

Antifungal Potential of Aqueous and Ethanolic Extracts of Cashew Apple Fruit, Stem Bark and Nut Shell Liquid on Fungal Rot Disease of Cocoyam

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Abstract. Post-harvest loss by fungal rot disease is a major problem associated with the availability and quality of cocoyam production in Nigeria. There is need for natural and ecofriendly antifungal agents to improve yield and storage. Fungi causing cocoyam rot were isolated on potato dextrose agar (PDA) using standard isolation method. Pure isolates were identified based on their macroscopic and microscopic morphology. Pathogenicity tests were carried out on healthy cocoyam cormels by inoculating with the isolated fungi. Ethanolic and aqueous extracts of different parts of the cashew plant were evaluated for antifungal activity against isolated fungi using the food poisoning technique. *Aspergillus niger*, *Mucor circinelloides* and *Rhizopus stolonifer* were isolated from rotten cocoyam. Pathogenicity test reveals that all three fungi induced rot in healthy cocoyam cormels after 5 days of inoculation with *Rhizopus stolonifer* being the most virulent. All extracts showed high degrees of antifungal activity, but ethanol extract proved to be more potent. The ethanolic cashew nut shell liquid extract (ECNSLE) gave the highest inhibitory effect of 99% 85% and 99% against *Mucor circinelloides*, *Rhizopus stolonifer* and *Aspergillus niger* respectively. The least effective was aqueous cashew stem bark extract (ACSBE) with inhibition percentage of 96%, 79% and 91% against *Mucor circinelloide*, *Rhizopus stolonifer* and *Aspergillus niger* respectively. All extracts had higher inhibition values than the control ketoconazole (70%, 62%, and 67% against *Mucor circinelloides*, *Rhizopus stolonifer*, and *Aspergillus niger* respectively). The plant extracts in this study possess antifungal properties and can be further developed and used in the control of rot diseases in cocoyam. It has the advantage of being natural, without the challenges associated with chemical fungicides.

Key words: antifungal, fungi, inhibition, cocoyam, cashew.

Introduction

Cocoyam (*Colocasia esculenta* L) belonging to the family *Araceae* is an important group of tropical root crops providing energy for over 500 million people worldwide. It is an important staple food in many developing countries in Africa, Asia, and the Pacific (Nwauzoma et al., 2017: 40-44). Cocoyam is believed to be consumed mostly by the low-income earners and the economically vulnerable groups. Nigeria is presently the world's leading producer of cocoyam, accounting for 35% of the world's total production (Ubalua et al., 2016: 53-59). In Nigeria, two species, *Colocasia esculenta* (taro) and *Xanthosoma sagittifolium* (tannia) are mainly cultivated for the edible corms as a source of carbohydrate to supplement yam and cassava as well as for medicinal purposes (Ilondu et al., 2013: 1404). It is ranked among the five important root crops in Nigeria alongside yam, cassava, Irish potato and sweet potato (Agbele-moge, 2013: 7956).

However, diseases and pests in farm and storage pose serious problem in cocoyam production and utilization. In storage, serious losses due to rotting of the corms and

cormels by fungi are a major factor affecting adversely the quantity and quality of cocoyam for consumption and planting (Opara et al., 2015: 17-23). The use of chemicals has helped in control of rot, but due to problems such as; chemical residues, biodegradation, phytotoxicity, pollution, development of resistance in target organism, high cost, atimes nonavailability and hazard to man and environment makes them slow to adopt or total refusal by farmers (Okigbo and Odurukwe, 2009: 117-127). Hence, the need for alternative control methods is required.

Natural plant products are potential alternatives to chemical fungicides in plant disease management. Presently, considerable efforts are directed at exploring the potentials of botanicals (plantextracts) as alternatives or complimentary to synthetic chemicals. Botanicals have the advantage of not only being readily available and affordable but are also non-phytotoxic and easily biodegradable alternative fungicides and antibiotics, hence environment friendly (Okigbo and Nmeke, 2005: 804-807; Okigbo and Omodamiro, 2006: 117-127). More so, many plants are extensively used locally in traditional medicine for the treatment and control of disease, with broad – spectrum antifungal activities (Okigbo and Odurukwe, 2007: 154-165).

Originally, a tropical plant bearing both seed and apple fruit at maturity, cashew (*Anacardium occidentale*) is always a well adaptable tropical plant which exhibits green foliage at all season of the year. The bark, leaves and shell oil of the plant are used medicinally to treat different ailments (Agedah et al., 2010: 25-27). The anti-microbial activity of the cashew leaf extrac't has been documented by several researchers (Agedah et al., 2010: 25-27; Ifesan et al., 2013: 465-473). Different parts of the cashew plant have been used in the treatment of allergies, gastrointestinal track syndromes, cardiovascular problems, respiratory tract dysfunctions and in the treatment of cancerous growth and tumor (Arekemase et al., 2011: 968-973). However, reports on their efficacy on the control of cocoyam rots disease are sparse. Therefore, the objective of the present study is to evaluate the antifungal potential of both aqueous and ethanolic extracts of cashew apple fruit, stem bark and nut shell liquid in the management of cocoyam rot disease.

Materials and Method

Sample Collection

Tubers of cocoyam with symptoms of rot were obtained from new market in Wukari, Taraba State, Nigeria. The diseased cocoyams were packaged in polyethylene bags and taken to the microbiology laboratory, Federal University Wukari, where they were assessed for microbial presence. The cashew apple, cashew nut and cashew stem bark were all collected from Federal University Wukari farm, Wukari Local Government, Taraba State. The healthy ones were sorted out and washed under running tap water to eliminate dust and other foreign particles.

Ethanolic and Aqueous Extraction

The cashew apple fruit, stem bark and the cashew nut shell were grounded to the smallest possible sizes. 250g of samples were weighed and added into the extraction container. 300ml of ethanol or water (separately for ethanol and aqueous extraction) was then added and tightly covered. For ethanolic extraction, the mixture was allowed to soak for 24 hours before it was filtered and concentrated using a Rotary Evaporator at 100 °C. while for aqueous extraction, the mixture was placed in a water bath at 60°C for 1 hour and allowed to soak for 24 hours. It was then filtered and concentrated using an electric heater °C.

Isolation of Fungi

The isolation method used by Ezeonu et al. (2018: 18) as reported by Onyike and Maduewesi (1985) was employed in this study. A small section of the cocoyam tissue containing the advancing margin of rot and adjoining healthy tissue were cut using sterilized scalpel and cork borer sterilized with 70% ethanol. The peeled portions of the cocoyams taken by cork borer were placed on the solidified agar. One peeled portion was placed per plate and done in duplicates. The plates were incubated at 27 ± 2 °C for 7 days and examined daily for development of fungal growth.

Identification of Fungal Isolates

Sub-culturing of the isolates was done using inocula from the different organisms in the mixed cultures to obtain a pure culture. The resulting pure cultures were identified based on their macroscopically and microscopically characteristics. Colony colour, type (compact, loose, aerial hyphae), texture (velvety, cottony, coarse) shape and growth pattern were observed. Direct observation of culture under the light microscope ($\times 40$) by careful preparation of slides, staining with cotton blue-lactophenol was done. Detailed drawings of the diagnostic features and identification manual and guides according to Rippon (1958: 163); Alexopoulos (1962); Nelson et al. (1983: 193); Samson et al. (1984: 1-25); and Snowdon (1991: 416) were used.

Pathogenicity Test

The pathogenicity test was carried out to establish which of the fungal isolates caused the rot and to determine whether they could induce similar symptoms on inoculation and be re-isolated, thus fulfilling Koch's postulates. The method of Okigbo and Ikediugwu (2000: 351-355) were adopted for the pathogenicity study. The pure fungal isolates obtained from infected cocoyam tuber were used for inoculation. Healthy looking cocoyam tubers were surface-sterilized with 70% ethanol. Each healthy cocoyam was bored into about 1cm deep, with a sterile 6mm diameter cork borer at three different points on the cocoyam tuber surface (proximal, middle and distal regions). Another 6mm sterile cork borer was used to cut about 5mm of mycelia disc from edge of a 5 days old culture of each fungus isolate. The mycelia discs were used to inoculate the holes created by scooping out the cocoyam tissue. The scooped out tissue of the cocoyam tuber was replaced after 5mm pieces had been cut off to compensate for the thickness of the fungal culture. Two whole tubers were inoculated per fungus. The control set up consists of tubers that were similarly bored into and inoculated with sterilized PDA agar discs.

The wounds were sealed with petroleum jelly (to prevent drying and contamination) and inoculated tubers were placed in transparent polythene bags whose inside has been moistened with cotton wool soaked in sterile distilled water to maintain a high humidity. The inoculated cocoyam tubers were kept in the laboratory at room temperature for about 7 to 21 days. The tubers were assessed for rot development by cutting through the points of inoculation where rots developed. The pathogens were re-isolated as previously described and their cultural and morphological characteristics were compared with those of the original isolates. The percentage severity of rot (Sr %) was calculated thus:

$$Sr (\%) = \frac{FW - w}{w} \times 100$$

where, FW = Final weight of infected cocoyam tuber,
w = weight of rotted cocoyam tuber portion.

Antifungal Activity of the Extracts

Effect of plant extract on mycelia growth of the test fungi was studied using the food poisoning techniques (Sangoyomi, 2004: 166-167; Oniyike and Maduewesi, 1985: 74-

81). Briefly, 1ml of each plant extract was dispensed per petri dishes and 9ml of PDA was added to each of the petri dishes containing extract and carefully spread evenly over the plate, this gave rise to PDA –extract mixture with 10% extract concentration. This was used for the inhibition of mycelia growth. The plates were gently rotated to ensure even dispersion of the extracts. The agar-extract mixture was allowed to solidify and then inoculated at the center with a 4mm diameter mycelia dish obtained from the colony edge of 7-day old pure cultures of each of the three test fungi. Each treatment was in duplicates. The negative set up was blank agar plate (no extract) inoculated with the test fungi as described above. Petri-dishes dispensed with PDA and 1ml of ketoconazole dissolved in distilled water inoculated with each test fungus served as the positive control. All the plates were incubated at 28 °C for 5 days and examined daily for growth and presence of inhibition. Colony diameter was taken as the mean growth along two directions on two pre-drawn perpendicular lines on the reverse side of the plates. The effectiveness of the extract was recorded in terms of percentage inhibition, which was calculated according to the method described by Whipps (1987: 127-142).

$$\text{Percentage inhibition} = R1 - R2 \times \frac{100}{R1}$$

Where R1 is the farthest radial distance of pathogen in control plate, while R2 is the farthest radial distance of pathogen in extract-incorporated agar plates.

Results and Discussions

Fungal Pathogens Isolated from Samples of Cocoyam Cormels

The fungi pathogens isolated from spoilt cocoyam in this study were identified based on their cultural characteristics as *Aspergillus niger*, *Mucor circinelloides* and *Rhizopus stolonifer* (Fig. 1 and 2). The three fungi isolated in this study have previously been isolated from spoilt cocoyam by various researchers as well. *Aspergillus* and *Rhizopus stolonifer* were identified by Ugwuanyi (1996) who carried out an examination on root and associated fungal pathogens in cocoyam and discovered *Aspergillus niger*, *Botryodiplodia spp.*, *Corticium rolfsii* *Geotrichum candidum*, *Fusarium spp.* to be causes of rot. This rot due to *Aspergillus*, according to Ugwuanyi (1996) was extensive resulting in complete maceration of cocoyam tissues. Okigbo (2003: 19-23) isolated *Rhizopus* and *Mucor* species, which belonged to the group of fast growing fungi that cause rot in cocoyam. Onuegbu (1999: 279-272) also isolated *Aspergillus* and *Fusarium* species from spoilt cocoyams. Likewise, Anukworji et al. (2012: 33-47) isolated and identified *Aspergillus niger* and *Rhizopus stolonifer* from rot-infested tissues of cocoyam cormels. In addition, reports by Frank and Kingsley (2014: 553-562) showed that the above named organisms are actual pathogens of root and tuber crops. In a more recent study, Ezeonu et al. (2018: 18) isolated and identified *Aspergillus niger* and *Rhizopus stolonifer* as cocoyam rot causing pathogens. The isolation of more than one pathogenic organisms from a particular cormel confirms the possibility of multiple infections whose cumulative effect may cause rapid rotting of root and tuber crops. These collaborates with the reports of Anukworji et al. (2012: 33-47).

Table 1 deals with systematization of peculiarities of colonial morphology and microscopic characterizations of fungi associated with cocoyam spoilage.

Table 1. Colonial Morphology and Microscopic Characterizations of Fungi Associated with Cocoyam Spoilage

Isolates	Colonial characteristics	Microscopic characteristics
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Mucor circinelloides	On PDA, colonies were floccose (cottony in texture), pale greyish-rown. Growth rate was rapid, thus, colonies filled the entire petri-dish in 3 days. Colour on the reverse side was yellow. Colonies were incubated at 30 °C for 5 days.	Sporangiophores were hyaline, erect, non-septate and branched sympodially and circinate. Sporangia were terminal, dark-brown, finely echinulate to smooth and spherical (20-80 µm in diameter). Sporangiospores were hyaline or pale-brown. Collumellae were ellipsoidal and 4.5-7 x3.5-5 µm in size. Chlamydo spores were absent.
Rhizopus stolonifer	On PDA, Colonies are very fast growing, cottony to fluffy, white to yellow, becoming dark-grey.	Sporangiospores are hyaline, grey or brownish, globose to ellipsoidal, and smooth-walled, and erect, simple or branched, forming large, terminal, globose to spherical, multispored sporangia, without apophyses and with well-developed subtending columellae.
Aspergillus niger	On PDA, colonies had rapid growth rate. However, colonies were flat and compact with yellow basal felt covered by a dense layer of black conidial heads with powdery texture. The colour on the reverse side was pale yellow. Colonies were incubated at 30°C for 5 days.	Septate hyphae with Conidiophores were hyaline or pale-brown to black, erect, simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial heads split into over 4 loose conidial columns with over 4 fragments apically composed of catenulate conidia.

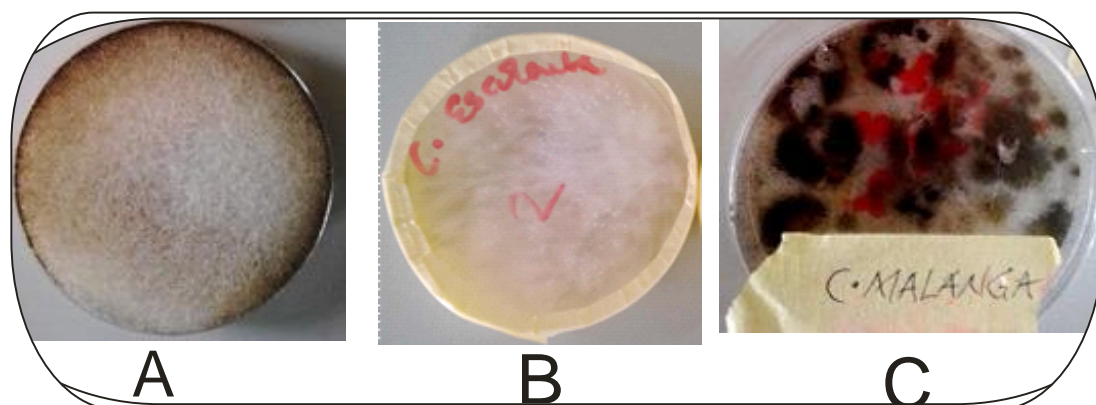


Fig. 1. The pure culture of: (A) *Rhizopus stolonifera*, (B) *Mucor circinelloides*, and (C) *Aspergillus niger* before treatment with the extracts

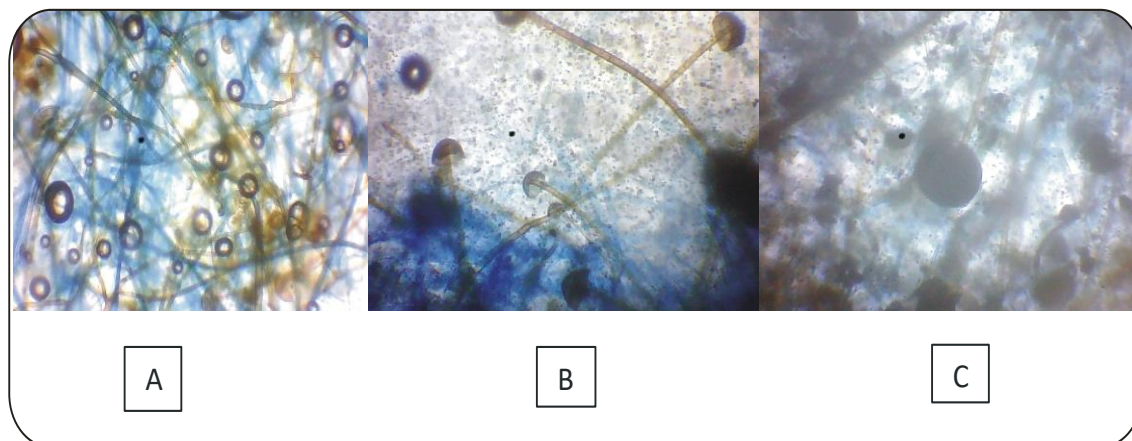


Fig. 2. Microscopic morphology ($\times 40$) of (A) *Mucor circinelloides* (B) *Rhizopus stolonifera* (C) *Aspergillus niger*

Pathogenicity Test

The pathogenicity test showed that all three test fungi *Rhizopus stolonifera*, *Mucor circinelloides*, and *Aspergillus niger* were pathogenic. Hence, causes rot in healthy cocoyam cormels as observed in the pathogenicity test. The nature of rot caused varied among the various pathogens. *Mucor circinelloides*, and *Aspergillus niger* showed soft rot, while *Rhizopus stolonifera* showed dried rot (Table 2). The most virulent among the four test fungi was *Rhizopus stolonifera* followed by *Mucor circinelloides*, while *Aspergillus niger* was the least pathogenic as evident in the weight loss (Fig. 3). There was obvious reduction in weight observed in corms of cocoyam exposed to fungal pathogenicity. Order of weight reduction from high to low in the cocoyam exposed to the fungi was 12.23 g (*R. stolonifera*), 8.10 g (*M. circinelloides*) and 4.03 g (*A. niger*). The reduction in weight of the control (uninfected cocoyam) was only 1.03 g. These results confirm the pathogenic nature of the fungi isolates. These fungi infect this crop before harvest, though, injuries caused after harvest by careless handling or by insects or other animal damage, and by direct penetration of the intact skin of the plant by these pathogenic organisms. Nevertheless, all these can be avoided by proper packaging and handling and use of clean planting equipment and healthy planting materials.

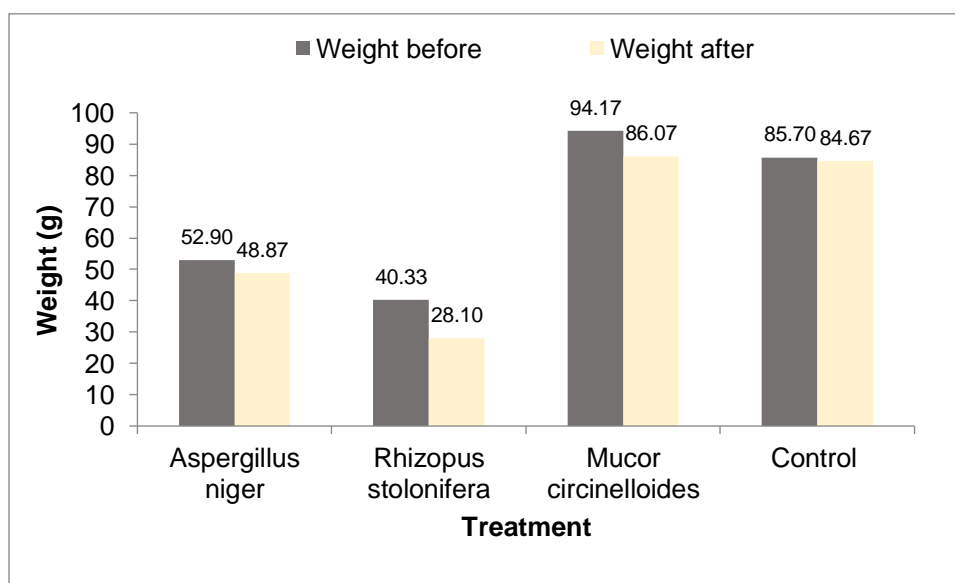


Fig. 3. The mean weight loss of cocoyam after pathogenicity test

Table 2. Pathogenicity Test Result

Isolate	Symptoms of infection after 21days	Pathogenicity
<i>Aspergillus niger</i>	Soft rot,	++
<i>Rhizopus stolonifera</i>	Dry rot	+++
<i>Mucor circinelloides</i>	Soft rot	+++
+++ = highly pathogenic, ++ = moderately pathogenic		

Antifungal activity of the extracts on fungi isolated from cocoyam

Analysis of variance at 95% showed that all the parts (fruit, bark and seed) of cashew plant used in this study significantly ($P < 0.05$) inhibited the growth of the fungal organisms causing cocoyam rot (Table 3). All tested plant parts using both extraction methods appeared to be more active against *Mucor circinelloides*. The ethanolic cashew nut shell liquid extract (ECNSLE) gave the highest inhibitory effect of 99% and 99% against *Mucor circinelloides* and *Aspergillus niger* respectively, followed by the aqueous cashew nut shell liquid extract (ACNSLE) with inhibitory effect of 99% and 98% against same organisms; while for the *Rhizopus stolonifer*, (Fig. 4) the aqueous cashew nutshell liquid extract (ACNSLE) gave the highest inhibitory effect of 97% compare to the ethanolic extract with inhibitory effect of 85%. Meanwhile, ketoconazole which was used as the positive control showed 70%, 62%, and 67% of inhibition against *Mucor circinelloides*, *Rhizopus stolonifer*, and *Aspergillus niger* respectively. Results obtained in this study suggest that the ethanolic extract is more effective than both the ketoconazole and the aqueous extracts.

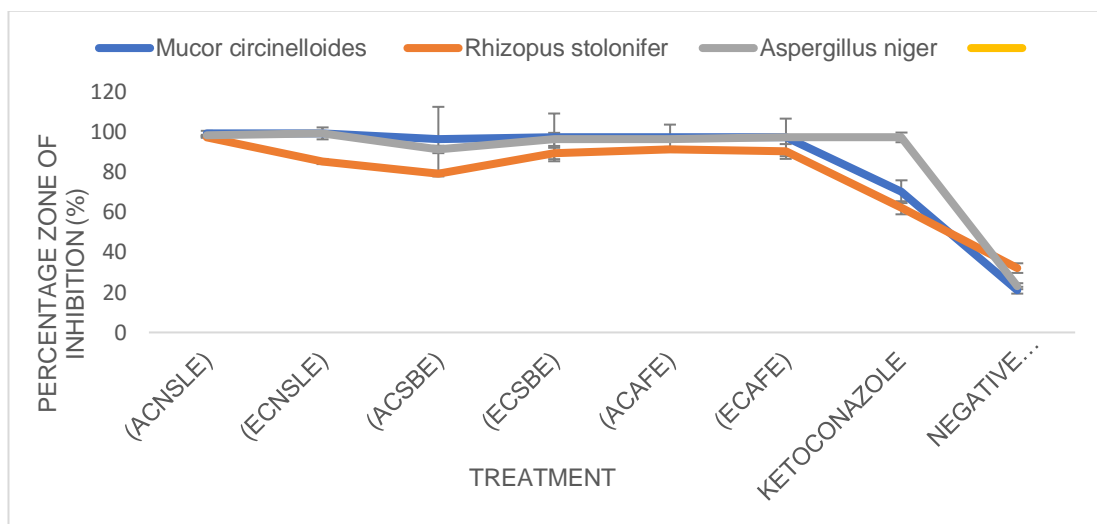


Fig. 4. Graph Showing the Mean Percentage (%) Zone of Inhibition of Extracts on all test organisms

For the cashew stem bark (CSB), the ethanolic cashew stem bark extract (ECSBE) demonstrated inhibitory effects of 97%, 89%, and 96% compare to the aqueous cashew stem bark extract (ACSBE) which gave a less percentage inhibitory effects of 96%, 79%, and 91% against *Mucor circinelloides*, *Rhizopus stolonifer*, and *Aspergillus niger* respectively. Ethanolic extracts of the cashew stem bark is more effective against *Mucor*

circinelloides and *Aspergillus niger* than the aqueous cashew stem bark which is more effective against *Rhizopus stolonifer*.

The ethanolic cashew apple fruit (ECAFE) also showed high inhibitory effects of 97%, 90%, and 97% against *Mucor circinelloides*, *Rhizopus stolonifer*, and *Aspergillus niger* respectively. This is not significantly different from the results obtained in the aqueous cashew apple fruit extract (ECAFE), which is 97%, 90%, and 97% against *Mucor circinelloides*, *Rhizopus stolonifer*, and *Aspergillus niger* respectively (Fig. 4).

Table 3. Percentage Mean Inhibition of All the Extracts on all the Fungi

Extracts	<i>Mucor circinelloides</i>	<i>Rhizopus stolonifera</i>	<i>Aspergillus niger</i>
Aqueous Cashew Nutshell Liquid Extract (ACNSLE)	99 ± 0.14 ^a	97 ± 1.27 ^a	98 ± 0.30 ^a
Ethanolic Cashew Nutshell Liquid Extract (ECNSLE)	99 ± 1.27 ^a	85 ± 2.97 ^c	99 ± 0.99 ^a
Aqueous Cashew Stem Bark Extract (ACSBE)	96 ± 1.41 ^a	79 ± 16.20 ^d	91 ± 1.84 ^b
Ethanolic Cashew Stem Bark Extract (ECSBE)	97 ± 2.83 ^a	89 ± 11.88 ^c	96 ± 3.26 ^a
Aqueous Cashew Apple Fruit Extract (ACAFE)	97 ± 1.27 ^a	91 ± 6.36 ^b	96 ± 1.13 ^a
Ethanolic Cashew Apple Fruit Extract (ECAFE)	97 ± 3.68 ^a	90 ± 9.33 ^b	97 ± 0.99 ^a
Positive Control	70 ± 3.25 ^b	62 ± 5.66 ^e	67 ± 2.40 ^c
Negative Control	21 ± 2.41 ^c	32 ± 1.70 ^f	23 ± 1.50 ^d
***Values with similar letters of alphabet in the same column do not have significant difference. Percentage inhibition values are represented with Mean ± Standard Deviation.			

The antifungal activity exhibited by the various parts of cashew plants in this study is not totally surprising, as Kannan et al. (2009: 253-257) previously reported that cashew nuts possess good antifungal properties. Kannan et al. (2009: 253-257) also reported an inhibition of 82.14% against *A. niger*, which is close to the values obtained in this study against the same organism. In a more recent research, Garcia et al. (2017: 95-103) reported cashew nut oil to possess antifungal activities against various fungi species. The antifungal activity of both the bark, the nutshell, and the apple fruit is probably due to the presence of reported bioactive components such as triterpenoids, phenolic and volatile oils, which have been reported to be active against fungal isolates (Ifesan et al., 2013: 465-473; Aleopoulos, 1962: 119-159).

Conclusion

The different parts of the plant in this study possess potential antifungal properties against cocoyam rot disease causing fungi, although their efficacy differs for different parts and extraction solvent employed. Results obtained reveals that ethanolic extracts were more effective than aqueous extracts, this suggests that water probably did not dissolve all the principal compounds present in the plants, compared to ethanol.

Mold infestation contributes hugely to the problems encountered during post-harvest storage of cocoyam. This study reveals *Aspergillus sp.*, *Mucor sp.* and *Rhizopus sp.* as major causative agents of cocoyam rot disease leading to enormous loss of

cocoyam tubers despite its economic and nutritive value. The plant extracts in this study were found to be very effective in inhibiting mycelial growth; they can possibly be used in the storage of these cocoyam tubers to reduce post-harvest loss. It is important to adopt disease control practices that will be affordable by the bulk of resource-poor farmers in our part of the world; and it will ensure substantial contribution of the cocoyam to food supply and national economy.

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