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RESEARCH ARTICLE

GAS CHROMATOGRAPHY-MASS SPECTROMETRY OF N-BUTANOLIC LEAF EXTRACT OF CARICA PAPAYA

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Abstract

Crude extract of carica papaya leaf was defatted with n-hexane and subjected to solvent-solvent partitioning by successively using solvents of increasing polarity, ethyl acetate and n-butanol. Carica papaya extract in the various solvents were separately tested for antibacterial activities and the n-butanol fraction was found to be most active against the test organism. The fraction obtained from the n-butanol extract of C. Papaya by subjecting it to column chromatography and thin layer chromatography successively was analysed with GC-MS. GC-MS analysis was done to determine the compounds or group of compounds in the plant extract which may substantiate its use in herbal medicine. GC-MS of carica papaya leaf extract showed eight peaks, indicating the presence of eight compounds. Three of these compounds were identified based on the molecular structure, molecular mass and calculated fragments. The identified compounds are ascorbic acid 2,6-dihexadecanoate (vitamin C), Oleic acid ethyl ester (9-octadecenoic acid ethyl ester) and stearic acid (octadecanoic acid). Vitamin C is known to have an antioxidant, anti-inflammatory and antinociceptic (reduction of pains within neurones) activities. Moreover vitamin C has antibacterial activities and also enhances the quality of spermatozoa. Fatty acids and their esters like stearic acid and oleic acid ester are known to have antibacterial and antifungal properties. They interfere with bacterial growth and survival. Fatty acids and their esters have been identified as the active ingredients in herbal medicines and their presence in carica papaya leaf extract validates its use in traditional medicine.

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Introduction:-

Carica papaya (pawpaw) leaf has been in use in Nigeria and other parts of the world to treat bacterial and other tropical diseases, there is therefore a need to investigate them for their scientific values.

Carica papaya is native to the tropics of the Americas. It is distributed throughout Asia and Africa (Afolayan, 2003). It belongs to the family caricaceae. It has the following common names; pawpaw tree, papaya and papayer. The

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plant is fast in growing, erect, usually unbranched tree or shrub, 7 – 8m tall with latex, trunk of about 20cm in diameter (Anibijuwon & Udeze, 2009).



Carica papaya tree

In herbal medicine the leaf poultice is used on nervous pains and elephantoid growths. The leaf is smoked for asthma relief in various remote areas (Reed, 1976). The aqueous leaf extract showed pronounced inhibition demonstrating a high activity against the test bacteria (Anibijuwon & Udeze, 2009). The young leaves and to a lesser extent other parts of the plant contain carpain, an active bitter alkaloid which has a depressing action on the heart. The plant is also a strong amoebicide (Reed, 1976). The leaf extract is also used as a profilaxis against malaria (Satrija et al., 1994). The leaves are also used as soap substitute which are supposed to remove stains.

Imaga et al., (2009) and Indran et al., (2008) have proven that *Carica papaya* leaf extract is a potential anti sickling agent and has protective effect against gastric ulcer in rats.

The different parts of *Carica papaya* plant (the leaves, fruits, seeds and latex) can be eaten and are used for the treatment of different ailments including wound healing (Wiart, 2002; Nor Suhada et al., 2008). The efficacy of some of the traditional claims to this plant has been validated scientifically (Indran et al., 2008; Imaga et al., 2009). *Carica papaya* flowers have antibacterial activities (Zakaria et al., 2006). The seed extract oral administration could induce reversible male infertility and could be used for pharmaceutical development of a male contraceptive (Udoh et al., 2005). Medical research in animals models has confirmed the contraceptive and abortifacient capability of papaya and also found that papaya seeds have contraceptive effects on adult male langur monkeys and possibly in adult male humans as well (Lohiya et al., 2002). Unripe papaya is especially effective in large amounts or high doses but the ripe ones are not teratogenic and will not cause miscarriage in small amounts. Javanese believe that eating pawpaw prevent rheumatism. Dietary papaya reduces urine acidity in humans while the flowers are used against jaundice (Reed, 1976). Papaya seeds may be nephroprotective in toxicity induced kidney failures (Olagunju et al., 2009). The juice of the leaf extract has an antiproliferative effect on in vitro liver cancer cells probably due to its component of lycopene (Rahmat et al., 2006) or immune system stimulation (Dang, 2010). Red flesh papaya fruit is also rich in lycopene (Chandrika et al., 2009; Chandrika et al., 2010) a potent antioxidant. Lycopene is reported to be beneficial in cardio vascular ailments and cancer (Kohimeier et al., 1997; Rao & Agarwal, 2000).

Papaya seed extract has antibacterial activity against *Escherichia Coli*, *Staphylococcus aureus* and *Salmonella typhi* (Yismaw et al., 2008).

Preclinical phytochemical analyses showed that the leaf extracts contain alkaloids, tannins, saponins, glycosides and phenols (Anbijuwon & Udeze, 2009).

Papaya contains many biochemically active compounds. Two important compounds are chymopapain and papain which are supposed to aid digestion. Papain is also used in the treatment of arthritis (Anbijuwon & Udeze, 2009). Papain, a proteolytic enzyme, has a wealth of industrial uses such as in making of beer, tenderization of meat and tanning of hides (James, 1983). In medicine papain is used in combating dyspepsia. In liquid preparation, it has been used to reduce enlarged tonsils. Phytochemicals in papaya may suppress the effect of progesterone (Oderinde et al., 2002).

The red fleshed fruit of *carica papaya* contain provitamin A carotenoids, Beta-carotene, B –cryptoxanthin and B-Carotene epoxide (Bendich 1991).

This study aims to investigate through bioassay guided isolation studies the active agents of *Carica papaya* (pawpaw) leaf that may substantiate its use in herbal medicine.

Materials and Methods:-

Leaves of *carica papaya*, were freshly collected around September from Vom in Jos south L.G.A of Plateau state, Nigeria and were identified at the Federal Department of Forestry, Jos.

Assay For Active Principles In The Plants' Extracts:

The fresh leaves of *carica papaya* was oven dried at 60°C (degrees centigrade) for 5 days. The plant sample was pulverized and 500 g of the powdered leaf was soaked overnight in n-Hexane to defat (remove the fats and oil). The n-Hexane was squeezed out of the plant material and the solvent recovered by distillation. The extract from n-hexane was dried down on a water bath. The solid mass obtained was weighed, labeled and stored in a cool dry place. The defatted plant material (marc) was then re dissolved with 500 ml of distilled water and extracted with 2 liters of methanol in an air tight clean flat bottom container for 7 days. This was done at room temperature with

occasional stirring and shaking. The extract was then first filtered through a fresh plug of cotton wool and finally through a Whatman No 4 filter paper. The methanol was recovered by distillation using soxlet apparatus and the methanolic extract dried down on a water bath and the solid mass obtained weighed and stored appropriately. 5g of the crude methanolic extract was dissolved in distilled water and solvent-solvent partitioning was done according to the protocol designed by Kupchan and Tsou (1973), and modified by Van-Wagenen (1993). The methanolic solution was fractionated successively using solvents of increasing polarity, ethyl acetate and n-butanol. The solvents were recovered by distillation and the fractions evaporated to dryness using a water bath. The fractions were weighed, labelled appropriately and stored in a cool dry place.

Screening for Antibacterial Activities:

Punch Hole Diffusion Method:

An inoculum size of 10^8 cfu/ml (prepared using the McFarland's standard) of the clinical isolates of a gram-negative bacteria *Salmonella typhi* was prepared according to the method of Bauer et al., (1966).

The nutrient agar plates were each inoculated by flooding with the inoculum of the different isolates. The plates were dried in an incubator at 37°C and seven holes were bored on each of the seeded plates, using a 6mm diameter sterile cork borer. A drop of molten nutrient agar was put into each hole to seal off from the bottom of the plate. Using an automatic pipette, 50µl of carica papaya crude extract in the various solvents were used to fill the holes corresponding to their respective labels. Sterile distilled water and nalidixic acid (5µg/ml) were used as negative and positive controls. All the plates were incubated aerobically at 37°C for 24hrs. Diameters of the zones of inhibition were measured in millimeters (mm) and recorded.

Column Chromatography of the n-Butanolic Fraction of the Plant Extract:

The leaf extract in n-hexane, methanol, ethyl acetate and n-butanol fractions were tested for antibacterial activities. The n-butanolic fraction of the leaf extract was found to have the highest antibacterial activities. The n-butanolic fraction of the leaf extract was subjected to column chromatography as follows. Qualikems silica gel 60-120 mesh was dissolved with ethyl acetate to get a slurry. The column (40cm high and 3cm in diameter) was lined with cotton wool at the base. The slurry was gradually (avoiding air bubbles) poured into the column until the column is well packed. The top of the gel is lined with cotton wool. The column is allowed to equilibrate for one hour. The n-butanolic extract was loaded into the column and a layer of cotton wool used to line the extract on top, such that the extract is sandwiched between the layers of cotton wool. 200ml of ethyl acetate was gradually poured into the column. Ethyl acetate (EA) and methanol are mixed in ratios of increasing order of polarity according to the protocol shown below. Ethyl acetate is fraction 1, 90ml of EA and 10ml of methanol is fraction 2 etc. Fraction 11 is 100ml of methanol. The flow rate is 18 minutes per 100ml. Each fraction was collected into a porcelain evaporating dish and dried down using a water bath and transferred into an appropriately labeled bijour bottle. The bottles are well stoppered and stored in the refrigerator.

Table 1:- Protocol showing the ratio of EA and methanol used.

	1	2	3	4	5	6	7	8	9	10	11
EA(ml)	200	90	80	70	60	50	40	30	20	10	-
Methanol(ml)	-	10	20	30	40	50	60	70	80	90	100

Thin Layer Chromatography of carica papaya Fraction 5:

The different fractions of the column chromatography of the leaf extract were also subjected to antibacterial testing and fraction 5 was found to be most active. Fraction 5 was therefore subjected to TLC. The TLC plate was prepared using 30g of silica gel dissolved with 60ml of water to get a slurry. The 20×20 glass plate was loaded in the TLC plate maker. The slurry was spread on the plate using the plate maker machine. The plate was allowed to dry at room temperature before transferring them to the oven at 110°C for 1hour. Prior to use the plate was activated in the oven at 110°C for 30 minutes. The solvents used were chloroform, acetic acid and distilled water in the ratio of 7:2:1. The solvents were mixed (elutant) and poured into the electrophoretic tank such that the depth of the elutant was not more than 1cm. 20µl of the sample (ie fraction 5 of carica papaya leaf) was spotted on the plate 2cm from the bottom of the plate (ie the origin). After spotting, it was allowed to dry for 5minutes. The plate was immersed into the tank such that 1cm of plate was inside the elutant. The plate was vertically placed in the tank and the lid placed over it to make airtight. Development was for 1 hour 45 minutes. The retardation factors (Rf) of the sample was recorded and the picture of the TLC plate documented in appendix 5.

Gas Chromatography Mass Spectrometry (GCMS) of the TLC Spots:

The TLC gave a picture of a single spot as the sample did not separate into different components. The single spot of the sample was scraped into the elutant and subjected to GCMS. The results were recorded.

Table 2:- Showing the compounds identified in the GC-MS analysis of n-butanolic leaf extract of carica papaya.

S/No.	Peak No.	Peak area	Peak area %	Retention time	Compound name	Common name	Molecular formular	Molecular weight
1	5	11971893	16.67	20.555	Ascorbic acid 2,6-dihexadecanoate	vitamin C	C ₃₈ H ₆₈ O ₈	652
2	6	20727852	28.86	23.293	9-octadecenoic acid ethyl ester	Oleic acid ethyl ester	C ₂₀ H ₃₈ O ₂	310
3	7	10214822	14.22	23.632	Octadecanoic acid	Stearic acid	C ₁₈ H ₃₆ O ₂	284

Table Showing the compounds identified in the GC-MS analysis of n-butanolic leaf extract of carica papaya. Three compounds matched 100% with those from the NIST library (See appendix). These compounds are ascorbic acid 2,6-dihexadecanoate (vitamin C), 9-octadecenoic acid ethyl ester (Oleic acid ethyl ester) and Octadecanoic acid (Stearic acid).

Discussion:-

Methanolic extract of carica papaya leaf was produced and further subjected to exhaustive sequential extraction along polarity gradient of solvents using n-hexane, ethylacetate, n-butanol, methanol and water. The extracts in these five solvents were tested for their antibacterial activities and n-butanolic fraction was found to have the most antibacterial action on the organism tested (appendix 1).

Then n-butanolic fraction of the plant extract was subjected to column chromatography and eleven fractions were obtained for the plant under study. These fractions were subjected to antibacterial screening and fraction 5 of had the highest antibacterial activity (appendix 2). This fraction was then subjected to thin layer chromatography. The single spot obtained (Appendix 4) was used for GC- MS analysis.

GC-MS chromatogram of the n-butanolic leaf extract of Carica papaya, pawpaw, (Appendix 3) showed eight peaks indicating the presence of eight compounds. Three of these compounds were identified based on the molecular structure, molecular mass and calculated fragments (Table 2). The spectrum of the unknown component was compared with the spectrum of the component stored in the database of National Institute Standard and Technology (NIST). These compounds are Ascorbic acid 2,6-dihexadecanoate (vitamin C), Oleic acid ethyl ester (9-octadecenoic acid ethyl ester) and stearic acid. The GC-MS was done in order to determine whether this plant species contain any individual compound or group of compounds, which may substantiate its traditional use as an herbal medicine in the treatment of bacterial infections. The chemical constituents present in herbs are a part of the physiological functions of living flora and hence they are believed to have better compatibility with the human body than synthetic drugs.

Vitamin C has been reported to have an antioxidant, anti-inflammatory and antinociceptive (reduction in pains within neurons) properties. It exhibits antibacterial activity against Staphylococcus aureus, Escherichia coli etc. It also enhances sperm quality and prevents sperm agglutination thus making them more motile with forward progression (Ogunlesi et al., 2010).

The other compounds are fatty acids and their derivatives, namely Octadecanoic acid (Stearic acid) and 9-octadecenoic acid ethyl ester (Oleic acid ethyl ester). Fatty acids (FAs) are ubiquitous molecules typically found bound to other compounds such as glycerol, sugars or phosphate groups to form lipids. FAs can be released from lipids, typically by enzyme action, to become free fatty acids (FFAs), which have diverse and potent biological activities. Fatty acids are the primary constituents of edible oils and medicinal herbs and are reported to possess the ability to interfere with bacterial growth and survival Benkendorff et al (2005). Many fatty acids are known to have antibacterial and antifungal properties Knapp and Melly (1986). Stearic acid, oleic acid and many other fatty acids have been reported to have potential antibacterial and antifungal activities Agoramoorthy et al (2007). Indeed, FFAs are often identified as the active ingredients in ethnic and herbal medicines (Yff et al 2002, McGaw et al 2002). Stearic acid is a saturated fatty acid. It is the most common saturated fatty acid that occur in nature following

palmitic acid. Stearic acid has been reported to have antimicrobial activities (McGaw et al 2002, Seidel and Taylor, 2004). Oleic acid is a long chain monounsaturated acid and a potent antibacterial (Dilika, et. al., 2000, Zheng, et. al., 2005). Antimicrobial activity of fatty acids was stated to be dependent on chain length and unsaturation degree (Benkendorff, et. al., 2005, Knapp and Melly, 1986). Long-chain unsaturated fatty acids exhibit inhibitory activity against many bacteria (Farrington, et. al., 1992). Examples of long chain unsaturated fatty acids are linoleic and oleic acids, and are reported as potent antibacterial agents (Dilika, et. al., 2000, Zheng, et. al., 2005). Some studies have been undertaken to understand the mechanism of antimicrobial action of fatty acids and it was concluded that fatty acids and their esters exhibited non-specific modes of action. Davidson et. al., (1992) and Lunde et. al., (2009) stated that while the antibacterial mechanisms of fatty acids and their esters may be unknown, these compounds resemble the bipolar membrane of the bacterial cell wall in having both a hydrophilic and hydrophobic tail. This similarity suggests that the fatty acids could possibly target bacterial and fungal cell walls thus killing them by penetrating and disrupting normal function of the cell wall. Long-chain unsaturated fatty acids inhibit FabI, whereas long-chain saturated fatty acids were not active. In the antibacterial assay using whole cells, unsaturated fatty acids showed greater inhibition than saturated fatty acids, which is consistent with the results seen by several other investigators (Freese et. al., 1973, Greenway and Dyke 1979). The GCMS chromatogram of carica papaya leaf extract with a rich content of vitamin C, long chain fatty acid (stearic acid) and an unsaturated fatty acid ester (Oleic acid ethyl ester) may substantiate its use as a herbal treatment for many microbial diseases.

Summary of Findings:

The n-butanolic leaf extract of carica papaya is rich in many phytochemical compounds and have antibacterial efficacies. The n-butanolic fraction has more antibacterial efficacy than the other solvents used for extraction in this study, suggesting that this solvent extracted more of the antibacterial bioactive agents.

Among the bioactive agent of this plant extract are vitamin C, stearic acid and oleic acid ester as seen in the GC-MS analysis.

Conclusion:-

This study has given evidence and scientific backing to the use of this plant material in the treatment of many diseases especially bacterial infections in Nigeria. The use of this plant as herbal medicine should therefore be encouraged.

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Appendix:**Appendix 1:**

Antibacterial Activities of *Carica papaya* leaf Extracts in Different Solvents (Zones of inhibition in mm).

Concentration (mg/ml)	25	75
Aqueous	12	12
n-Butanol	16	20
Methanol	15	15
Ethyl acetate	08	12
n-Hexane	12	12
+ve Control	18	18
-ve Control	-	-

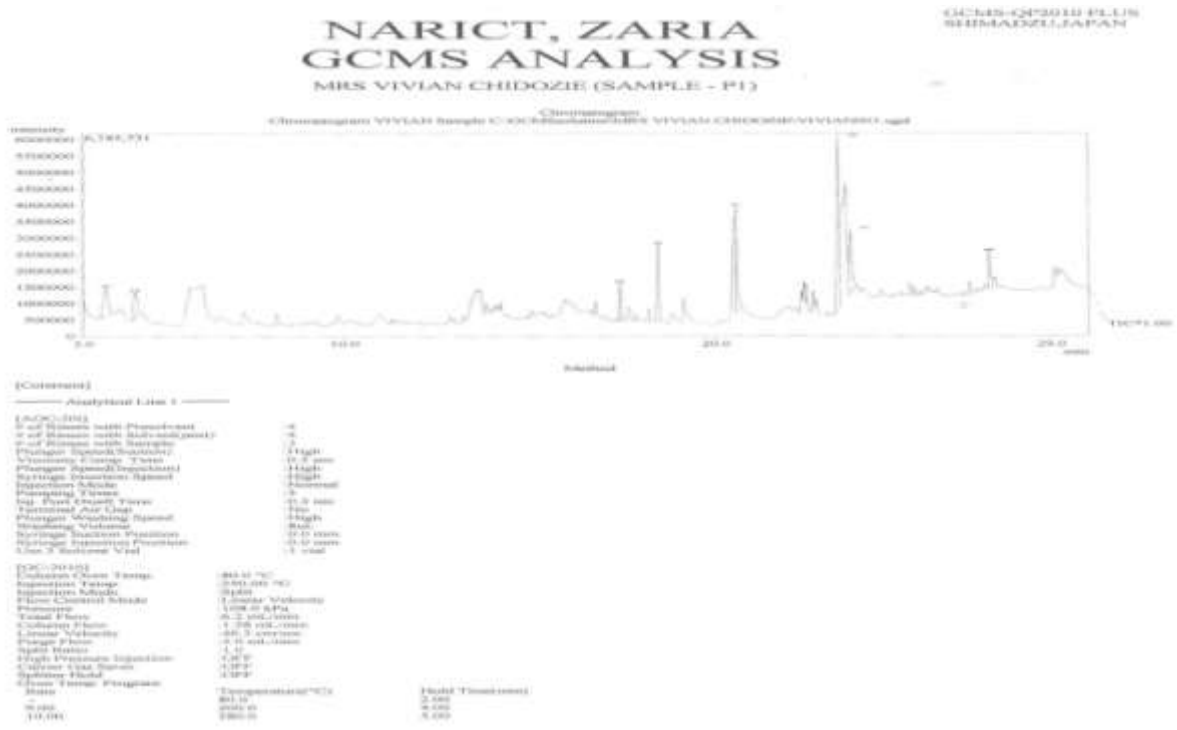
Appendix 2:

Antibacterial Screening of the Eleven Column Chromatographic Fractions *Carica papaya* leaf extract. (Zones of inhibition in mm).

Fraction	1	2	3	4	5	6	7	8	9	10	11	+	-
Zones of inhibition	18	18	18	18	20	16	16	16	17	18	18	25	-

Key: + = Positive control, - = Negative control.

Appendix 3: GC-MS Analysis of the TLC Spot of Pawpaw Fraction 5



Appendix 4: Picture of thin layer chromatography (TLC).

