# *Original Research Article*

## COMPARATIVE STUDY ON PHYTOCHEMICAL PROPERTIES AND ANTIFUNGAL ACTIVITIES OF DIFFERENT PLANT EXTRACTS ON INFECTED FRUIT OF *CARICA PAPAYA* L.


## ABSTRACT

The comparative studies of phytochemical properties and antifungal activities of different plant extracts; *Ageratum conyzoides* L., *Phyllanthus amarus* Schum. & Thonn, *Senna alata* (L.) Roxb. and *Mangifera indica* L. on infected fruit of *Carica papaya* L was carried out using standard methods for analysis of all the tested parameters. The test was conducted using the selected plant extracts at five different concentrations (100 - 500mg/ml) against the isolated fungi. Molecular analysis was done to identify the isolated fungi. The results of the qualitative phytochemical screening revealed the presence of alkaloid, tannins, saponins, phenols and flavonoid in all the plant extracts. The quantitative phytochemical screening results showed varying quantities of alkaloids, tannins, saponins, flavonoids and phenols present in the extracts. The quantitative screening showed that *A. conyzoides* had the highest quantity of the constituents present in its extracts. Four fungal pathogens were isolated from paw-paw fruits and were identified as *Rhizopus* spp., *Aspergillus glavus, Aspergillus flavus* and *Penicillium* spp. The results of the antimicrobial assay on fungi isolated from infected pawpaw fruits showed that inhibition of fungal growth increased with a corresponding increase in concentration for all the fungal isolates. *Penicillium* spp showed resistance to all the extracts. *A. conyzoides* showed the highest inhibition from 200mg/ml for the isolates followed by *P.amarus*. Molecular analysis identified the isolates as *Rhizopus arrhizopus, R. delemar, R. oryzae* and *Aspergillus clavatus*. The phylogenetic tree showed that *Rhizopus oryzae, R. delemar, R arrhizus* are closely related. *A. conyzoides* and *P. amarus* can be used to minimize the infection of paw-paw fruit by microbes.


## INTRODUCTION

The damage to crops caused by fungal pathogens has necessitated the use of range of antifungal control agents. Among pesticides used to protect crops, fungicides were perceived until recently as relatively safe. However, the 1986 National Academy of Sciences (NAS) report on pesticide residues on food indicated that fungicides pose more of a carcinogenic risk than insecticides and herbicides together [1]. Furthermore, the use in crop protection of many synthetic fungicides that have various degrees of persistence has now been cautioned due to their carcinogenicity, teratogenicity and other residual toxicities. Several of the synthetic fungicides are reported to

cause adverse effects on treated soil ecosystems because of their non-biodegradable nature [2,3]. Synthetic fungicide residues are suspected to present a significant health risk to consumers, and demand is increasing to find safe alternatives. Additionally, continued use of fungicides leads to an increase in resistance by plant pathogens, creating a need for finding biological alternatives with these pesticides. Present activities to find both natural and synthetic fungicides focus on finding compounds that are safe to humans, environment and delicate ecosystems [4,5].

New antifungal compounds with distinct modes of action need to be identified because of increasing incidence of fungal resistance to existing antibiotics [6]. Plant secondary metabolites have great potential as a source of effective antifungal agents [7]. Plant-derived compounds such as hydroquinones and naphthoquinones (lapachol, juglone), sesquiterpenes (cinnamodial, capsidiol) and alkaloids (berberine) have shown diverse activities as antimicrobial and antifungal. An advantage to the approach of using ethnobotanical leads to identify compounds with antimicrobial activity [8]. Leaves of *Vitis vinifera* are rich in tannins, flavonoids, procyanidins and also contain organic acids, lipids, enzymes and vitamins [9, 10, 11, 12]. The quantitative analysis of compounds found in leaves has also been evaluated by [13]. Grape-vine leaves possesses a resistance towards several fungus diseases as *Plasmoparaviticola, Oidium tukeri*and *Botrytis cinerea* which cause downy mildew, powdery mildew and fruit rot, respectively [14]. Flavonoids including quercetin and quercetin- 3-O-[β-xylosyl-(1-2)-α-rhamnoside] 4'-O-α-rhamnoside as bioactive compounds were secluded from *Zizyphus spina-christi* leaves [15]. Also, various gallic acid derivatives were isolated from the leaves of *Punica granatum* such as 1,2,3-tri-*O*-galloyl-β-glucopyranose,1,2,4-tri-O-galloyl-β-glucopyranose, 1,3,4-tri-O-galloyl-β-glucopyranose, 1,2,6-tri-O-galloyl-β-glucopyranose and 1,4,6-tri-O-galloyl-β-glucopyranose [16, 17, 18].

*Carica papaya* L (Pawpaw) also called Idekpe in Yoruba, Gwanda in Hausa, Okur in Igbo and Bobo in Ibibio belongs to the family Caricaceae constituting of four genera and thirty one species. The *Papaya* originated in Tropical South America, possibly in the area between Mexico and Central America in the 15th century [19]. It is one of the leading fruits in respect of acreage and per hectare production. Recently, 1.25 metric tons of *Papaya* were produced from an area of about 1.24 thousand hectares of land with an average yield of 7ton/ha. In some countries like Nigeria, yield of *Papaya*, in fact, is far below compared to other countries of the world [20]. Several species of Caricaceae have been used as remedy against a variety of diseases [21]. *Carica papaya* is a nutraceutical plant having a wide range of pharmacological activities. The whole plant has its own medicinal value. Papaya is a powerhouse of nutrients and is available throughout the year. It is a rich source of three powerful antioxidants (vitamin C, vitamin A and vitamin E), the minerals (magnesium and potassium), the B vitamin (pantothenic acid and folate) and fiber [22]. The black seeds of

papaya are edible and have a sharp, spicy taste. They are sometimes ground and used as a substitute for black pepper.

Despite the nutritional and health importance attached to pawpaw, the availability of the fruit is reduced due to high level of postharvest loss [23]. Spoilage in pawpaw can also be referred to as rot or decay. Spoilt pawpaw fruits is characterized by excess softening, mycelia growth, loss of moisture, unpleasant odour, shrinkage and total drying up of water in the fruits [24]. The spoilage may be caused by microorganisms, insects and rodents attack, physical injury such as bruising and freezing as well as chemical breakdown of the fruit may also lead to deterioration in quality of the fruit.

Numerous natural products of plant origin are pesticidal and have the potentials to control fungal diseases of crops considerable effort has been directed and devoted to screening plants in order to develop new natural fungicides as alternatives to existing synthetic fungicides, which are associated with problems such as phytotoxicity, vertebrate toxicity, pest resistance and resurgence, widespread environmental hazards and high cost [25].

There is a compelling need to find alternative plant pathogenic fungi control measures other than commercial synthetic fungicides. As with many tropical crops, pawpaw fruits are beset with problems of field and storage rot. *C. papaya* L. is host to various species of pests and pathogens. Plant pathologists have reported about 39 arthropods that infest papaya [26]. Papaya fruit fly (*Toxotrypana curvicauda*) is one of the principal insect pests that affect *C. papaya* throughout the tropical and sub-tropical areas. Others include red spider mite (*Tetranychus urticae), Brevipalpauscar lifornicus* which cause damage by penetrating plant tissue. Nematodes namely *Rotylenchulus reniformis*, *Meloidogyne* spp, *Helicotylenchus dihysteria*, have been reported to cause root disease in papaya. Fungal pathogens such as *Phytophthora palmivora* causes root and fruit rot in papaya, *Collectricum gloerosporoides* causes anthracnose, *Asperisporium caricae* causes black spot in papaya. The losses are mainly due to decay, physiological disorders and mechanical injury. According to [27, 28], *Aspergillus niger*, *Rhizopus nigricans, Aspergillus flavus, Rhizopus oryzae* and *Fusarium moniliforme* of fungal origin are responsible for post-harvest losses in pawpaw. Besides the economic losses to pawpaw fruit marketers, the rotten fruits could cause serious health hazards to consumers [27]. Some of the harmful metabolites produced by pawpaw spoilage organisms include: Ochratoxins, Fumonisins and Aflatoxins produced by *Aspergillus* spp. These toxins when ingested by humans may cause severe effects in respiratory tract which may lead to bronchitis and liver dysfunction [29, 30]. Thus the effects of these toxins are of serious global consequence particularly in the developing countries where there are shortages of food and medical infrastructures. According to [31], *C. papaya* L. is mainly grown (> 90%) and consumed in

developing countries. It is fast becoming an important fruit internationally both as a fresh fruit and as processed products. Thus, spoilage of such fruits further undermines the food scarcity and health of such percentage of the human population that rely on it as a source of vitamins. Chemical pesticides and fungicides have been used to increase yield and protect pawpaw fruits crops. However, chemicals pose hazards to ecosystem through induced resistance against target organisms and undue inundation of the environment with organic pollutants. Reports regarding this area of research is rather scanty, hence the essence of the research.

## MATERIALS AND METHODS

### Collection and Identification

Fresh samples of four (4) plants were collected from Obio Etoi: *Ageratum conyzoides, Phyllanthus amarus, Mangifera indica* and *Senna alata*. The plants were identified and authenticated by an AKSU (Akwa Ibom State University) curator Mr Felix Udo with the following herbarium number: *Phyllanthus amarus* (AKSUH/E0015), *Senna alata* (AKSUH/F006), *Mangifera indica* (AKSUH/A0043) and *Ageratum conyzoides* (AKSUH/A0011). The voucher specimens were deposited in the herbarium for future references.

### Preparation of Plant Extracts

The leaves were processed and air dried at room temperature. After which, they were pulverized. They were macerated in 50% ethanol and were allowed to stand for 72 hours. After, it was filtrated and the residue discarded while the filtrate was concentrated using a water-bath. The concentrated extracts were stored in beakers and labelled accordingly.

### Qualitative Analysis on Phytochemical Constituents

### Test for Alkaloids

About 0.5g each of the plant extract was stirred with 5ml in 5% Hydrochloric acid (HCI) on a steam bath and then filtered. 1ml of the filtrate was treated with a few drops of Mayer's reagent and a second 1ml portion treated similarly with Dragendorff's reagent. Turbidity or precipitation of cream and pink or red colouration respectively indicated in test. The third 1ml portion treated similarly with Dragendorff's reagent. Turbidity or precipitation of cream and pink or red colouraiton respectively indicated in we test. The third 1ml portion was treated with a few drops of picric acid. While a yellow precipitate was taken as preliminary evidence for the presence of alkaloids [32].

**Saponins Test**

**(a)Frothing Test**

About 0.5g of each extract was shaken vigorously with distilled water in a test tube. Frothing that persisted on warning was taken as preliminary evidence for the presence of saponnins [32].

**(b)Sodium Bicarbonate Test**

About 0.5g of each extract was added with 5% sodium bicarbonate and Fehling's solution A & B and boiled. Presence of brown precipitate was termed as a positive test [33].

**Tannins Test**

**(a)Ferric Chloride Test**

About 0.5g of each plant extract was stirred with 10mls of distilled water and filtered. To the filtrate was added 5% Ferric chloride reagent. A blue-black, green or blue green precipitate was taken as evidence for the presence of tannins [33].

**(b)Bromine Water Test**

About 5 drops of each plant extract solution was mixed with 10lm distilled water and bromide water. Decolourisation of bromine water indicated the presence of tannins [33].

**Phlobatannins Test**

**(a)Hydrochloric Acid Test**

About 0.5g of each plant extract was dissolved in water and filtered. The filtrate was boiled with 1% hydrochloric acid. Deposition of a red coloured precipitate was taken as a positive test [32].

(b)About 5ml of each plant extract solution was mixed with 3 drops of 40% formaldehyde and 6 drops of 1% hydrochloric acid and heated, then cooled. To the cooled solution were added hot water, warm ethanol and warm 5% potassium hydroxide. A bulky precipitate which leaves coloured residue indicated the presence of phlobatannins [33].

**Anthraquninones Test**

**(a)Borntrager's Test for the free Hydroxyanthraquinones**

About 0.5% of each plant extract was shaken with 10ml benzene and filtered. To the filtrate were added 10% ammonia solution and the mixture shaken. The presence of a pink, red or violet colouration in the ammonical (lower) phase indicated the presence of free anthraquinones [33].

(b)**Borntrager's Test for combined Anthraquinones**

About 0.51% of each plant extract was boiled with 10ml dilute Sulphuric acid and filtered while hot. The filtrate was shaken with 5ml benzene and 10% ammonia solution was added to the separate benzene layer. A pink or violet colouration in the ammonia (lower layer) indicated the presence of anthraquinone derivatives [32].

**Flavonoids Test**

**Shinoda Reduction Test**

Few pieces of magnesium metal were added to 5mls of each plant extract solution. The solution was obtained by using concentrated hydrochloric acid to dissolve the extract. The formation of orange (Flavanones), red (Flavonols), crimson or magenta colouration was taken as evidence of preliminary presence of flavonoids [33].

**Cardiac Glycosides**

**(a)Salkowskis Test (TERPENOIDS)**

About 0.5% of each plant extract was dissolved in 2ml of chloroform. Concentrated sulphuric acid was carefully added by running it down the side of the test tube. A reddish brown colour at the interphase indicated the presence of algycone portion of cardiac glycosides [32].

**(b)Keller – Killiani Test**

About O.5g of each plant extract was dissolved in 2ml glacial acetic acid. This was then underplayed with 1ml concentrated sulphuric acid. A brown ring obtained at the interphase indicates the presence of deoxy-sugar characteristic of cardenolide. A violet ring may appear below the brown ring while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer [32].

**(c)Liebermann Test (STEROIDS)**

About 0.5g of each plant extract was added 3mls of chloroform and filtered. 10 drops of acetic anhydride was added to the filtrate along with 2 drops of concentrated sulphuric acid. A reddish

brown or violet ring at the interphase is a positive test while a bluish colour could be suspected to be steroids [33].

**(d)Kedde Test**

About 1ml of 8% solution of the extract in methanol was mixed with 1ml of 2% solution of 3, 5- dinitrobenzoic acid in methanol and 1ml of a 5.7% aqueous NaOH. An immediate violet colour indicated the presence of cardenolides in the extract, the colour fading gradually through reddish brown to brownish – yellow with the precipitation of a whitish crystalline solid. This test indicates the presence of lactone ring in the cardenolide.

**(e)Legal Test**

The extract was dissolved in pyridine and a few drops of 2% sodium nitroprusside together with a few drops of 20% NaOH were added. A deep red colour which faded to a brownish yellow indicated the presence of cardenolides [32].

**Test for Cyanogenetic Glycosides**

To test for cyanogenetic glycoside qualitatively the materials is well broken and placed in a small flask, a suitably impregnated strip of filter paper is suspended by means of a cork. The paper may be treated in either of the following ways to give a color reaction with free hydrocyanic acid, either sodium picrate (yellow), which is converted to sodium isopurpurate (brick-red).

About 0.5g of the fine powder was placed on each of the three test tubes labeled A. B. C. Powder in test tube A and B were mixed with a little water and sodium picrate test paper was suspended in each of the three test tubes. The test tubes were then stopped immediately. Test tube B was placed in a boiling water-bath for about three to five minutes while test tube A & C were kept at warm temperature. A brick red color on picrate paper indicated the presence of cyanogenetic glycosides.

**Quantitative analysis on Phytochemical Constituents**

**Alkaloids**

About 5g of the plant sample is prepared in a beaker and 200ml of 10% $CH_3CO_2H$ in $C_2H_5OH$ is added to the plant sample. The mixture is covered and allowed to stand for 4hours. The mixture then filtered and the extract is allowed to become concentrated in a water bath until it reaches 1/4 of the original volume. Concentrated $NH_4OH$ is added until the precipitation is complete. The whole solution is allowed to settle and the precipitate is collected and washed with dilute $NH_4OH$ and then filtered. The residue is alkaloid which is then dried and weighed.

**Saponins**

The samples were ground and 20g of each plant sample is put into a conical flask and 100 ml of 20% $C_2H_5OH$ is added to the plant sample. The sample is heated over a hot water bath for 4hours with continuous stirring at about $55^0$C. The mixture is then filtered and the residue re-extracted with another 200ml of 20% $C_2H_5OH$. The combined extracts are reduced to 40ml over a water bath at about $90^o$C. The concentrated is then transferred into a 250ml separator funnel and 20ml of $(CH_3CH_5)_2O$ is added to the extract and shaken vigorously. The aqueous layer is recovered while the $(CH_3CH_5)_2O$ layer is discarded and the purification process is repeated. 60ml of n-$C_4H_9OH$ is added and the combined n-$C_4H_9OH$ extracts is washed twice with 10ml of 5% NaCl. The remaining solution is then heated in a water bath and after evaporation; the samples are dried in the oven to a constant weight.

**Tannins**

Quantity of tannins is determined by using the spectrophotometer method. 0.5g of plant sample is weighed into a 50ml plastic bottle. 50ml of distilled is added and stirred for 1hour. The sample is filtered into a 50ml volumetric flask and made up to mark. 5ml of the filtered sample is then pipetted out into test tube and mixed with 2ml of 0.1 M $FeCl_3$ in 0.1 M HCl and 0.008 M $K_4Fe\ (CN)_6\ 3H_2O$. The absorbance is measured with a spectrophotometer at 396nm wavelength within 10minutes.

**Flavonoids**

About 10g of plant sample is repeatedly extracted with 100ml of 80% aqueous methanol at room temperature. The whole solution is then filtered through filter paper and the filtrate is later on transferred into a water bath and solution is evaporated into dryness. The sample is then weighed until a constant weight.

**Preparation of culture medium and isolation of fungi isolates from diseased (infected) *Carica papaya* (paw paw):**

Sabouraud Dextrose Agar (SDA) medium was prepared by dissolving 6.2g of SDA in 100ml of sterile water and autoclaving at $121^o$ C for 10 minutes. On cooling to $45^o$C, 0.5ml of streptomycin was added into the medium and about 20ml was poured into petridishes aseptically. Plates were left on the bench to set.

Different fungi colonies on the diseased *Carica* were uprooted and planted on the sterile SDA plates and incubated at $28^o$C for 7days.

**Purification of fungal isolates**

Fresh SDA was prepared and dirty colonies of the fungi was transplanted into the fresh medium and incubated for 5 to 7days.Pure isolates were stocked on fresh SDA plates.

**Identification of fungal isolates**

The pure fungal isolates were identified using cultural and morphological characteristics such as pigmentation, colony growth pattern, conidal morphology etc. The technique of Okeke and Manga (2008), Barnett and Hunter (2005) were also adopted for the identification of the isolated fungi using lactophenol in cotton blue stain. The identification was carried out by placing a drop of the stain on a slide with a sterile inoculating needle, a small portion of the aerial mycelia from the test organisms were uprooted and placed in a drop of the stain and covered with a cover slip gently. A little pressure was applied to eliminate air bubbles. The slides were then mounted and viewed under the light microscope at x10 and x40 objective lenses. The morphological characteristics and appearance of the fungi organisms were identified in accordance with [34]. The organisms were *Aspergillus flavus, penincillium species, Rhizopus species* and *Aspergillus glavus.*

**Preparation of isolates for anti-fungal Assay.**

The test organisms were inoculated with sterile SDA broth and incubated at 28$^{o}$C for 5 days. The inoculum of the test organisms were prepared from fully sporulated culture. The colonists were shaken and 2.5% tween 80 added to facilitate the uniform distribution of the spores of the fungal isolate.

The spores were dislodged by vigorous shaking for 15minutes. 1ml of each stock suspension was diluted into 99ml sterile distilled water. 0.5ml from few stocks was used to inoculate the bored SDA plate (the 0.5ml was equivalent to McFarland std).

Screening of the extracts for antifungal activities using well in agar diffusion techniques Okeke *et al*, (2001). 0.5ml of each diluted organism was aseptically spread on the surface of the SDA plates using sterile hocking stick. These were allowed to stand for 30mins for pre-diffusion into the agar plates. A sterile cork borer of 5mm was used to bore holes on the agar plates. The extracts were dissolved using sterile water to prepare different concentration of 100mg/ml, 200mg/ml, 300mg/ml, 400mg/ml and 500mg/ml. 0.5ml volume of each diluted extract was used to fill the agar wells made in the SDA plates. The plates were allowed to stand for 1 hour to allow the extract diffuse into the medium. Nystatin was used as standard drug for fungal activities. All plates were incubated at 28$^{0}$C for 5-7 days.

Antifungal activities of the extracts were determined by measuring the zone of inhibition in millimeters (mm).

**Effects of the extract on fresh pawpaw fruits**

The extracts used were numbered 1- 4. Extract no 1 represent *Senna alata,* extract 2 represent *Phyllanthus amarus,* extract 3 represent *Mangifera indica,* extract 4 represent *Ageratum conyzoides.*

Crude and aqueous extract were rubbed on the surface of fresh pawpaw fruits and stored on the bench for several days. The control fruit which had no extract rubbed on it showed more fungal growth than all other fruits with different extracts rubbed on them. Fungal growth was observed to be scanty on all the experimental fruits. Two extracts showed more growth inhibition strength, extract 2 (*Phyllanthus amarus)* and extract 4 (*Ageratum conyzoides*) with (*Ageratum conyzoides*) showing the highest inhibition.

**Statistical Analysis**

Statistical analysis was done using two way analysis of variance (ANOVA) involving two factors: the four (4) test plants and the five (5) phytochemicals. ANOVA analysis for the sensitivity of fungal strains was also done.

**Molecular Analysis**

The molecular analysis was carried out at the Genomics Training Center and Laboratory Limited, Uyo, Akwa Ibom State.

**DNA Extraction Protocol**

The DNA extraction was carried out according to the manufacturer's protocol (Zymo Research Quick-DNA Fungal and Bacteria Kit).

60mg of the fungal isolate was added to a ZR Bashing Bead lysis tube then 750ul of Bashing Bead buffer was added to the tube. A bead beater was used at maximum speed for not less than 5 minutes. The ZR Bashing Bead lysis tube was centrifuged in a microcentrifuge at 10,000g for 1 minute. 400ul of the supernatant was transferred to a zymo-spin filter in a collection tube and centrifuge at 8,000g for 1 minute. 1,200ul of genomic lysis buffer was added to the filtrate in the collection tube from the last step. 800ul of the mixture from step 5 was transferred to a zymo-spin IICR column in a collection tube and centrifuged at 10,000g for 1 minute. The flow through was discarded and the last step repeated. 200ul DNA pre-wash buffer was added to the zymo-spin ICCR column and centrifuged at 10,000 g in a new collection tube for 1 minute. 500ul g-DNA wash buffer was added to the zymo-spin ICCR column and centrifuged at 10,000g for 1 minute. The zymo-spin ICCR column was transferred to a clean 1.5 ml microcentrifuge tube and 100ul DNA elution buffer was added directly to the column matrix, centrifuged at 10,000g for 30 seconds to elute the DNA.

**DNA Quantification**

DNA concentration was determined using a Spectrophotometer (Gene Quant Pro). The concentration of DNA was 380 ng. The absorbance of total genomic DNA (gDNA) was quantified by measuring optical density (OD) at 260nm and 280nm. The concentration of DNA calculated from the absorbance at 260/280 nm is shown in the table below:

**Table 1:DNA Concentration and Purity of Fungi Samples**

| Sample ID | DNA Concentration (ug/ul) | DNA Purity (260/280) |
|---|---|---|
| **1** | 391 | 1.82 |
| **2** | 345 | 1.89 |
| **3** | 371 | 1.80 |
| **4** | 368 | 1.88 |

**Gel Electrophoresis**

The presence and quality of gDNA was also evaluated by agarose gel electrophoresis. DNA was quantified on 1.5% agarose gel. Electrophoresis was conducted in a 1X TAE (Tris-base glacial acetic acid, EDTA) gel buffer at 120 volts for 20 minutes. The gel was stained with 7 ul of Safe View dye. The gel was visualized under an EV trans illuminator.

**PCR Amplification**

**PCR Reagents**

The PCR master mix contains PCR amplification buffer, MgCl, DmSO, DNTPs and Taq polymerase. Other reagents include forward primer, reverse primer, dd$H_2O$ and template DNA.

**Cocktail Mix**

The PCR final reaction volume was made up to 20ul, using 4ul solisbiodyne master mix, 0.5 forward primer, 0.5 reverse primer, 2ul of sample and 13ul nuclease free water.

**Table 2: ITS Primer and sequence**

| Primer name | Sequence (5' – 3') |
|---|---|
| ITS4 | TCCTCCGCTTATTGATATGS |
| ITS5 | GGAAGTAAAAGTCGTAACAAGG |

**PCR Conditions**

PCR reaction conditions conducted at BIO-RAD thermocycler were the following: initial denaturation at 94$^{o}$C for 30 seconds, 30 cycles of denaturation at 94$^{o}$C for 30 seconds, annealing at 51$^{o}$C for 30 seconds, initial elongation at 68$^{o}$C for 1 minutes and final elongation at 68$^{o}$C for 5 minutes. Amplicons were separated on 1.5% agarose gel electrophoresis for 20 minutes at 120 V. DNA ladder of 100 bp was used as molecular weight pattern.

**Sequencing and Sequence Analysis**

PCR products obtained with ITS primers were purified and sequenced in forward and reverse direction to determine the sequence of nucleotides for the ITS gene in each isolate. Sanger sequencing was carried out on ABI Prism 3130X1 Genetic Analyzer (Applied Biosystems) and Big Dye terminator V3.1 kit (Applied Biosystems Inc.). Sequencing reaction was set up in 10.0µl volume using 1.0µl of 1ml Big Dye terminator√3.1 (Applied Biosystems), 1.5µl of Big Dye buffer (5x), 1.5µl of 2.5pmole of the reverse or the forward primer, 1.0µl of the purified template DNA, generated from the sequencing reaction products, and 5.0µl of GIBCO water (Invitrogen Corporation). Sequencing PCR profile consists of an initial denaturation of 96$^{o}$C for 1min after performing rapid ramp to 96$^{o}$C and was subjected to 25 cycles of the following program: 96$^{o}$C for 10sec, 50$^{o}$C for 5sec, 60$^{o}$C for 4min and performed rapid thermal ramp for 4$^{o}$C and held forever. The sequencing reaction products were cleaned up through ethanol purification method consisting of 1.0µl 125mM EDTA, 1.0ul 3M NaOAc2 and 25.0µl 100% ethanol, before addition of 9µl of Hi-Di formamide (Applied Biosystems) to each of the purified sequencing PCR product. The mixtures were denatured for 5min at 95$^{o}$C prior to the sample analysis on an ABI Prism 3130X1 Genetic automated sequencer (Applied Biosystems) using the default settings.

Obtained raw sequences were edited by trimming both ends and removing bad chromatograms. Nucleotide sequences were aligned by Clustal W (Thompson *et al.,* 1994). BLAST algorithm was conducted on NCBI with aligned sequences for each isolate to determine its sequence similarity and identity.

**Phylogenetic Analysis**

The phylogenetic tree constructed using MEG7, with the maximum-likelihood method based on the Tamura-Nei model. Branch support was assessed by bootstrap resembling with 500 replications.

**RESULTS**

**Qualitative Phytochemical Screening**

The qualitative analysis of the test plants revealed the presence of phytochemicals such as alkaloids,flavonoids,saponins,steroids,tannins,terpenoids,anthraquinone,cardiac glycosides,phlobatannins and phenols.However Cardiac glycoside and Phlobatannin were not detected in *Phyllanthus amarus*,steroids and Anthraquinone was not detected in *A.conyzoides*,Phlobatannin was not detected in *Mangifera indica*,while in *Senna alata*, Steroids,Terpenoids and Phlobatannin were not detected as shown in Table 2.

**Table 3: Qualitative Phytochemical Screening of Test Plants**

| S/N | Phytochemicals | *Phyllantus amarus* | *Ageratum conizoides* | *Mangifera indica* | *Senna alata* |
|---|---|---|---|---|---|
| 1. | Alkaloid | + | + | + | + |
| 2. | Flavanoid | + | + | + | + |
| 3. | Saponins | + | + | + | + |
| 4. | Steroids | + | **ND** | + | ND |
| 5. | Tannins | + | + | + | + |
| 6. | Terpenoids | + | + | + | **ND** |
| 7. | Anthraquinone | + | **ND** | + | + |
| 8. | Cardiac glycoside | **ND** | + | + | + |
| 9. | Phlobatannins | **ND** | + | ND | **ND** |
| 10. | Phenols | + | + | + | + |

**LEGEND: + = Positive, ND = Not Detected.**

**Quantitative Phytochemical Screening of the Test Plants**

The quantitative analysis of test plants showed varying quantities of different phytochemicals in each plant as shown in the table below.Phenol recorded the highest quantity in *A.conyzoides*(83.08)mg/ml followed by *P.amarus* which recorded 73.16mg/ml.The least quantity was recorded against Tannin in *A.conyzoides*(0.02)mg/ml followed by *Mangifera indica* (2.37)mg/ml.

**Table 4: Quantitative Phytochemical Screening of the Test Plants**

| Plant Constituents | *Ageratum conyzoides*(mg/mL) | *Mangifera indica* (mg/mL) | *Phyllanthus amarus* (mg/mL) | *Senna alata* (mg/mL) |
|---|---|---|---|---|
| Alkaloid | 38.40 | 2.80 | 34.82 | 27.43 |
| Tannins | 0.02 | 2.37 | 30.12 | 6.20 |
| Saponins | 10.01 | 4.05 | 3.581 | 2.30 |
| Flavonoids | 24.21 | 5.20 | 17.27 | 7.73 |

| | | | | |
|---|---|---|---|---|
| Phenols | 83.08 | 12.20 | 73.16 | 39.73 |

**Antimicrobial assay of Plant Extracts on Fungi isolated from Infected *Carica papaya* fruits**

The inhibitory effect of *Senna alata* extract on the different fungi isolated from infected paw-paw fruits was observed at the highest concentration used (500mg/ml). There was no clear inhibition at 100 - 400mg/ml concentration. The zones of inhibition for the different fungi at 500mg/ml are shown in Table 4. The inhibitory effect of *P. amarus* extract on the different fungi isolated from infected paw paw was observed from 300mg/ml concentration of the extract for *A. flavus* and *Rhizopus* spp. The inhibition zone increased as the concentration increased from 300 – 500mg/ml for all tested organisms except *Penicillium* spp. The different dimensions of the inhibition zones of *P. phyllanthus* are shown in Table 5. The inhibitory effect of *Mangifera indica* extract was observed at the highest concentration used (500mg/ml) against Rhizopus spp. only, there was no observed inhibition for all other fungi isolated. Table 6 shows the zones of inhibition for *M. indica*. The inhibitory effect of *Ageratum conyzoides* extract on fungi isolated from infected pawpaw was observed from as low as 200mg/ml concentration against *Aspergillus* spp. *Penicillium* spp. could not be inhibited at all while *Rhizopus* spp. could be inhibited from 300mg/ml concentration upward, inhibition zone for *Aspergillus flavus* recorded 6mm at 200mg/ml concentration, 8mm at 300mg/ml, 12mm at 400mg/ml and 16mm at 500mg/ml. *Aspergillus glavus* was inhibited with inhibition zone recording 7mm at 200mg/ml, 10mm at 300mh/ml, 14mm at 400mg/ml and 18mm at 500mg/ml zones of inhibition for *Rhizopus* spp was 6mm at 300mg/ml, 8mm at 400mg/ml, 12mm at 500mg/ml. There was no inhibition for *Penicillium* spp at all concentrations. The different zones of inhibition showed by *A. conyzoides* against the isolated fungi are shown in table 7

**Table 5: Effect of ethanol extract of *Senna alata* on fungi isolated from infected *C. papaya* fruits**

| **Isolates** | **Concentration Zone of Inhibition (mm)** | | | | |
|---|---|---|---|---|---|
| | 100mg/mL | 200mg/mL | 300mg/mL | 400mg/Ml | 500mg/mL |
| *Aspergillus flavus* | - | - | - | - | 9 |
| *Aspergillus glavus* | - | - | - | - | 6 |
| *Penicillium* spp. | - | - | - | - | - |
| *Rhizopus* spp. | - | - | - | - | 8 |

**Table 6: Effect of ethanol extract of *Phyllanthus amarus* on fungi isolated from infected *C. papaya* fruits**

| **Isolates** | **Concentration Zone of Inhibition (mm)** | | | | |
|---|---|---|---|---|---|
| | 100mg/mL | 200mg/mL | 300mg/mL | 400mg/mL | 500mg/mL |
| *Aspergillus flavus* | - | - | 6 | 8 | 11 |
| *Aspergillus glavus* | - | - | - | 6 | 8 |
| *Penicillium* spp. | - | - | - | - | - |
| *Rhizopus* spp. | - | - | 7 | 10 | 13 |

**Table 7: Effect of ethanol extract of *Mangifera indica* on fungi isolated from infected *C. papaya* fruits**

| Isolates | Concentration Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | 100mg/mL | 200mg/mL | 300mg/mL | 400mg/mL | 500mg/mL |
| *Aspergillus flavus* | - | - | - | - | - |
| *Aspergillus glavus* | - | - | - | - | - |
| *Penicillium* spp. | - | - | - | - | - |
| *Rhizopus* spp. | - | - | - | - | 7 |

**Table 8: Effect of ethanol extract of *Ageratum conyzoides* on fungi isolated from infected *C. papaya* fruits**

| Isolates | Concentration Zone of Inhibition (mm) | | | | |
|---|---|---|---|---|---|
| | 100mg/mL | 200mg/mL | 300mg/mL | 400mg/mL | 500mg/mL |
| *Aspergillus flavus* | - | 6 | 8 | 12 | 16 |
| *Aspergillus glavus* | - | 7 | 10 | 14 | 18 |
| *Penicillium* spp. | - | - | - | - | - |
| *Rhizopus* spp. | - | - | 6 | 8 | 12 |

**Antifungal activity at different Concentrations**

The antifungal activity of the test plant extracts at different concentrations are summarized below with corresponding graphs to show the antifumgal activity of each extract at various concentrations. There was no activity for the experiment carried out at 100mg/ml concentration of the extracts. Activities were observed from 200 -500 mg/ml as shown in Tables 9 -

**Table 9: Antifungal activity at 200mg/mL**

| Plant Extracts | Isolates Zones of Inhibition (mm) | | | |
|---|---|---|---|---|
| | ***A. flavus*** | ***A. glavus*** | ***Pen.* Spp.** | ***Rhiz.* Spp.** |
| *S. alata* | 0 | 0 | 0 | 0 |
| *P.amarus* | 0 | 0 | 0 | 0 |
| *M. indica* | 0 | 0 | 0 | 0 |
| *A.conyz* | 6 | 7 | 0 | 0 |

**Table 10: Antifungal activity at 300mg/mL**

| **Plant Extracts** | **Isolates Zones of Inhibition** | | | |
|---|---|---|---|---|
| | ***A. Flavus*** | ***A. glavus*** | ***Pen. Spp.*** | ***Rhiz. Spp.*** |
| *S. alata* | 0 | 0 | 0 | 0 |
| *P.amarus* | 6 | 0 | 0 | 7 |
| *M. indica* | 0 | 0 | 0 | 0 |
| *A.conyzoides* | 8 | 10 | 0 | 6 |

**Figure 2 Antifungal activity at 300mg/mL for the ethanol extracts of *S. alata, P. amarus, M. indica* and *A. conyzoides***

**Table 11: Antifungal activity at 400mg/mL**

| **Plant Extracts** | **Isolates Zones of Inhibition** | | | |
|---|---|---|---|---|
| | ***A. flavus*** | ***A. glavus*** | ***Penicillium. Spp.*** | ***Rhizopus. Spp.*** |
| *S. alata* | 0 | 0 | 0 | 0 |
| *P. amarus* | 6 | 8 | 0 | 10 |
| *M. indica* | 0 | 0 | 0 | 0 |
| *A. conyzoides* | 12 | 14 | 0 | 8 |

**Table 12: Antifungal activity at 500mg/mL**

| **Plant Extracts** | **Isolates Zones of Inhibition** | | | |
|---|---|---|---|---|
| | ***A. flavus*** | ***A. glavus*** | ***Pen. Spp.*** | ***Rhiz. Spp.*** |
| *S. alata* | 9 | 6 | 0 | 8 |
| *P.amarus* | 11 | 8 | 0 | 13 |
| *M. indica* | 0 | 0 | 0 | 7 |
| *A. conyzoides* | 16 | 18 | 0 | 12 |

**Table 13: Two-way Analysis of variance**

| | Sum of sqrs | df | Mean square | F | p (same) |
|---|---|---|---|---|---|
| Plant extract: | 81152.1 | 2 | 40576.1 | 3.845 | 0.03015 |
| phytochemicals: | 204018 | **19** | 10737.8 | **1.018** | 0.465 |
| Error: | 401003 | **38** | 10552.7 | | |
| Total: | 686173 | **59** | | | |

**significant at p >0.05**

**Table 14: Two-way Anova for antifungal properties**

| | Sum of sqrs | df | Mean square | F | p (same) |
|---|---|---|---|---|---|
| Plant types | 181.25 | 3 | 60.4167 | 4.759 | 0.02969 |
| Sensitvity: | 181.25 | 3 | 68.75 | 5.416 | 0.02098Error: |
| | 114.25 | 9 | 12.6944 | | |
| Total: | 501.75 | 15 | | | |

Significant at p > 0.05

The plates below show the zones of inhibition for the different plant extracts against the isolated fungi. The four plates are numbered 1 -4 representing the four isolated fungi. The five spots on each plate show the extracts at the five different concentrations used. The clear zones around the bored wells with the extracts show the inhibition zones. They are labelled A to D indicating the different extracts being used (Plate 1).

**Plate 1: A: Inhibition zones for *S. alata*; B: Inhibition zones for *P. amarus*; C: Inhibition zones for *M. indica*; D: Inhibition zones for *A. conyzoides***


**Plate 2: Application of the different extracts on the fresh non-infected pawpaw. 1: *S. alata* crude extract, 2: *P. amarus* crude extract, 3: *M. indica* crude extract, 4: *A. conyzoides* crude extract**

**Plate 3: Effect of the extracts on the pawpaw after 8 days**
**1: *S. alata* crude extract, 2: *P. amarus* crude extracts, 3: *M. indica* crude extract, 4: *A.* conyzoides crude extract**

**Table 15: ISOLATE IDENTIFICATION**

| ISOLATE | DESCRIPTION | % IDENTITY | E-VALUE | ACCESSION NO. |
|---|---|---|---|---|
| I ITS 4 | *Rhizopus arrhizus* strain | 99.83 | 0.0 | gi/1735638417/MN423300.1 |
| | *Rhizopus arrhizopus* isolate | 100 | 0.0 | gi/1531397084/MK267423.1 |
| II ITS 4 | *Rhizopus delemar* | 100 | 0.0 | gi/1788250264/LC514332.1 |
| | *Rhizopus oryzae* strain | 100 | 0.0 | gi/1473251417/MH864361.1 |
| ITS 5 | *Rhizopus delemar* | 100.00 | 0.0 | gi/788250263/LC5143331.1 |
| | *Rhizopus oryzae* strain | 100.00 | 0.0 | gi/l731444961/kp068977.1 |
| ITS 4 | *Aspergillus clavatus* isolate | 100.00 | 0.0 | gi/1359341396/MF152923.1 |
| ITS 5 | *Aspergillus clavatus* strain | 99.11 | 7e-167 | gi/1858336229/MT620752.1 |
| ITS 4 | *Rhizopus delemar* | 91.45 | 0.0 | gi/1788250264/LC514332.1 |

**Figure 5: The phylogenic tree showing Evolutionary relationships of taxa**

The evolutionary history was inferred using the UPGMA method [1]. The optimal tree with the sum of branch length = 2.20015600 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [2] and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons [3]. The

analysis involved 13 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 305 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].

## DISCUSSION

Plants are important source of potentially useful structures for the development of new agents. They synthesize bioactive compounds which are of great potential in agriculture and anti-insect activity (Emitaro *et al*., 2020). Phytochemical screening of the various ethanol leaf extracts of *P. amarus*, *A. conyzoides*, *M. indica* and *S. alata* revealed the presence of alkaloids and flavonoids which cuts across the evaluated extracts. Saponins and Tannins were reported in all extracts as well. Terpenoids were found present in all also except in *S. alata* (Table 1). The antifungal studies showed that four (4) fungus were isolated from rotten pawpaw fruit and were confirmed through pathogenicity test to be pathogens causing rot in the fruit. This agrees with the works of Maga and Zakawa (2018) as they reported *Mucosspp, R. stolonifera* and *A. niger* to be responsible for fruit rot in *C. papaya*.

From this study four different fungi associated with the rot of C. *papaya* were noted. The molecular result revealed the identification of two genera i.e *Rhizopus* and *Aspergillus.* The identified species were *Aspergillus clavatus*, *Rhizopus delemar* and *Rhizopus arrhizopus*. These include *Aspergillus flavus*, *Aspergillus glavus*, *Penicillium spp* and *Rhizopus spp*. These isolates confirmed previous reports of Gupta and Pathak, 1986 and Bayiewu, 1994 that isolated *A. flavus*, *Rhizopus* spp, and *Botryodiplodia* and *Cuvularia spp*.

The leaf extracts of *A. conyzoides* produced zones of inhibition as 16mm (*A. flavus*), 18mm (*A. glavus*) and 12mm (*Rhizopus spp*) at 500mg/mL followed by *Phyllanthus amarus* as 6 mm (*A. flavus*), 8 mm (*A. glavus*) and 10 mm (*Rhizopus spp*) (Figure 4 and Table 4). [35], reported the presence of precocene II, nobiletin and polymethoxy flavones which are flavonoid compounds with marked antifungal activity. The zone of inhibition by the extract increases with an increase in concentration. [36], reported the antifungal activity of *A. conyzoides* and *Chormolaenaodorata* on post-harvest fruit rot. Some of these compounds are effective super oxide antioxidants with ability to inhibit mycelial growth by reacting with cell wall components of these fungi.

The growth inhibitory effect of different fungi by plant extracts proved to be dependent on the concentration, the type of extract and the plant tested. Results obtained from *A. conyzoides* extract are in agreement with previous studies that showed the antifungal activities of this plantagainst devastating pathogen on variety of economic plant [37] which showed that these extracts inhibit the development of *Phytophtora megakarya* (responsible for the brown rot of cocoa). A wide range of allelochemicals including alkaloids, flavonoids, terpenoids and benzofuran have been isolated from *A. conyzoides* which might result in the stronger inhibitory activity.

Results obtained in this study showed that the extracts of *Agratum conyzoides* and *Phyllanthus amarus* inhibited the growth of three test organisms in varying degrees. The isolates were *A. glavus, A. flavus* and *Rhizopus spp.*

The results of the present study also showed the presence of various concentrations of phytochemicals. Alkaloids was recorded highest in *A. conyzoides* (38.4mg/ml), followed by *P. amarus* (34.82mg/ml) while *M. indica* and *S. alata* had 2.8mg/ml and 27.43mg/ml respectively. Flavonoids were also recorded highest in *A. conyzoides* (24.21mg/mL), followed by *P. amarus*

(17.27mg/mL), *S. alata* had 7.73mg/mL and *M. indica* had 2.8mg/mL respectively. Extract of *M. indica* showed no antifungal activity on the tested organisms except on *Rhizopus spp* at a very high concentration of 500mg/mL of the extract.

The present study also revealed *invitro* antifungal activity against all tested organisms except *Penicillium* spp which was resistant to all the plant extracts. Most susceptible organisms were *Rhizopus* spp, *Aspergillus flavus* and *Aspergillus glavus* in which its of interest that the plant extract be used for the preservation of shelf-life of *C. papaya* fruit.

**Conclusion**

From the result of the research, the molecular analysis identified and named correctly the pathogens as *A. clavatus, Rhizopus delemar, R. oryzae* and *R. arrhizus* respectively using the phylogenetic as shown in the phylogenetic tree. Therefore, ass pathogens responsible for *C. papaya* fruit rot. *A. conyzoides* was able to inhibit the growth of the pathogens followed by *P. amarus*. These plants extracts can be said to have antifungal effect owing to the presence of various phytochemicals. *A. conyzoides* and *P. amarus* can be used to minimize the spoilage of fruit by microbes.

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