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A comparative study of the separation and chemical composition of unfermented, fermented and chloroform extracts of ripe *Carica papaya* seeds (Homestead Variety)

O. T. Oyelowo[a*], J. B. Aisoni[b], O. A. Morenikeji[b], A. F. Bolarinwa[c]

[a] Department of Physiology, Faculty of Basic Medical Sciences, University of Lagos, Nigeria
[b]Department of Zoology, Faculty of Science, University of Ibadan, Nigeria
[c] Department of Physiology, Faculty of Basic Medical Sciences, University of Ibadan, Nigeria

***Corresponding author: ooyelowo@unilag.edu.ng, +2348036733891**

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ABSTRACT: The renewed interest in papaya is as a result of its major compounds, such as those associated with antifertility, antibacterial, antihelminthic activities. The ripe *Carica papaya* seed was used in this study. The chemical composition of unfermented fermented, and chloroform extracts of *Carica papaya* seed of Homestead variety was analysed using Gas Chromatography-Mass Spectroscopic (GCMS) techniques. Data analysis and identification of compounds were performed using NIST Mass Spectral Library Software for correct matching. The separation yielded a total of 31 compounds from the three samples of papaya seed. In the unfermented papaya seed extract, 12 compounds were identified, in the fermented papaya seed extract, 9 compounds were identified while in the chloroform extracted papaya seed, 10 compounds were identified. The major components of the unfermented seeds extract were acetamide (17.44%), octadecanoic acid (13.73%), benzenemethanamine (12.32%) and oleic acid (12.31%), while the major components of the fermented seeds were oleic acid (57.47%), cis-vaccenic acid (13.09%), and n-hexadecanoic acid (13.40%). The main compounds of the chloroform extract were oleic acid (41.36%), palmitic acid (15.73%), cis-13-octadecenoic acid (15.50%), trans-13- octadecenoic acid (5.44%) and stearic acid (3.05%). The compounds identified in the three papaya seed samples in this study, with their associated activity according to literature search, are as follows: antifertility (Oleic, n-hexadecanoic, palmitic, 6-Octadecenoic and cis-13-Octadecenoic acids), antibacterial (Oleic acid, Palmitic acid, Petanoic acid 4-methyl, and Cyclononasiloxane octadecamethyl) and antihelminthic (n-hexadecanoic acid). The compound n-hexadecanoic acid was identified in the fermented papaya seed. The information provided by this study should initiate further chemical investigations into the fermented extraction of the ripe *Carica papaya* seed.

Running Title: Identification of chemical compounds in the unfermented, fermented, and chloroform extracts of ripe papaya seeds.

Keywords: *Carica papaya* seed, antibacterial, antifertility, anthelminthic

Introduction

The papaya (*Carica papaya* L.) is among the most significant tropical plants grown in the world and so it is an important agricultural fruit (Abreu *et al*., 2015). It is native to America and has spread to many other parts of the world over time. The largest producers of papaya are Brazil, Mexico, Nigeria, India, Indonesia, and the Dominican Republic (Abreu *et al*., 2015).

Carica papaya is an herbaceous plant that grows fast and can reach as tall as 8 feet. It lives for about 5-10 years. All the parts of the papaya plant are either useful as sources of food for humans and animals or health and economic benefits (Anitha *et al*.,2018). The black seed of papaya is edible with a spicy flavour. It is sometimes ground and used as a substitute for black pepper, also when dried, papaya seeds can be ground over protein-rich meals to improve digestion (Arvind *et al*., 2013). The addition of papaya seed flour to hamburgers improves their technological characteristics without impairing their sensory quality (Azevedo &Campagnol, 2014). The seeds of papaya are also used as a food condiment called 'daddawa' in Nigeria (Rabi *et al*., 2013). The seed of papaya is coated with a mucilaginous substance. When the seeds are processed either to be eaten or to be used as a medicine, they could either be fermented (Dakare *et al*., 2011) or unfermented (Anaga & Onehi, 2010; Ayoola &Adeyeye, 2010). Fermentation is one of the oldest food processing methods (Abdulazeez & Sani, 2011). The method is widely used as a means of food preservation in developing countries especially in places where canning, freezing, and refrigeration are either unavailable or not accessible. Fermentation helps nutritional quality, enhances protein digestibility, micronutrient bioavailability, and degrades anti-nutritional factors. Other processes of papaya seed extraction involve extracting the seeds with water (aqueous) or other chemicals such as chloroform.

The renewed interest in papaya fruit is as a result of its rich compounds, especially those with antifertility, antibacterial, and anthelminthic activities. The antifertility, antibacterial, and antihelminthic properties are the most important properties attributed to the seed of papaya. The seed contains a bioactive substance known as benzyl isothiocyanate (BITC) which has antihelminthic activity (Barrosso *et al*., 2016) as well as other applications ranging from vascular relaxation to the inhibition of cancer proliferation. The seeds do not naturally contain BITC. The production of BITC occurs when the benzyl glucosinolate, present in the interior of the seeds contacts the myrosinase enzyme, present on the seed's surface. The myrosinase enzyme catalyzes the BITC production (Li *et al*., 2014). The occurrence of antihelminthic activity depends on how the seeds are processed. The BITC production occurs when the benzyl glucosinolate, present in the interior of the seeds, contacts the myrosinase enzyme, present in the seed's surface. This is important as it reveals a very effective defence mechanism in evolution. The seed to be broken by an animal generates a substance that has toxic effects on some organisms. This also reveals the importance of breaking the seed hull before eating it (Barrosso *et al*., 2016). Thus, the importance of breaking the seed hull (e.g. through fermentation) before eating the seed comes to play. The effects of different extracts of *Carica papaya* seed on male fertility, female reproduction, pregnancy and offspring outcome abound in literature and these effects depending on the seed processing technique, dose and duration include an increase in implantation sites and a significant decrease in foetal weights; vaginal bleeding; absence of foetal growth, litter size, and foetal body weight; increased post-natal weights; and delayed somatic landmarks (Oyelowo *et al*.,2014; Senthil *et al*., 2016; Shrivastava *et al*., 2011).

The composition of papaya differs from region to region and this depends on different geographical locations. In Nigeria, the exotic varieties available are Homestead, Pink solo, Kapoho solo, and JS.22 (NIHORT 2011).

As a result of the continuous consumption and medicinal use of the papaya seed, this work aims to separate and compare the chemical compounds of the fermented, unfermented and chloroform extracts of the ripe or mature *Carica papaya* seed (Homestead variety) and identify the main compounds possessing antihelminthic, antifertility and antibacterial properties.

Materials and Methods

Plant material

The Homestead variety of *C. papaya* seed was obtained from ripe fruits in farmland in Ibadan, identified and authenticated at Forestry Research Institute of Nigeria (FRIN), Ibadan, Nigeria by a plant taxonomist. The voucher specimen (FRIN 108041) was deposited at FRIN.

Preparation of *Carica papaya* seed

To obtain Sample A- an unfermented seed, one set of fresh seeds was shade- dried and coarsely powdered, after which, 1.0 kg of the ground seeds were soaked in 5 liters of distilled water for 48 hours at room temperature and was filtered using Whatman No1 filter paper. The extract was stored in a refrigerator and later characterized by GC-MS analysis.

Sample B-fermented seeds: To obtain the fermented seed extract, a set of seeds, was shade-dried and dehulled by cracking. Afterward, the seeds were winnowed to remove the seed coat. The seeds were then rinsed about five times with tap water to eliminate the fruit material. It was afterward boiled for two hours and while still hot the seeds were spread out and incubated at 37˚C and fermented for 72 hours. The extract was stored in a refrigerator and later characterized by GC-MS analysis.

The third set of seeds (Sample C- seeds extracted with chloroform) was shade-dried for a minimum of three weeks. Powdered seeds (1 kg) were extracted with chloroform (5.0 L), using a Soxhlet extractor (Pėrez-Gutiėrrez *et al*., 2011) for 4 hours and the extract was cooled to room temperature and filtered. The solvent was removed under reduced pressure by a rotatory evaporator and the extract was dried for 12 hours (yield, 6.5% by weight). The extract was stored in a refrigerator and later characterized by GC-MS analysis.

Experimental Design

The analysis was done as follows. Fatty acid methyl esters were prepared according to the AOAC-IUPAC Method 969.33 (AOAC 1990). Chloroform extract (90 mg) and 1 N solution of NaOH in methanol (4 mL) were placed in a round-bottom flask, with the mixture stirred and heated at boiling point for 15 minutes. Afterward, BF3-MeOH (5 mL, 15% w/w) was added and heating continued for another 5 minutes. Subsequently, 2 mLs of Iso-octane were added and the mixture was stirred for 5 minutes. This was extracted with 2 mLs of hexane. The organic phase was dried over anhydrous Na2SO4. The analysis was done with an Agilent Technologies (USA) 6890N GC equipped with an HP-5MS column with dimensions (30 m in length; 25 mm internal diameter; 0.25 μm film thicknesses) equipped with an Agilent EM 5973 detector, at 150 °C. The carrier gas used was helium, at a flow rate of 1 mL/minute and the split ratio was 2:1. The column temperature was programmed at an initial 60 °C for 3 minutes and it was gradually increased to 170 °C, at a temperature rate of 3 °C/minute. The temperature was maintained at 1 minute. Afterward, the temperature was raised to 330 °C, at a rate of 10 °C/minute, and maintained for 10 minutes. The injector temperature was 330 °C and 1 μL of the organic phase were injected twice. The interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library.

Results and Discussion

The analysis of the unfermented fermented, and chloroform extracts of mature *Carica papaya* seed was homogeneous in composition, especially regarding the presence of various fatty acids, among others. Despite the different solvents and processes involved, the different seed samples had similar components such as undecylic, oleic, cis-vaccenic, and trans-13 octadecenoic acids. A comparison between the unfermented and fermented seeds in terms of their chemical classes revealed that both samples differed significantly from one another although they both had fatty acid components.

The unfermented seed sample was dominated by alkane hydrocarbons and fatty acids which accounted for 25.01% of the total composition. The unfermented sample also had 16.60% amides, 8.30% benzyl group, and 16.60% siloxane. The fermented sample accounted for 77.70% fatty acids, 11.10% anabolic-androgenic steroid, and 11.10% benzyl group. The chloroform extract of papaya seed accounted for 90.0% fatty acids and 11.01% carboxylic acid. Apart from the similarity in fatty acid composition, the unfermented and fermented seed samples also had benzyl group components. The fatty acids contained in the chloroform extract of papaya seed was about 3-4 times more than the unfermented sample of papaya seed. The chloroform extract of the papaya seed was also dominated by fatty acids and the main components were oleic acid (41.36%), palmitic acid (15.73%), cis-13-octadecenoic acid (15.50%), trans-13- octadecenoic acid (5.44%) and stearic acid (3.05%).

Twelve compounds were identified from the unfermented papaya seed and were dominated by acetamide (17.44%), followed by octadecanoic acid (13.73%), benzenemethanamine (12.32%) and oleic acid (12.31%) all representing the major components (Table 1). In the fermented papaya seed sample, nine compounds were identified and oleic acid (57.47%), cis-vaccenic acid (13.09%), n-hexadecanoic acid (13.40%) were the major components (Table 2). Ten compounds were identified from the chloroform extract of the papaya seed and the main components were oleic acid (41.36%), palmitic acid (15.73%), cis-13-octadecenoic acid (15.50%), trans-13- octadecenoic acid (5.44%) and stearic acid (3.05%) (Table 3).

Table 1: Chemical Compounds Identified in the Unfermented *C. papaya* Seed (Homestead Variety)

S/N	Compound Name	Retention time (min)	Peak area (%)	Molecular formulae
1	Pentanoic acid, 4-methyl-	6.28	5.91	$C_6H_2O_2$
2	Undecane	9.60	8.99	$C_{11}H_{24}$
3	Dodecane	12.36	3.33	$C_{12}H_{26}$
4	Tridecane	14.95	8.55	$C_{13}H_{28}$
5	Propanamide,	20.18	5.31	C_3H_7NO
6	Acetamide, N-(phenylmethyl)-	21.96	17.44	$C_8H_{11}N$
7	Benzenemethanamine	24.82	12.32	$C_{10}H_{15}N$
8	Oleic acid	31.78	12.31	$C_{18}H_{34}O_2$
9	9-Octadecenoic acid	31.69	0.94	$C_{19}H_{36}O_2$
10	Octadecanoic acid	31.95	13.73	$C_{19}H_{38}O_2$
11	Cyclononasiloxane, Octadecamethyl	34.31	0.84	$C_{18}H_{54}O_9Si_9$
12	Hexasiloxane	34.34	0.22	$C_{12}H_{38}O_5Si_6$

Table 2: Chemical Compounds Identified in the Fermented *C. papaya* Seed (Homestead Variety)

S/N	Compound Name	Retention time (min)	Peak area (%)	Molecular formulae
1	19 -Norepiandrosterone	14.07	0.57	$C_{18}H_{28}O_2$
2	Benzothiophene-2- carboxylic acid	14.34	0.04	$C_9H_6O_2S$
3	11-Octadecenoic acid, methyl ester	14.82	0.55	$C_{19}H_{36}O_2$
4	Methyl ricinoleate	14.93	0.99	$C_{18}H_{34}O_3$
5	Methyl 12-hydroxy-9-octadecenoate	19.93	2.60	$C_{19}H_{36}O_3$
6	n-hexadecanoic acid	16.49	13.40	$C_{16}H_{32}O_2$
7	Cis-vaccenic acid	18.30	13.09	$C_{18}H_{34}O_2$
8	Oleic acid	18.64	57.47	$C_{18}H_{34}O_2$
9	6-Octadecenoic acid	18.74	7.31	$C_{18}H_{34}O_2$

Earlier studies conducted on the composition of papaya seeds revealed that they are a rich source of proteins (27.3-28.3%), lipids (28.2-30.7%), and crude fibers (19.1-22.6%) while other studies reported appreciable quantities of calcium and phosphorus in the seeds as well as the presence of toxicants, such as glucosinolates (Li *et al.*, 2014). Recent studies have however been able to identify the specific components. Reports on different extracts of papaya characterized by GC-MS from different regions of the world revealed that despite the different species of papaya used and different extraction processes, the composition of the papaya seed revealed many fatty acids. For instance, a study by Pérez-Gutiérrez *et al.*, (2011) reported that the chloroform extract of C.papaya seed (Mandol variety) had nineteen compounds as identified by GC-MS analysis, and the major components were fatty acids, being oleic acid (49.70%), palmitic acid (24.15%), and stearic acid (8.52%). Another study reported nine components in a petroleum ether seed extract of Batek Batu *C. papaya* variety, with oleic acid (76.90%), palmitic (13.40%), stearic acid (4.60%) and linoleic acid (3.20%) being the major components (Pérez-Gutiérrez *et al*., 2011). In yet another study, (Pérez-Gutiérrez *et al*., 2011), seven constituents were analysed and the major components were oleic acid (76.75%), palmitic acid (12.89%), linoleic acid (4.11%), and stearic acid (3.96%) from the acetone extract of seeds of the Mamey variety of C.papaya which were all fatty acids. In this study, fatty acids dominated the unfermented, fermented, and chloroform extract of papaya seed as well. Furthermore, the major fatty acid that has been implicated in the composition of extractions of papaya seed is oleic acid. A study by Malacrida *et al*., (2011), reported 71.30% of oleic acid in the papaya seed component from Brazil. The Hong Kong/Sekaki variety of papaya seeds has been reported to possess 73.5% oleic acid (Yanty *et al*.,2014). Researchers like Pérez-Gutiérrez *et al*., (2011) have also reported a high percentage of the oleic acid composition. In this study, the unfermented papaya seed possessed 12.31% oleic acid, the fermented papaya seed possessed 57.47% oleic acid as its highest component as well as chloroform extract which possessed 41.36% of oleic acid as its highest component. Among the compounds identified in the unfermented and chloroform extracts of papaya seed, which from literature search are associated with antibacterial activities, oleic acid occupied the highest peak area. Also, among the compounds identified in the fermented and chloroform extracts of papaya seed, which from literature search are associated with antifertility activities, oleic acid also occupied the highest peak area. The peak area of the oleic acid (57.47%) was highest in the fermented papaya seed extract. The role of oleic acid regarding its antibacterial, antifertility, and antihelminthic activities in fermented papaya seed require further research.

Table 3: Chemical Compounds Identified in the Chloroform Extract of *C. papaya* Seed (Homestead Variety)

S/N	Compound Name	Retention time (min)	Peak area (%)	Molecular formulae
1	Stearic acid	21.1	3.05	$C_{18}H_{36}O_2$
2	Myristic acid	21.55	1.79	$C_{14}H_{28}O_2$
3	Pentadecanoic acid	21.86	1.76	$C_{19}H_{34}O_2$
4	Undecylic acid	22.07	1.76	$C_{11}H_{24}$
5	Palmitic acid	23.06	15.73	$C_{16}H_{32}O_2$
6	Oleic acid	46.58	41.36	$C_{18}H_{34}O_2$
7	Cis-13-octadecenoic acid	47.77	15.50	$C_{18}H_{34}O_2$
8	Trans-13-octadecenoic acid	23.33	5.44	$C_{19}H_{36}O_2$
9	Cis-vaccenic acid	24.11	2.52	$C_{18}H_{34}O_2$
10	Cis-10-nonadecenoic acid	24.53	2.41	$C_{19}H_{36}O_2$

The compounds found in the three extracts of papaya seeds have widespread biological and medicinal applications. The presence of Pentatonic acid, 4-methyl; Oleic acid; Cyclononasiloxane, Octadecamethyl acid, and Palmitic acid were observed in the unfermented, fermented and chloroform extract samples. Studies have reported that these compounds have been associated with antibacterial activities (Awa *et al*., 2012; Bokaeian *et al*., 2014; Khan *et al*., 2012; Song *et al*., 2015). The presence of Oleic acid; n-hexadecanoic acid; 6-Octadecenoic acid; Palmitic acid; Cis-13-octadecenoic acid were observed in the unfermented, fermented and chloroform extract samples. These compounds have been associated with antifertility activities in the literature (Karthikeyan *et al*., 2015; Kumar *et al*., 2010; Lampiao, 2011; Njoku *et al*., 2019; Oyelowo & Adegoke 2016; Oyelowo & Bolarinwa 2017; Rashed *et al*., 2014; Sreekumar *et al*., 2014; Vijisaral & Arumugan, 2014). The presence of n-hexadecanoic acid was detected in the fermented extract sample. This compound has also been associated with antihelminthic activities in the literature (Ishnava & Konar, 2020). The antihelminthic property of *Carica papaya* seed has been attributed only to benzyl isothiocyanate (BITC) previously (Li *et al*., 2014). The n-hexadecanoic acid found in the fermented papaya seed in this study might be another compound that may be useful in destroying parasitic worms.

Conclusion

This study compared the separation and chemical composition of unfermented, fermented, and chloroform extracts of *Carica papaya* seeds (Homestead variety). The study revealed that the three seed extracts were dominated by fatty acids and the foremost among them was oleic acid. In this study, the n-hexadecanoic acid identified from the fermented papaya seed, according to literature, might be another compound that may be useful in destroying helminths. Information provided by the study is of great importance for further chemical investigations into the fermented extraction of the ripe *Carica papaya* seed.

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Conflict of Interest

The authors declare no conflict of interest.

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