric method based on the enzymatic reduction of rezasurin (Alamar Blue dye) to risofurin (pink dye). The test were carried out in triplicate in 96 wells micro-plates. In fact, two-fold serial dilutions of extracts were performed in a Muller Hinton broth to obtain volumes of 100 µL per well. One hundred microliters of a bacterial suspension (1.5×10^6 CFU/mL) were added into each well containing the test substances to obtain final concentration range from 4000-500 µg/mL. The percentage of DMSO control was 1% and showed no effect on bacterial growth. Ciprofloxacin was used as a positive control and the plates were covered and incubated at 37ºC for 24 h. MICs were determined by addition of 20 µL of Resazurin (1.5 mg/mL) to the wells (in duplicate) and incubated at 37ºC for 30 min. Membrane dehydrogenases from viable cells reduce the dark blue-colored dye to Resorufin pink. The MIC was determined as the lowest concentration of test substance that have the blue colored dye (no reduction of resazurin). MBCs were determined by sub-culturing 25 µL aliquots of the inhibitory cups not having received Resazurin into the wells of sterile plates containing 175 µL of Muller Hinton broth. The plates were then covered and incubated at 37ºC for 48 h. Cell viability was determined by the colorimetric method as above. The lowest concentration of the test substances showing blue color was considered MBC. Negative control constituted of culture medium and the inoculum. The sterility control only contained the culture medium.

2.4 Evaluation of the cytotoxicity assay of the active *C. papaya* fractions

Cytotoxicity of active fractions was assessed according to the protocol described by Bowling *et al*., 2012 [24]. African green monkey normal kidney Vero cells (ATCC CRL 1586) were maintained in T-25 flasks using complete Dulbecco's Modified Eagle's Medium, supplemented with, 10% fetal bovine serum, 0.2 % sodium bicarbonate (w/v) and 1% (v/v) penicillin-streptomycin. Cells were kept at 37°C for 72 h in 5% CO_2 incubator. The medium was renewed each 72 h and the cell density was monitored under an inverted microscope Etaluma (520) until formation of a monolayer. Confluent culture (nearly 90%) was trypsinized (0.05% Trypsin-EDTA), then centrifuged at 1800 rpm for 5 min and the resulting pellet was suspended in culture medium. Mixtures at 10^4 cells per well were seeded (100µL) in 96-well culture plates (Costar, USA) and incubated overnight to allow cell adhesion. Thereafter, 10 µL of active fractions were diluted at the concentrations of 1000, 200, 40.8 and 1.6 µg/mL. Thereafter, they were added to plate wells in duplicate. The plates were incubated in a humidified and 5% CO_2 atmosphere at 37°C for 48 h. Podophyllotoxin at 20 µM was added as positive control and wells containing untreated cells was included as 100% growth control. Ten microliters of a stock solution of Resazurin at 0.15 mg/mL in sterile PBS were added to each well, and incubated for an additional 4 h. Fluorescence was then read using a Magelan Infinite M200 fluorescence multi-well plate reader (Tecan) with an excitation and an emission wavelengths of 530 and 590 nm, respectively. Percentage of cell viability was calculated with regard to the negative control, and subsequently used to determine the concentration that reduced 50% of cell viability (CC_{50}) by non-linear regression using the GraphPad Prism 5.0 software (San Diego, California).

2.5 General Experimental Procedures for phytochemical study

Melting points were determined on Electrothermal I A 9000 series digital melting point apparatus and are uncorrected. The UV spectra were recorded on UV-570/ VIS/ NIP and Shimadzu UV-24012A double-beam spectrophotometers. IR measurements were obtained on a PerkinElmer (model 1600) FTIR spectrometer. The 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY HSQC and HMBC) NMR spectra were recorded in DMSO-d_6 and MeOH-d_4 using Bruker 600 (600 MHz for ^{1}H-NMR, 150 MHz for ^{13}C-NMR) spectrometers. ESIMS were obtained using a MSQ Thermofinnigan instrument. Chemical shifts are stated in parts per million (ppm) from the internal standard, tetramethylsilane (TMS). Flash column chromatography was performed using silica gel 60 (Merck, 0.040−0.063 mm). TLC was conducted on pre-coated Merck Kieselgel 60 F_{254} plates (20 × 20 cm, 0.25 mm). Spots were checked on TLC plates under UV light (254 nm), and developed with vanillin or $KMnO_4$ reagents, followed by heating.

3. Results and discussion

3.1 Results of the antibacterial activity

Yield of the extraction of the different fractions are illustrated in Table 1.

Table 1: Yield of extraction of the aqueous and methanolic fractions of *Carica* papaya leaves

Fractions code	Leaves weight (g)	Fraction weight (g)	Yield (%)
FH_2O[a]	280	20.2	7.2
FMeOH[b]	280	13.8	4.92

[a]FH_2O: water fraction; [b]FMeOH: Methanol fraction

From the results presented in table 1, the yield of the aqueous fraction (7.2 %) was higher than that of the methanolic fraction (4.92 %).

3.1.1 Antimicrobial Susceptibility Test

Evaluation of the antibacterial activity of the different fractions of *C. papaya* leaves was determined by measuring Minimum Inhibitory Concentration (MICs) and Minimum Bactericidal Concentrations (MBCs). The results are presented in Table 2.

Table 2: MIC and MBC (µg/mL) of the fractions of leaves *C. papaya*

Fractions code	*Salmonella. enteretidis CPC*		*Salmonella. typhi CPC*		*Shigella. flexneri NR 518*		*Eschericia. coli ATCC 25922*		*Klebsiella. pneumonia ATCC 700603*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
FH_2O	1000	4000	1000	4000	1000	>4000	500	>4000	2000	1000
FMeOH	>4000	>4000	>4000	>4000	1000	>4000	>4000	>4000	>4000	>4000
Isolated compound	1000	4000	1000	4000	1000	>4000	500	>4000	2000	1000
Ciprofloxacin	0.0195	0.0390	0.0390	0.313	0.0195	0.063	0.0195	0.039	0.0195	0.0625

Cp: *Cariaca papaya*; L: leaves, FH_2O: water fraction; FMeOH: methanol fraction; ND: not determined; >4000 means no inhibition was observed

The susceptibility of the tested fractions of *C. papaya* leaves was carried out against five bacterial strains (Table 2). The results indicated that MIC and MBCs varies according to the strains and fractions. The lowest MIC value obtained from the aqueous fraction of *C. papaya* leaves (FCpLH_2O) with a value of 500 µg/mL against *E. coli*. In addition, the same fraction showed a MIC value at 1000 µg/mL against *S. enteretidis*, *S. typhi* and *S. flexneri*. Moreover, MIC at 2000 µg/mL was obtained against *K. pneumonia*. We found that the higher inhibition was attributed to aqueous fraction of *C. papaya* (FH_2O) with a MBCs value at 1000 µg/mL against *K. pneumonia*. However, the methanolic fraction of *C. papaya* leaves (FCpLMeOH) only demonstrated inhibition against *S. flexneri* with MIC at 1000 µg/mL. Subsequent phytochemical investigation of aqueous fraction have been carried out.

The balance between active substance, fractions and it cytotoxicity is very important in the development of new antimicrobial. This is necessary to avoid the undesirable effect. For this reason, aqueous fraction and isolated compound were evaluated for their cytotoxicity on Vero cell line. We finally evaluated the cytotoxicity of active fractions of *C. papaya* as reported in Table 3.

Table 3: Median Cytotoxic Concentration of fractions (CC_{50}) of *C. papaya* in Vero cells (ATCC CRL 1586)

Fractions and isolated compound	CC_{50} (µg/mL)
Leaves aqueous fraction of *C. papaya*.	>1000
Isolated compound.	>1000

According to the American National Cancer Institute (NCI), the extract are consider as cytotoxic whether the CC_{50}< 30 µg/mL. The CC_{50} values were >1000µg/mL for aqueous fraction and isolated fraction. According to the NCI, no fraction are cytotoxicity.

3.1.2 Discussion of the antibacterial activity

The aim of this work was to evaluate the antibacterial activity of the phenol-rich fractions of *C. papaya* leaves, carry out phenolic substances screening and study cytotoxicity.

Aqueous fraction of leaves demonstrated a higher extraction yield (7.2 %) compared to the methanolic fraction. This fact showed that polyphenol constituents of *C. papaya* leaves are more soluble in water than in methanol. Parameters as solvent type and method of extraction are fundamental factors to consider for optimizing extraction yield [25].

Among the tested fractions, the aqueous fraction was revealed to be the most active against the five bacterial strains tested. *E. coli* was the more sensitive strain with a MIC value at 500 µg/mL. However, the methanolic fraction displayed an activity only against *S. flexneri* with the MIC at 1000 µg/mL. According to the Tamokou *et al.* [26], the antimicrobial activities of extract are consider to be highly active if the MIC<100 µg/mL, significantly active when 100<MIC <512µg/mL, moderately active when 512<MIC<2048µg/mL, weakly active if MIC> 2048µg/mL and not active when MIC>10 000 µg/mL. For this, aqueous fraction inhibited moderately active on all microorganism except *E. coli* that inhibited significantly active. Otherwise, methanol fraction is

moderately active on *S. flexneri* and no active on all other microorganism. Microorganisms studied herein are the most important enteric human pathogens worldwide and causes infectious diarrhea [27, 28]. Comparing results found with those in a previous works, alcoholic *C. papaya* leaves extracts usually demonstrated the stronger antimicrobial activity [17, 29]. In our work; the aqueous fraction rich in phenols was more effective. This finding suggests that solvent extraction technique affects secondary metabolites extraction and bioactivity [30].

The qualitative chemical analysis show high presence of flavonoid and polyphenols in FLH_2O and FLMeOH, respectively. This corroborate the work of Fatma *et al*., [31]; Agung *et al*., [15] who demonstrated the presence of the phenolic compounds in the extracts of leaves. It is known that phenolic compounds undertake their antibacterial mechanism specifically on the cytoplasmic membrane of bacterial cells that is particularly associated with the number and position of the compound hydroxyl groups [32]. Potential membrane alteration is an indication that the integrity of bacterial membranes were disrupted and will be followed by entry of more antibacterial agents. Furthermore, flavonoids cause bacterial cell death through disruption of the cytoplasmic membrane that leads to the loss of potential membrane and interferes with metabolic activity such as synthesis of DNA, RNA and proteins [33, 34].

3.2 Results and discussion of the phytochemical investigation

The aqueous fraction of *C. papaya* leaves ($FCpLH_2O$) was chromatographed over a silica gel and sephadex LH20 column to afford one new flavonoid (**1**) 6"-*O*-acetyl-3'-*O*-methyl orientin**.** The structure elucidation of the new compound was carried out by means of ESIMS and NMR spectroscopy and compared with previously reported data.

Compound 1 was obtained as yellow powder and gave positive reaction with Neu reagent [22]. Compound 1 gave a pseudo-molecular ion peak at *m/z* 503.4 $[M-H]^-$ in negative ESI-MS (figure: 1) consistent with the molecular formula of $C_{24}H_{24}O_{12}$. The IR spectrum of 1 disclosed vibration bands due to hydrogen bonded hydroxyl (3242 cm^{-1}), acetyl group (1720 cm^{-1}), chelated carbonyl (1648 cm^{-1}) and bands attributable to aromatic ring at 1575 and 1510 cm^{-1} (figure :2). The UV spectrum showed absorptions at λ_{max} 329 and 275 nm suggesting the presence of flavone moieties.

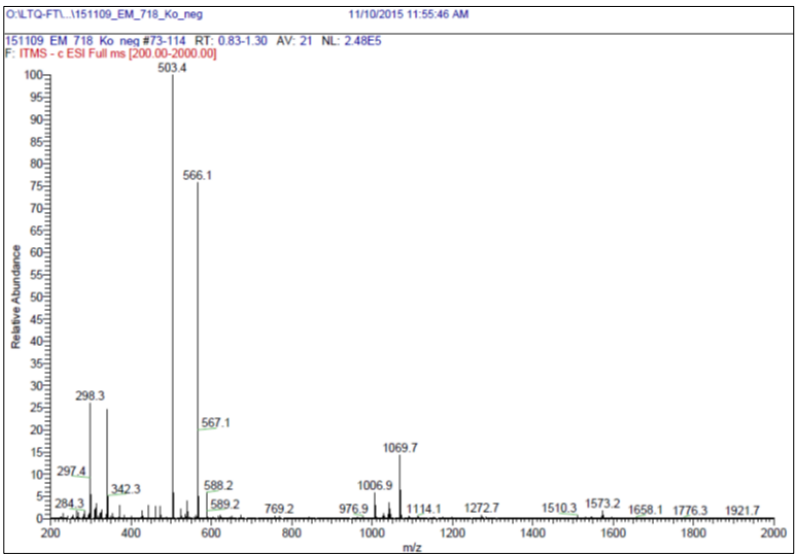


Fig 1: ESIMS Spectrum of compound 1

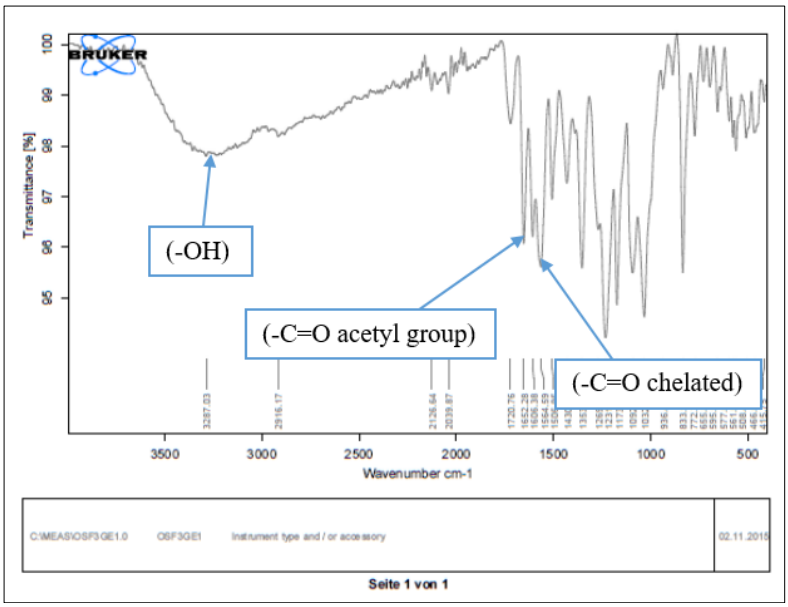


Fig 2: IR spectrum of compound 1

The ^{1}H NMR spectrum of 1 (Table 4) and (figure: 3) showed and intramolecular hydrogen-bonded hydroxyl proton signal at δ_H 13.16 suggesting that 1 is a 5-hydroxyflavonoid [35]. This spectrum also showed characteristic signal for one flavone structures [35] at δ_H 6.65 (1H, s, H-3). In the aromatic region, the^{1}H NMR spectra exhibited one ABX-type proton systems at δ_H 8.35 (1H, dd, J=9. 0 and 3. 0 Hz, H-6'), 7.89 (1H, d, J=3 Hz, H-2') and 7.02 (1H, d, J=9.0Hz, H-5') for the B-ring. The remaining singlet at δ_H 6.22 (1H, s, H-6) could be assigned to proton of the pentasubstituted ring A. The upfield portion of the ^{1}H NMR spectra present methoxy resonance at δ_H 3.87. It was also observed one anomeric signal at δ_H 5.24 (*d*, *J* = 7.8 Hz, H-1") characteristic of a *β*-linked sugar [36, 37] and one singlet of 3 protons at δ_H 1.97 (3H, s, H_3COCO-6′′).

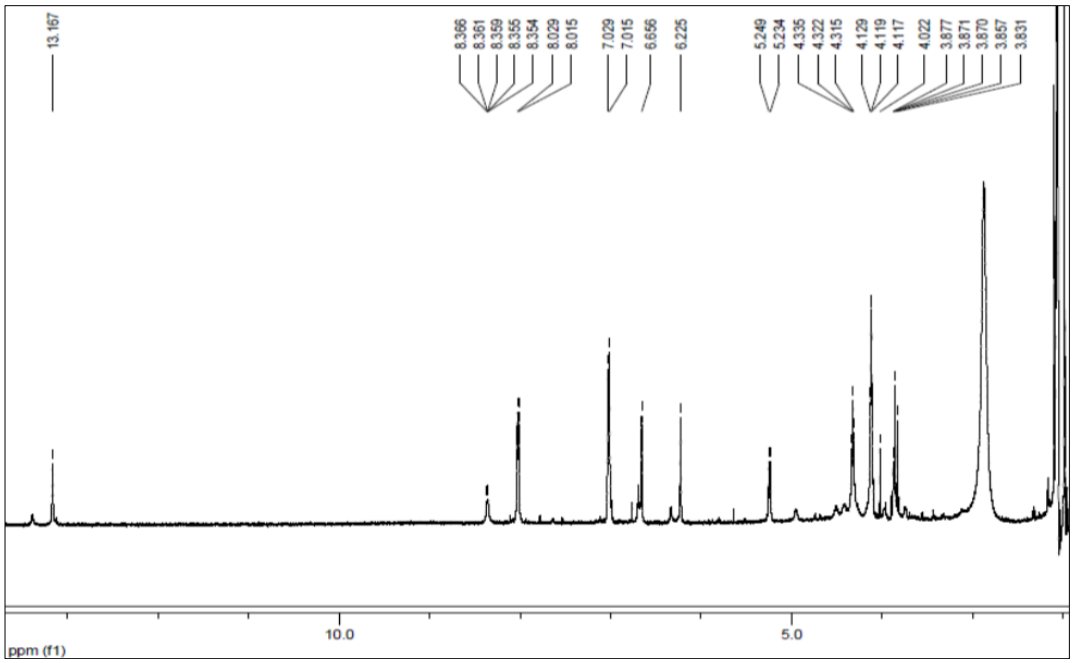


Fig 3: ^{1}H NMR (600MHz, MeOH-*d*$_4$) spectrum of compound 1

The ^{13}C NMR data of **1** (Table 4) and (figure: 4) supported the flavone based flavonoid structure, with 24 carbon resonances representing one carbonyl carbon at *δ*c 182.9 ppm, one sugar residue, one methoxyl and one acetoxyl groups with the respect signal at $\delta_{C/H}$ 55.6/3.87 ppm and 169.9/1.97 ppm. Six carbon signals between 63.9 and 76.6 ppm were assigned to one sugar residue, suggesting that **1** is a flavone *C*-glucoside system [37].

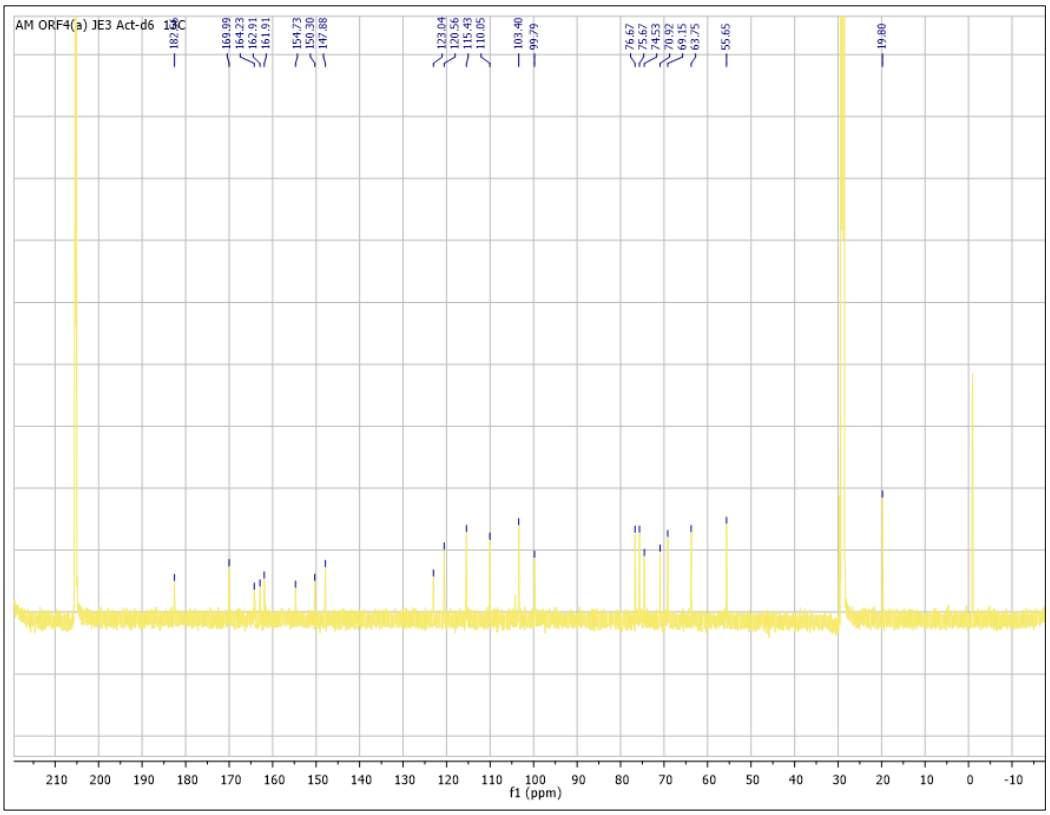
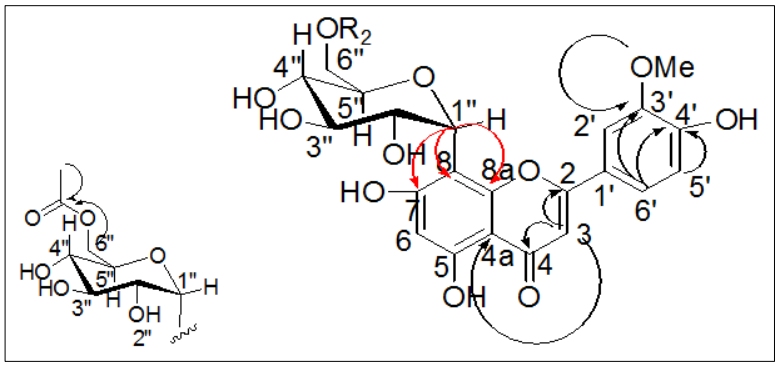


Fig 4: ^{13}C NMR (150MHz MeOH-*d*$_4$) spectrum of compound 1

The HMBC correlations (figure: 5) between the methoxy proton signals (δ_H 3.87) and C-3' (δ_C 147.8) suggested the position of methoxyl group at C-3'. Furthermore, key HMBC correlations were observed between the anomeric proton at δ_H 5.24 ppm with carbon atom signals at δ_C 103.6 ppm (C-8), 154.7 ppm (C-8a), and 162.9 ppm (C-7), indicating that, glucoside was attached at C-8 of flavone aglycone unities. These data suggested the presence of the orientin skeleton moiety in 1 [36, 37]. The location of the acetyl group at C-6" was established by HMBC experiments, where HMBC correlations between the diastereotopic protons at (δ_H 4.33 and 4.31 (H-6") and the ester carbonyl carbon atom at (δ_C169.9)/ COO- 6"(I) were observed (Scheme 1).



Scheme 1: Keys HMBC Correlations of compound 1

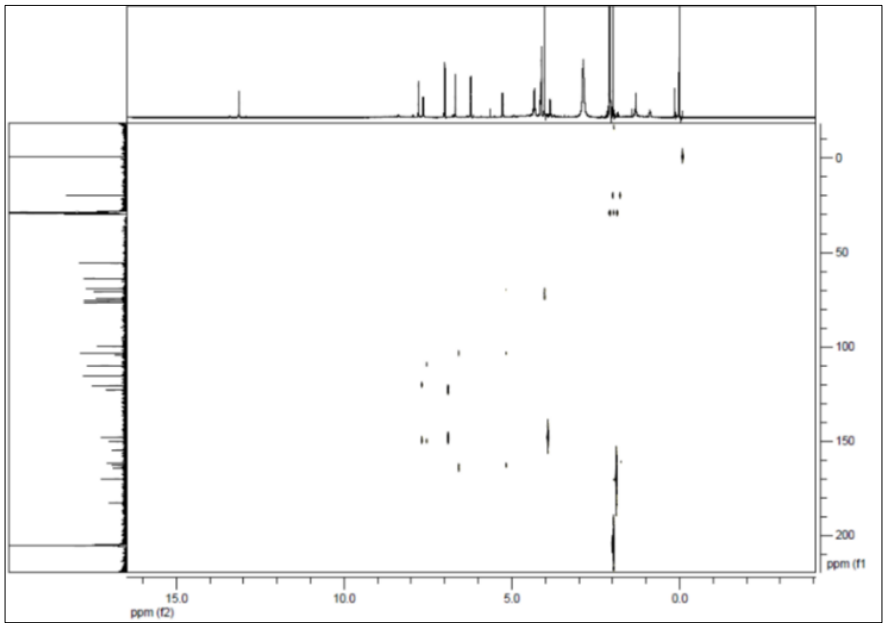


Fig 5: HMBC spectrum of compound 1

From the foregoing observations, compound 1 was characterized as 6"-*O*-acetyl-3'-*O*- methyl orientin.

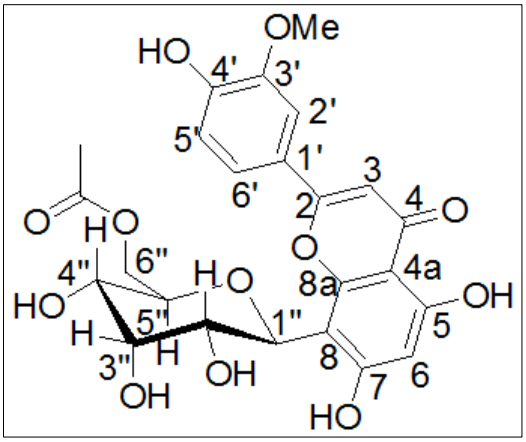


Table 5: ^{1}H NMR (600MHz) and ^{13}C NMR (150MHz) data of 1

Position	δ_C (in ppm)	δ_H (J in Hz)
2	164. 2	-
3	103. 4	6.65 (1H; s)
4	182. 9	-
4a	104. 7	-
5	161. 9	-
6	99. 7	6.22 (1H; s)
7	162. 9	-
8	103. 6	-
8a	154. 7	-
1′	120. 5	-
2′	110. 0	7. 99 (1H; d ; *J* = 3. 0)
3′	147. 8	-
4′	150. 3	-
5′	117. 3	7. 02 (1H;d; *J* = 9. 0)
6′	125. 0	8. 35 (1H;dd; *J* =9. 0;3.0)
1″	74. 5	5. 24 (1H;d; *J* = 9. 6)
2″	69. 5	4. 20(1H; m)
3″	75. 6	3. 66(1H; m)
4″	70. 9	4. 46(1H; m)
5″	76. 6	3. 98 (1H; m)
6″	63. 9	4.33(1H;m) ; 4.31(1H;m)
H_3COCO-6″	169. 9	1. 97 (3H;s)
3′-OMe	55. 6	3. 87 (3H;s)

4. Conclusion

The aqueous fraction of *Carica papaya* leaves have potent antibacterial eff ects against all bacterial strains tested. This result showed that 6''-*O*-acetyl-3'-*O*-methyl orientin is responsible for the antimicrobial activity of *Carica papaya* leaves and may be considered as antimicrobial lead compound.

5. Acknowledgement

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Authors' contributions

All authors (MA, NM, KE, MD. TE, BB and DD) contributed in the experimental studies, data acquisition, and data analysis. MA, NM, TE, BB and KE contributed to the concepts of ideas, experimental design, literature study, manuscript preparation and editing. MA did the manuscript review and submission. All authors read and agreed its publication

Conflict of interests

None to declare.

Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or redundancy) have been completely observed by the authors.

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