

CHEMICAL COMPOSITION AND ANTI-MICROBIAL EVALUATION OF VARIOUS FRACTIONS OF THE METHANOL SEED EXTRACT OF *CARICA PAPAYA* L (CARICACEAE)

Patrick Igbina², Omotoso Abayomi E*¹, Uwumarongie H.O³

¹Department of Pharmaceutical & Medicinal Chemistry, Faculty of Pharmaceutical Sciences, University of Port Harcourt, Port Harcourt, **Nigeria**

²Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Benin, Benin City, Edo State, **Nigeria**

³Department of Pharmacognosy, Faculty of Pharmacy, University of Benin, Benin City, Edo State, **Nigeria**

*Corresponding Author: Phone: +2348035662535; Email: abayomi.omotoso@uniport.edu.ng, abatoseb2001@yahoo.com

ABSTRACT

Carica papaya is used in traditional medicine in the management of malaria, typhoid fever and other infectious diseases. Hence, the objectives of this study were to determine the chemical constituents of the methanol, petroleum ether and ethyl acetate fractions of the methanol seed extract of *Carica papaya*, as well as evaluate the antibacterial activity against *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Escherichia coli*, *Candida albican* and *Microsporum audiounii*. Chemical constituents were assessed using Gas Chromatography-Mass Spectrometric (GC/MS) technique and mass spectra of the compounds were matched with the National Institute of Standard and Technology (NIST) library. The antibacterial activity was evaluated using the agar well diffusion technique, where the inhibition zone diameters (IZD) and minimum inhibitory concentrations (MICs) of the fractions were determined. Gas Chromatography-Mass Spectrometric (GC/MS) technique revealed the presence of useful chemical constituents in the various fractions of the methanol seed extract. The fractions showed activity against *B. Subtilis* with MICs of 0.08 mg/mL, 0.2 mg/mL and 0.4 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively; followed by *S. aureus* with MICs of 0.1 mg/mL, 0.4 mg/mL and 0.6 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively; followed by *E. coli* with MICs 0.2 mg/mL, 0.6 mg/mL and 0.8 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively. The least among the bacteria was observed with *K. pneumonia* with MICs of 0.4 mg/mL, 0.8 mg/mL and 1.0 mg/mL for the methanol, petroleum ether and ethyl acetate fractions respectively. The fractions exhibited low activity against the tested fungi. *C. albican* had MICs of 0.4 mg/mL, 1.0 mg/mL and 0.8 mg/mL for the methanol, petroleum ether and ethyl acetate fractions respectively; while *M. audiounii* had MICs of 0.8 mg/mL, 2 mg/mL and 1.0 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively. This study revealed the presence of useful chemical constituents in the various fractions (methanol, petroleum ether and ethyl acetate), as well as, authenticated the ethnomedicinal use of the seeds of *C. papaya* in the management of various infectious diseases.

Keywords: Herb, Infectious diseases, Phytochemicals, Enzymes.

INTRODUCTION

In recent years, growing demand for herbal products has led to increase in volume of plant materials traded across countries especially Asian and African continents (Kafaru, 1994). *Carica papaya* is native to tropical America. Different parts of the plant including seeds, latex and fruit have demonstrated medicinal value. The latex from unripe papaya fruit

contains enzymes papain and Chymopapain, cysteine endopeptidases, chitinases and an inhibitor of serine protease. Phytochemical analysis of *Carica papaya* leaf and root extracts revealed the presence of alkaloids, glycosides, flavonoids, tannins, phenols and steroids (Natarjan and Theivanai, 2014; Emokpae *et al.*, 2016).

The health benefits of *C. papaya* may be attributed to its high content of Vitamins A, B and C, proteolytic enzymes like papain and Chymopapain which have antiviral, antifungal and antibacterial properties. The seeds of *Carica papaya* have been shown to increase platelet count and are good for dengue fever patient (Fenny *et al.*, 2012). From literature, a lot of studies have been carried out on the use of papaya plant especially the leaf, in the treatment of typhoid fever and malaria. Most of these studied plant preparations have been found to be active biologically, and as such, can be employed in the therapeutic management of diseases and infections. Presently, there seem not to exist any published study on the use of petroleum ether and ethyl acetate fractions of the plant, with a focus on the antimicrobial effect of the seeds of the plant; which this study seeks to investigate.

MATERIALS AND METHODS

Materials

All solvents were of the Analar grade. They were obtained from BDH Chemicals, England. Microbiological media were obtained from BIOTEC, India and they include MacConkey agar, Blood agar base, Mannitol salt agar, Mueller Hinton agar, Sabouraud Dextrose agar, Nutrient broth and Peptone broth.

COLLECTION AND IDENTIFICATION OF PLANT MATERIAL

The seeds of *Carica papaya* were collected from fruit vendors at New Benin Market, Benin City, Edo State, Nigeria. They were identified by Pharm. H. O. Uwumarongie of the Department of Pharmacognosy, University of Benin, where a voucher specimen was deposited and assigned number UBN/PCG/986. They were washed, dried in an oven and grounded into fine powder using a mechanical grinder.

PREPARATION OF FRACTIONS OF THE METHANOL SEED EXTRACT

The powdered seed (400.02 g) was extracted with 2.5 litres of 95% methanol using Soxhlet extractor apparatus for 72 hr. After exhaustive extraction, the seed extract was filtered and concentrated using rotatory evaporator at 45°C. This was further reduced to dryness on a thermostatically regulated hot water bath at 40°C to yield 10.42% extract.

At time of use, the methanol extract was re-dissolved in 300 mL of methanol and partitioned with 500 mL each of petroleum ether and ethyl acetate, to obtain the methanol, petroleum ether and ethyl acetate fractions of the methanol seed extract. These were also reduced to dryness to obtain 32.06%, 36.06% and 28.86% yield of methanol, petroleum ether and ethyl acetate fractions.

GAS CHROMATOGRAPHY AND MASS SPECTROMETRY (GC-MS) ANALYSIS

Methanol, petroleum ether and ethyl acetate fractions of the methanol seed extract of *Carica papaya* were sent for GC-MS analysis at Shimadzu training centre for analytical instruments (STC), Lagos. GC-MS analysis was carried out with Shimadzu Japan gas chromatography 5890-11 with a fused GC column (OV-101) coated with polymethyl silicon (0.25 mm × 50 m) and the conditions were as follows: temperature programming from 60 - 280°C held at 60°C for 1 min, and at 160°C for 2 min (rate 10/min), at 220°C for 2 min (rate 10/min) and finally at 280°C for another 2 min (rate 10/min). The injection temperature was 220°C. GC-

MS analysis was conducted using GC-MS-QP2010 Plus Shimadzu Japan with column oven temperature of 60°C. The carrier gas was Helium, with a pressure of 100.2 Kpa and 20.7 mL/min, column flow was 1.61 mL/min, injection mode was split, flow control mode was linear velocity, purge flow was 3.0 mL/min and split ratio was 10.0. Also, ion source temperature was 200°C, interface temperature was 250°C, equilibrium time was 3.0 min, solvent cut time was 2.5 min., detector gain was 0.00 KV, detector gain mode was relative and the threshold was 1000. For the mass spectrometry, start time was 3.0 min., end time was 27.0 min, event time was 0.5 s, scan speed was 1250, and start m/z was 50 while end m/z was 600. The mass spectrum was also equipped with a computer fed mass spectra data bank. Using computer searches on a NIST Ver2.1 MS data library and comparing the spectrum obtained via GC-MS, compounds present in the various fractions of the methanol seed extract of *Carica papaya* were identified.

SOURCE OF TEST MICROORGANISMS

Microorganisms used in this study were obtained from stock cultures of clinical isolates sourced from the Department of Pharmaceutical Microbiology Laboratory, Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria. The microorganisms include *Staphylococcus aureus*, *Klebsiella pneumonia*, *Bacillus subtilis*, *Escherichia coli*, *Candida albicans* and *Microsporum audouinii*.

PREPARATION OF CULTURE MEDIA

All microbiological media used were reconstituted and prepared according to manufacturer's prescription. Specific mass of the agar/broth were weighed and dissolved in a known volume of distilled water. This was then dispensed into bottles/vessels, after which it was sterilized by autoclaving at 15 psi (121°C) for 15 minutes. It was allowed to cool to 40°C, then dispensed aseptically into sterile petri dishes or used as desired.

PREPARATION OF TEST MICROORGANISM

Prior to use, the test microorganisms were authenticated and subculture from stock into sterile nutrient broth (bacteria) or Sabouraud dextrose broth (fungi), and incubated overnight at 37°C for bacteria and at room temperature (20 - 25°C) for 72 hr for fungi. After incubation, overnight broth was adjusted to 0.5 McFarland standard; to give an inoculum size of approximately 10⁸cfu/mL. A further two fold serial dilution (1:100) using normal saline solution was done to yield approximately 10⁶ cfu/mL.

PREPARATION OF STOCK SOLUTIONS AND DILUTIONS OF FRACTIONS

The methanol, petroleum ether and ethyl acetate fractions were constituted by weighing 1.0 g each of the sample and dissolving in 10 mL of 10% Tween-80 to obtain stock concentrations of 100 mg/mL each. Other concentrations were made by appropriate dilutions. The antibiotic (Ciprofloxacin 1 µg/mL) and the antifungal (Ketoconazole 10 µg/mL) were also prepared. A twofold serial dilution of the stock solution of the antibacterial drug (100 µg/mL) was done to obtain a concentration of 1µg/mL, whereas a single fold serial dilution of the stock Ketoconazole was done to obtain 10 µg/mL.

ANTIMICROBIAL SENSITIVITY TESTS

Overnight broth culture of each pure isolate was adjusted to 0.5 McFarland turbidity standard and diluted (1:100) to give approximately 1x10⁶ cfu/mL microbial suspension. Antimicrobial susceptibility tests were then carried out using agar well diffusion method (Vinothkumar *et al.*, 2010) with some modifications. Wells of 7 mm in diameter were made into previously seeded nutrient/Sabouraud agar plates using a sterile cork borer. Each well was filled with

different volumes of the stock concentration of the various fractions corresponding to 30, 20, 10 and 5 mg/mL concentrations. The same quantity of 10% Tween-80 served as negative control while the standard drugs (1µg/mL of Ciprofloxacin for bacteria plates and 10 µg/mL of Ketoconazole for fungal plates) served as positive controls. The plates were left to stand for 1 hr on the workbench so as to allow diffusion of extract into the agar, before incubating bacterial plates overnight at 37°C and the fungal plates at room temperature (20-25°C) for 72 hr. The inhibition zone diameter was observed and measured in millimetres (mm). The experiments were replicated in triplicates and the mean inhibition zone diameters (IZDs) calculated.

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC)

The modified Agar dilution method (NCCLS, 2003; Lalitha *et al.*, 2004) was used to determine the MIC of the various fractions of the methanol seed extract of *Carica papaya* on susceptible organisms. From the stock concentration of 100 mg/mL, lower concentrations were prepared to obtain a range of concentrations of between 0.01 - 4 mg/mL. Then a loopful volume of two fold dilution of 0.5 McFarland turbidity standard of microbial suspensions were spotted on the surface of the agar plates at marked segments containing different concentrations of the methanol, petroleum ether and ethyl acetate fractions respectively. The plates were incubated at 37°C for 24 hr (for bacteria) and at room temperature (20 - 25°C) for 48 - 72 hr (for fungi). In all cases, the lowest concentration at which there was no observable bacterial/fungal growth was recorded as the MIC.

RESULTS

YIELDS OF PLANT EXTRACT AND SOLVENT FRACTIONS

The results showing the yield upon extraction of *C. papaya* seed using methanol and the subsequent fractionation of the extract into various fractions are presented in Tables 1 and 2 respectively.

Table 1: Yield of the methanol seed extract

Extraction Solvent	Weight of plant material (g)	Weight of extract (g)	Percentage yield (%)
Methanol	400.02	41.67	10.42

Table 2: Yield of various solvent fractions

Fractions	Weight of fractions (g)	Percentage yield (%)
Methanol	13.61	32.66
Petroleum ether	15.03	36.06
Ethyl acetate	12.03	28.86

GC-MS ANALYSIS

The result of the GC-MS and the spectra of the various fractions of the methanol seed extract of *Carica papaya* are attached as appendix.

ANTIMICROBIAL ASSAY

The results of the antimicrobial activities of the fractions of the methanol seed extract of *C. papaya* against the test microorganisms are presented in Tables 3 to 6. Table 3 shows that the methanol fraction was active against the test microorganisms to varying degrees, with the

highest inhibition zone diameter (IZD) of 25.5 ± 0.5 mm against *B. subtilis*, whereas the least IZD of 9.5 ± 0.5 mm was shown by the ethyl acetate fraction against *K. pneumonia* at 5 mg/mL. The fungus, *M. audiounii*, was less susceptible compared to *C. albicans* which was more susceptible to the inhibitory effect of the methanol and petroleum ether fractions, at the concentrations used. Table 6 shows the minimum inhibitory concentrations (MICs) of the methanol, petroleum ether and ethyl acetate fractions respectively.

Table 3: Antimicrobial activities of the methanol fraction at different concentrations

Organisms	Inhibition zone diameters (mm)						
	Fraction concentrations (mg/mL)				CIP 1µg/mL	KET 10µg/mL	Tween-80 (10%)
	5	10	20	30			
<i>E. coli</i>	11.0±1.0	14.5±1.5	19.0±1.0	21.0±0.0	32.5±1.5	–	–
<i>B. subtilis</i>	14.5±2.5	17.0±1.0	22.0±0.0	25.5±0.5	33.0±0.0	–	–
<i>S. aureus</i>	13.0±2.0	15.5±1.5	22.5±1.5	24.0±0.0	33.5±1.5	–	–
<i>K. pneumoniae</i>	13.0±0.0	15.0±1.0	20.0±0.0	22.0±0.0	32.0±1.0	–	–
<i>C. albicans</i>	13.0±0.0	14.5±0.5	16.0±0.0	20.5±1.0	–	32.5±1.5	–
<i>M. audiounii</i>	11.0±1.0	13.0±3.0	14.5±0.5	17.5±0.5	–	30.5±2.5	–

Key: Values are expressed as Mean ± SEM. n = 3. – = No activity, CIP = Ciprofloxacin, KET = Ketoconazole.

Table 4: Antimicrobial activities of the petroleum ether fraction at different concentrations

Organisms	Inhibition zone diameters (mm)						
	Fraction concentrations (mg/mL)				CIP 1µg/mL	KET 10µg/mL	Tween-80 (10%)
	5	10	20	30			
<i>E. coli</i>	10.5±0.5	14.0±1.0	16.0±0.0	20.5±0.5	30.5±2.5	–	–
<i>B. subtilis</i>	12.0±1.0	15.5±2.5	19.0±1.0	23.0±0.5	34.0±1.0	–	–
<i>S. aureus</i>	11.5±0.5	14.0±0.0	18.0±1.0	22.0±0.0	33.5±1.5	–	–
<i>K. pneumoniae</i>	10.0±1.0	11.5±0.0	17.0±1.0	19.0±1.0	31.0±0.0	–	–
<i>C. albicans</i>	11.5±0.5	13.5±1.5	16.0±0.0	19.0±1.0	–	32.5±0.5	–
<i>M. audiounii</i>	10.0±0.0	11.5±0.5	14.0±3.0	16.5±0.5	–	31.0±2.0	–

Key: Values are expressed as Mean ± SEM. n = 3. – = No activity, CIP = Ciprofloxacin, KET = Ketoconazole.

Table 5: Antimicrobial activities of the ethyl acetate fraction at different concentrations

Organisms	Inhibition zone diameters (mm)						
	Fraction concentrations (mg/mL)				CIP 1µg/mL	KET 10µg/mL	Tween-80 (10%)
	5	10	20	30			
<i>E. coli</i>	11.0±1.0	12.0±1.0	14.0±0.0	17.0±1.0	31.0±3.0	–	–
<i>B. subtilis</i>	11.0±1.0	12.0±1.0	14.0±0.0	17.0±1.0	34.0±1.0	–	–
<i>S. aureus</i>	11.5±1.5	13.0±1.0	16.0±0.0	19.0±1.0	33.0±2.0	–	–
<i>K. pneumoniae</i>	9.5±0.5	11.5±2.5	13.0±1.0	17.5±0.5	30.0±2.0	–	–
<i>C. albicans</i>	13.0±1.0	15.5±0.5	17.0±0.0	18.5±3.0	–	33.5±0.5	–
<i>M. audiounii</i>	11.0±1.0	12.5±0.5	14.0±0.0	16.5±0.5	–	32.5±1.5	–

Key: Values are expressed as Mean ± SEM. n = 3. – = No activity, CIP = Ciprofloxacin, KET = Ketoconazole.

Table 6: Minimum inhibitory concentrations (MICs) of the fractions against the test organisms

Organisms	MICs (mg/mL)		
	Methanol fraction	Petroleum ether fraction	Ethyl acetate fraction
<i>E. coli</i>	0.2	0.6	0.8
<i>B. subtilis</i>	0.08	0.2	0.4
<i>S. aureus</i>	0.1	0.4	0.6
<i>K. pneumonia</i>	0.2	0.8	1
<i>C. albicans</i>	0.4	1	0.8
<i>M. audiounii</i>	0.8	2	1

GC-MS spectra

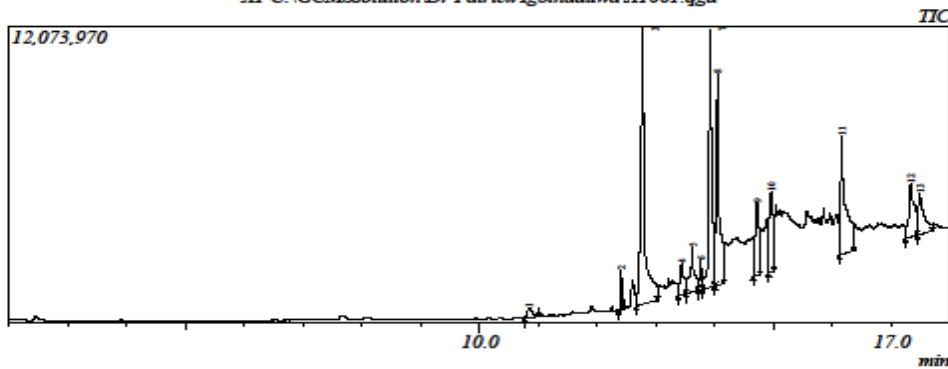
30/07/2016 08:20:07

GCMS-QP2010SE SHIMADZU,JAPAN

SHIMADZU TRAINING CENTRE FOR ANALYTICAL INSTRUMENTS (STC) LAGOS

Sample Information
 Analyzed by : SBen OS
 Analyzed : 30/07/2016 02:40:05
 Sample Type : Unknown
 Level # : 1
 Sample Name: AI
 Sample ID : AI
 IS Amount : [1]=1
 Sample Amount : 1
 Dilution Factor : 1
 Vial # : 1
 Injection Volume : 1.00
 Data File : C:\GCMSolution\Dr Patrick Igbina\AI1001.qgd
 Orig Data File : C:\GCMSolution\Dr Patrick Igbina\AI1001.qgd
 Method File : C:\GCMSolution\Dr Patrick Igbina\Dp.qgm
 Orig Method File : C:\GCMSolution\Dr Patrick Igbina\Dp.qgm
 Report File :
 Tuning File : C:\GCMSolution\System1\Tune1\Ben1 03-05-2016.qgt
 Modified by : Admin
 Modified : 30/07/2016 07:38:13

AI C:\GCMSolution\Dr Patrick Igbina\AI1001.qgd



Peak#	R. Time	I. Time	F. Time	Area	Area%	Height	Height%	A/H	Mark	Name
1	10.845	10.767	11.008	2573179	1.14	417839	0.81	6.16	V	Tetradecanoic acid
2	12.407	12.367	12.450	2450879	1.08	1640065	3.18	1.49	V	Hexadecanoic acid, methyl ester
3	12.775	12.667	13.025	51317716	22.68	11329303	21.95	4.53	V	n-Hexadecanoic acid
4	13.431	13.375	13.508	5924505	2.62	1226383	2.38	4.83	V	1-(+)-Ascorbic acid 2,6-dihexadecanoate
5	13.620	13.508	13.717	9542577	4.22	1836399	3.56	5.20	V	Oleic Acid
6	13.759	13.717	13.792	2885335	1.28	1195405	2.32	2.41	V	1-(-)-Ascorbic acid 2,6-dihexadecanoate
7	13.926	13.792	14.000	38624696	17.07	10489191	20.32	3.68	V	9-Octadecenoic acid, (E)-
8	14.042	14.000	14.150	29808555	13.17	8601588	16.66	3.47	V	Octadecanoic acid
9	14.716	14.658	14.775	14453215	6.39	2940464	5.70	4.92	V	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-
10	14.964	14.917	15.008	14107103	6.23	3261087	6.32	4.33	V	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-
11	16.156	16.117	16.367	29229656	12.92	4809062	9.32	6.08	V	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)-
12	17.332	17.242	17.442	13725964	6.07	2193796	4.25	6.26	V	9-Octadecenoic acid (Z)-, 2,5-dihydroxy
13	17.485	17.442	17.700	11656071	5.15	1678153	3.25	6.95	V	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-
				226299451	100.00	51618735	100.00			

Library

DISCUSSION

Carica papaya Linn (Caricaceae) as a herbal remedy has been used in ethno-medicine for a long time. It is used in the management of malaria, typhoid fever and other infectious diseases. This work was carried out to evaluate the antimicrobial potentials of different fractions of the methanol seed extract of *Carica papaya* and determine the bioactive compounds in them.

The result of the antimicrobial activities of the partitioned fractions of *C. papaya* seeds shows that the methanol fraction was active against the test microorganisms to varying degrees, with the highest inhibition zone diameter (IZD) of 25.5 ± 0.5 mm against *B. subtilis* at 30 mg/ml; whereas, the least IZD of 9.5 ± 0.5 mm was shown by the ethyl acetate fraction against *K. pneumonia* at 5 mg/mL. The fungus *M. audiounii* was less susceptible compared to *C. albicans* which was more susceptible to the inhibitory effect of the petroleum ether fraction at the concentrations used.

From the MIC results, highest activity was seen against *B. subtilis* with MICs of 0.08 mg/mL, 0.2 mg/mL and 0.4 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively, while the least among the bacteria was *K. pneumonia* with MICs 0.4 mg/mL, 0.8 mg/mL and 1.0 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively. The partitioned fractions had low activity against the tested fungi. *C. albicans* had MICs of 0.4 mg/mL, 1.0 mg/mL and 0.8 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively, and *M. audiounii* had MICs of 0.8 mg/mL, 2 mg/mL and 1.0 mg/mL for methanol, petroleum ether and ethyl acetate fractions respectively.

The GC-MS chromatogram of extract of seeds of *Carica papaya* combined, gave 62 peaks as shown in the appendix. But in all, 24 different compounds were identified. From the compounds identified in the extract, n- hexadecanoic acid was found to be in highest percentage (14.73%).

From the antimicrobial test conducted, it was observed that the methanol fraction had more activity compared to the other solvent fractions. This may be attributed to the potentiating effect of some compounds that were present in the methanol fraction but absent in the other fractions. These compounds include; benzeneacetamide, 1-(+)-Ascorbic acid 2, 6 - dihexadecanoate, citronellol epoxide (R or S) and oleic acids. Oleic acid is known to possess excellent antimicrobial activity and was present in the methanol extract only.

The different fractions (methanol, petroleum ether and ethyl acetate) all showed significant antimicrobial activity. There is no documented use of fatty acids as an antimicrobial agent. However studies have shown that most fatty acids especially the unsaturated fatty acids possess antimicrobial activities (Kabara *et al.*, 1972), of which oleic acid a long chain unsaturated fatty acid is the most researched on. It has been documented that the antibacterial activities of fatty acids are usually attributed to long chain unsaturated fatty acids such as oleic acid (Sun *et al.*, 2003). In other study, it was noted that long chain unsaturated fatty acids (C16 - C20) are bactericidal to important pathogenic microorganisms including methicillin resistant *Staphylococcus aureus* (Farrington *et al.*, 1992).

Amongst the diverse and potent biological activities of free fatty acids (FFAs) is the ability to kill or inhibit the growth of bacteria. The antibacterial properties of FFAs are used by many organisms to defend against parasitic or pathogenic bacteria. Though while the antibacterial

mode of action of fatty acids is still poorly understood, the prime target of fatty acids is the cell membrane, where fatty acids disrupt the electron transport chain and oxidative phosphorylation. Besides interfering with cellular energy production, fatty acid action may also result from the inhibition of enzyme activity, impairment of nutrient uptake, generation of peroxidation and auto-oxidation degradation products or direct lysis of bacterial cells (Desbois and Smith. 2010).

The broad spectrum activity of *Carica papaya*, non-specific mode of action of identified constituents and safety makes them attractive as antibacterial agents for various applications in medicine, especially where the use of conventional antibiotics is undesirable or prohibited. Moreover, the evolution of inducible FFA - resistant phenotypes is less problematic than with conventional antibiotics. The result of this work thus shows the potential for biomedical exploitation of antibacterial FFAs, especially those from natural sources.

CONCLUSION

From the work carried out on the fractions (methanol, petroleum ether and ethyl acetate) obtained from the methanol seed extract of *C. papaya*, it was evident that they all possess significant antimicrobial activity. The GC-MS results suggest that the antimicrobial activity of *C. papaya* seeds could be attributed largely to the presence of relative high proportions of various fatty acids.

ACKNOWLEDGEMENT

The technical help of Mr. Dowe E. and Mr. John Uwadiae of the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Benin, Benin City, Edo State, Nigeria; is appreciated and thankfully acknowledged.

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