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ANTIFUNGAL POTENCIES OF LEAF EXTRACTS OF *Carica papaya* ON FUNGI IMPLICATED IN SOFT ROT OF YAM

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Abstract
Antifungal potency of leaf extracts of *Carica papaya* on fungal pathogens implicated in soft rot of post-harvest yam was evaluated. The cup-plate agar method was used to determine the inhibition zone diameters of cold and hot ethanolic leaf extracts of *Carica papaya* on fungal pathogens implicated in soft rot of yam, namely; *Rhizopus nigricans* and *Mucor circinelloides*. Though cold extracts showed higher zones of growth inhibition (effects) than hot extracts for each of the concentrations tested, there was no significant difference ($p>0.05$) between them; hot extract showed no antifungal effect on *Mucor circinelloides*. For the cold extract, there was no significant difference ($p>0.05$) in the zones of growth inhibition between *Rhizopus nigricans* and *Mucor circinelloides*, while significant difference ($p<0.05$) between their zones of growth inhibition was noticed for the hot extracts. Different concentrations (100mg/ml, 50mg/ml, 25mg/ml, and 12.5mg/ml) showed significant difference ($p<0.05$) in their zones of growth inhibition on *Mucor circinelloides* for the cold extracts, and on *Rhizopus nigricans* for the hot extract. The minimum inhibitory concentrations obtained for the cold extract were 0.06mg/ml and 0.10mg/ml for *Rhizopus nigricans* and *Mucor circinelloides* respectively; while minimum inhibitory concentration obtained for the hot extract was 0.08mg/ml for *Rhizopus nigricans*. Phytochemical analysis of the leaf extract of *Carica papaya* revealed the presence of tannins, glycosides, alkaloids, and flavonoids. This study reveals that the antifungal and phytochemical potentials of leaf extracts of *Carica papaya* may justify its use as a feasible alternative to more toxic chemical methods employed in yam preservation.

Keywords: antifungal, soft rot, extracts, post-harvest, yam.

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1. INTRODUCTION

Yams are starchy staples in the form of large tubers produced by annual and perennial vines grown in Africa, the Americas, the Caribbean, South Pacific and Asia. There are hundreds of wild and domesticated *Dioscorea* species. White Guinea yam, *D. rotundata*, is the most important species especially in the dominant yam production zone in West and Central Africa. It is indigenous to West Africa, as is the Yellow yam, *D. cayenensis*. Water yam, *D. alata*, the second most cultivated species, originated from Asia and is the most widely distributed species in the world (IITA, 2011). Ninety seven percent (97%) of this come from Africa, the main producers being Nigeria with 71% of world production; Côte D'Ivoire 8.1%; Benin 4.3% and Ghana 3.5%. Nigeria alone produces three – quarters of the total world output of yams, making Nigeria the world's largest producer. Yam is the second most important root/tuber crop in Africa with production reaching just under one third the level of cassava. More than 95 percent (2.8 million ha) of the current global area under yam cultivation is in sub-Saharan Africa, where mean gross yields are 10 tons/ha. In Asia, production for 2004 stood at 226, 426 metric tons. Colombia (333 000 t) leads the production in South America followed by Brazil (230 000 t), while Japan (204 000 t) is the leader in Asia. Yams are also important in the Caribbean (e.g., Haiti with 197 000 t in 2005), and the South Pacific Islands. Ghana exports the largest quantity of yams (about 12 000 t) annually. (FAO, 2004).

In humid tropical countries of West Africa, yams are one of the most highly regarded food products and are closely integrated into social, cultural, economic and religious aspects of life (Ike and Inoni, 2006). Yam tubers consist of about 21% dietary fiber and are rich in

carbohydrates, vitamins B, C, riboflavin, thiamin and essential minerals like calcium, phosphorus, and iron (Ebenezer and Olufokunbi, 2004; IITA, 2009). Worldwide annual consumption of yams is 18 million tons, with 15 million in West Africa. Annual consumption in West Africa is 61 kilograms per capita. Average yam consumption per capita per day is highest in Bénin (364 kcal) followed by Côte d'Ivoire (342 kcal), Ghana (296 kcal), and Nigeria (258 kcal). African countries imported more than 2,000 tons in 2002, and exported 15,500, of which Nigeria exported 12% (FAO, 2004; IITA, 2011). Yams are boiled, roasted, or fried. In Africa they are also mashed into a sticky paste or dough (fufu) after boiling. Recently yam has also been processed into flour. Some species of yam have been used medically to treat disease like diabetes mellitus, to increase coronary flow and prevent hypercholesterolemia (Okigbo and Ogbonnaya, 2006).

In West Africa alone, yam loss is estimated to be about one million tons per annum. The magnitude of weight loss in stored yams increases rapidly after the first months. Transit losses of about 15-40% occur in some developing countries due mainly to inefficient storage and transport facilities. In Puerto Rico, post harvest losses of yams due to decay exceeded 50%. Processing losses during culinary preparation of peeled yam can amount to 10-15% (Opara, 2003). These losses are attributed by many researchers mostly to rot caused by bacteria, fungi and nematodes (Amusa *et al*., 2003). According to Okigbo (2004), fungi account for a greater loss than any other single cause. Minimizing physical damage of tubers during post-harvest operations has been shown to reduce the incidence of rot (Okigbo and Nmeka, 2005). The storage diseases of yam can be categorized into 3 based on the symptoms and the causal agents: Dry rot, Soft rot, and Wet rot (Amusa and Baiyewu, 1999).

Carica papaya otherwise called Papaya is widely distributed throughout tropical and subtropical regions. These regions are synonymous to the zone of major yam production. Worldwide, over 6.8 million tons of fruit were produced in 2004 on about 389,990 Ha (FAO 2004). The fruit is consumed world-wide as fresh fruit and as a vegetable or used as processed products (Teixeira da Silva *et al*., 2007). The leaves of Papaya have found use in various herbal solutions: as a tumour-destroying agent, an antiseptic, as a tonic and blood purifier. The tea, prepared with the green papaya leaf, promotes digestion and aids the in treatment of ailments such as chronic indigestion, overweight and obesity, arteriosclerosis, high blood pressure and weakening of the heart (Ayoola and Adeyeye, 2010). Papaya which is prevalent in yam producing regions is chosen in this study with a view to investigating the antifungal properties of its leaf extract on *Rhizopus nigricans Mucor circinelloides* which are common pathogens of soft rot disease of post harvest yam (Amusa and Baiyewu, 1999). Soft rot of yam accounts for 36.1% of post harvest diseases of yam (Ogaraku and Usman, 2008).

3. MATERIALS AND METHODS

Sample collection

Rotten yam tubers were collected from yam stored on horizontal bamboo platforms (improvised yam barn) at Okwuaba village in Okpofe Ezinihitte Mbaise Local Government Area of Imo State. Fresh leaves of *Glyphaea brevis* and *Spondias mombin* were collected at about midday at Okwuaba Okpofe. The leaves were identified and classified by Mr. G.C. Anosike of the Department of Forestry, Imo State Ministry of Agriculture, Owerri. The leaves were air dried at ambient room temperature (28^0C – 31^0C) until constant weight. The dried leaves were ground to powder using a mechanical grinder.

Extraction of plant materials

Ethanol (95%) was used for the extraction of the plant materials. For the cold ethanol extraction, 100g of each powdered plant material was seeped in 500ml of 95% ethanol for 48 hours, while hot ethanol extraction was

carried out by seeping 100g of each powdered plant material in 500ml of 95% hot ethanol which was maintained at 60^0C for 1 hour in a water bath (Osadebe and Ukwue, 2004). The slurries were filtered through folds of sterile cheese cloth. The filtrates were evaporated to dryness by forced air pressure using a rotary evaporator (Esimone *et al*., 1998) to a yield of about 12.5%w/w (with respect to the powdered plant material).

Preparation of plant extract diluents

As desribed by Nweze *et al*. (2004), 1000 mg (1 g) quantity of each ethanolic extract (hot and cold) was reconstituted with 5 ml of 10% dimethylsulphoxide (DMSO) to obtain a concentration of 200 mg/ml. Two fold serial dilution was used to obtain the following concentrations in sterile distilled water: 100 mg/ml, 50 mg/ml, 25 mg/ml, and 12.5 mg/ml. These were stored by refrigeration at 4^0C in sterile amber coloured bottles until required.

Isolation of Fungal Pathogens from Rotten Yam

This was carried out as described by Okigbo and Nmeka (2005). Briefly, the rotten yam tubers were rinsed in sterile distilled water and surface sterilized with 70% ethanol. The rotten yam tubers were then cut open with a sterile knife (flamed). About 3 pieces (3 mm diameter) of each infected tissues were picked with flamed sterilized forceps and inoculated on solid Sabouraud's Dextrose agar (SDA) in different sterile plates. The plates were incubated at ambient room temperature (29 – 31^0C) for up to 5 – 7 days and examined daily for growth of moulds. The isolates were then sub-cultured to obtain pure cultures of the organisms. They were eventually transferred to SDA slants and stored at 4^0C until required.

Identification of Fungal Isolates

The fungal isolates were identified as described by Ogbulie *et al.* (2001) using their growth (colonial) morphology on SDA and microscopic morphology. The colonial morphology on SDA was determined by observing the surface and reverse views of the fungi growing on each agar plate. The colour, shape, elevation, and spore head colouration were noted. The microscopic morphology was also determined. Two drops of lactophenol cotton blue were placed on a clean grease-free glass slide. Then sterile (flamed) inoculating needles were used to transfer a small portion of mycelial growth to the lactophenol. The fungal growth was then teased and covered with a clean grease-free coverslip and then examined microscopically using x10 and x40 objectives.

Preparation of inocula

All fungal isolates were aseptically inoculated onto sterile SDA slants prepared in McCartney bottles and incubated at ambient room temperature (29 - 31^0C) for 4 days to obtain young actively growing cultures consisting of mycelia and conidia / arthrospores / blastospores. The fungal growth on each agar slant was aseptically scrapped off and placed in 10 ml sterile saline (0.9% w/v) and shaken vigorously using a vortex mixer until the fungal filaments were broken into small colony forming units (cfu). Each suspension was standardized using a haemocytometer to obtain 10^4 - 10^6 cfu/ml and this used as the inoculum.

Testing for anti-fungal potency of the plant extracts

The antifungal effects of the plant extracts were examined using cup-plate agar diffusion technique as described by Esimone *et al*. (1998). About 0.2 ml of each standardized fungal suspension was aseptically introduced into a separate sterile petri dish, and then 20 ml of sterile molten SDA was added to each Petri dish while slowly rotating to ensure uniform distribution of the microorganisms. These were labeled and the medium was then allowed to solidify in the petri dish. Cups, 8 mm in diameter, were made in the solidified agar using a sterile cork borer. Equal volumes (0.04 ml) of the serially diluted plant extracts were introduced into the cups made on the solid agar using sterile Pasteur pipettes and labeled according to concentration. The dishes were incubated at ambient room temperature (29 – 31^0C) for 1 – 5 days. At the end of incubation,

the clear zones of growth inhibition that formed were measured using a transparent ruler with millimeter calibration. Each test was carried out in triplicates and the mean of the inhibition zone diameter (IZD) was calculated at the end of the incubation period. Controls were also set up for each experiment using 95% ethanol and 10% DMSO in place of plant extracts.

Determination of minimum inhibitory concentration

Computer generated graphs were obtained using Microsoft Excel software to plot the square of inhibition zone diameter against the logarithm of the concentration. The antilogarithm of the intercept on the log concentration axis gave the minimum inhibitory concentration values (Osadebe and Ukwue, 2004).

Determination of minimum biocidal concentration

The minimum biocidal concentration (MBC) otherwise known as minimum lethal concentration (MLC) was determined using the macro broth method of Shadomy and Espineal - Ingroff (1984). A two fold serial dilution in sterile Sabouraud's Dextrose broth (SDB) was carried out for each concentration of plant extract. Each plant extract was then serially diluted up to the tenth tube.

The eleventh tube served as the control tube. Each tube was then inoculated with 0.2 ml of the standardized inoculum of each test organism and then incubated for 7 days at 28^0C.

Subsequently, each of those tubes showing no growth (without turbidity) were vigorously shaken and 0.1 ml of the SDB withdrawn and introduced onto a freshly prepared SDA plate respectively, and then incubated for 7 days at ambient room temperature (29 – 31^0C). The minimum biocidal concentration (MBC) was the concentration of the extracts in the tubes with the highest dilution that gave no growth on the SDA plates after incubation.

Phytochemical analysis

Phytochemical tests were carried out using standard procedures to identify the constituents as described by Sofowora (1993), Trease and Evans (1989) and Harborne (1973). This was carried out in triplicates.

Test for Tannins

5.0 g of dried extract was stirred with 10 ml of distilled water. This was filtered and ferric chloride reagent was added to the filtrate. A blue-black precipitate was taken as evidence for the presence of tannins.

Test for Saponins

Froth test for saponins was used. 0.5 g of dried extract was shaken vigorously with 10 ml sterile distilled water in a test tube for 30 seconds. It was allowed to stand for 30 minutes. Honeycomb froth indicated the presence of saponins.

Test of Flavonoids

A portion of each powdered plant sample was heated with 10 ml of ethyl acetate over a steam bath for 3 minutes. The mixture was filtered and 4 ml of the filtrate was shaken with 1 ml of dilute ammonia solution. A yellow colouration showed a positive test for flavonoids.

Test for Alkaloids

20 ml of 5% tetraoxosulphate (vi) acid in 96% ethanol was added to 2 g of dried plant extract in a conical flask. This was boiled and filtered. Two drops of Meyer's reagent was added to 2 ml of the filtrate. A yellow precipitate indicated a positive test.

Test for Glycosides

10 ml of 50% H_2SO_4 was added to 10 ml of the extracts in a test tube. The mixture was heated in boiling water for 15 minutes. 10 ml of Fehling's solution was added and the mixture was boiled. A brick-red precipitate indicated a positive test.

Statistical analysis

Bar charts were computer generated using Microsoft Office Excel 2003. Computer generated ANOVA and Fisher's pair wise comparisons were obtained using Microsoft Office Excel 2003 and ezANOVA (v0.98) statistical software.

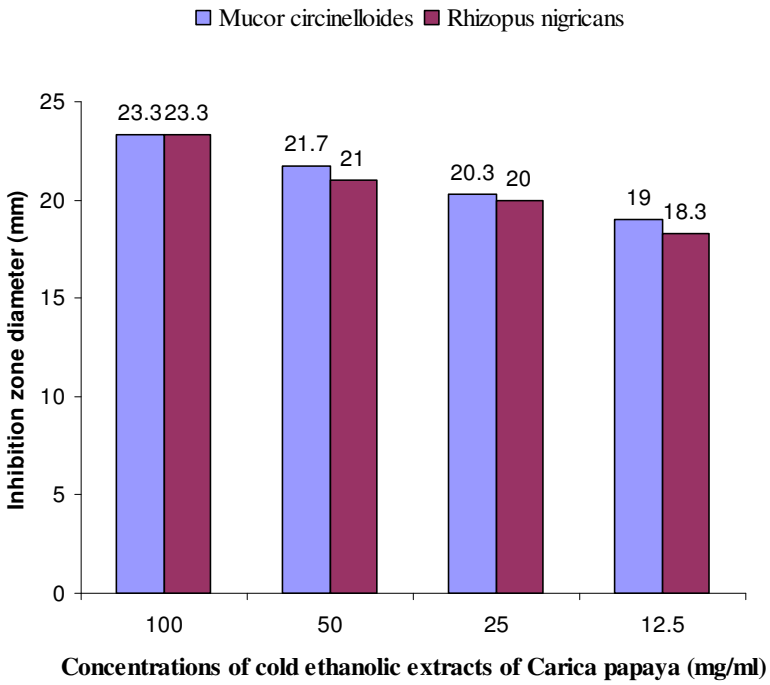

Figure 1 Relative effect of cold ethanolic leaf extracts of *Carica papaya* on *Mucor circinelloides* and *Rhizopus nigricans*

3. RESULTS AND DISCUSSIONS

The relative effects (zones of growth inhibition) of different concentrations of cold and hot ethanolic leaf extracts of *Carica papaya* on *Mucor circinelloides and Rhizopus nigricans* are shown in Figures 1 and 2. It was observed that the higher the concentration of the extract, the higher the zone of inhibition recorded. The ANOVA for the potency of leaf extracts on fungal isolates showed no statistically significant difference ($p>0.05$) between the cold and hot extracts for each concentration tested.

Figure 1 reveals that for the cold ethanolic leaf extract, *Carica papaya* showed inhibitory effects on both *Mucor circinelloides* and *Rhizopus nigricans*. However it was observed that *Mucor circinelloides* was more sensitive to the cold leaf extract than *Rhizopus nigricans*. The highest zone of growth inhibition recorded was 23.3mm for both *Mucor circinelloides* and *Rhizopus nigricans* (100gm/ml concentration). While the lowest zone of inhibition was observed in 12mg/ml concentration as 18.3mm for *Rhizopus nigricans*. The ANOVA for the sensitivity of *Mucor circinelloides* and *Rhizopus nigricans* at each concentration revealed no statistically significant difference ($p>0.05$) between them for the cold extracts. However, different concentrations of the cold extract showed statistically significant difference ($p<0.05$) in their zones of growth inhibition (effect) against *Mucor circinelloides*, and no statistically significant difference ($p>0.05$) in their zones of growth inhibition (effect) against *Rhizopus nigricans*. Figure 2 reveals that for the hot ethanolic leaf extract there was no effect on *Mucor circinelloides*. The highest zone of growth inhibition recorded for *Rhizopus nigricans* was at 100mg/ml (20.7mm), while the lowest zone of growth inhibition recorded was at 12.5mg/ml (17.0mm). The ANOVA for the sensitivities of *Mucor circinelloides* and *Rhizopus nigricans* revealed statistically significant difference ($p<0.05$) between them for the hot extracts. Also, different concentrations of the hot extract produced statistically significant difference ($p<0.05$) in the zones of growth inhibition (effect) against *Rhizopus nigricans*.

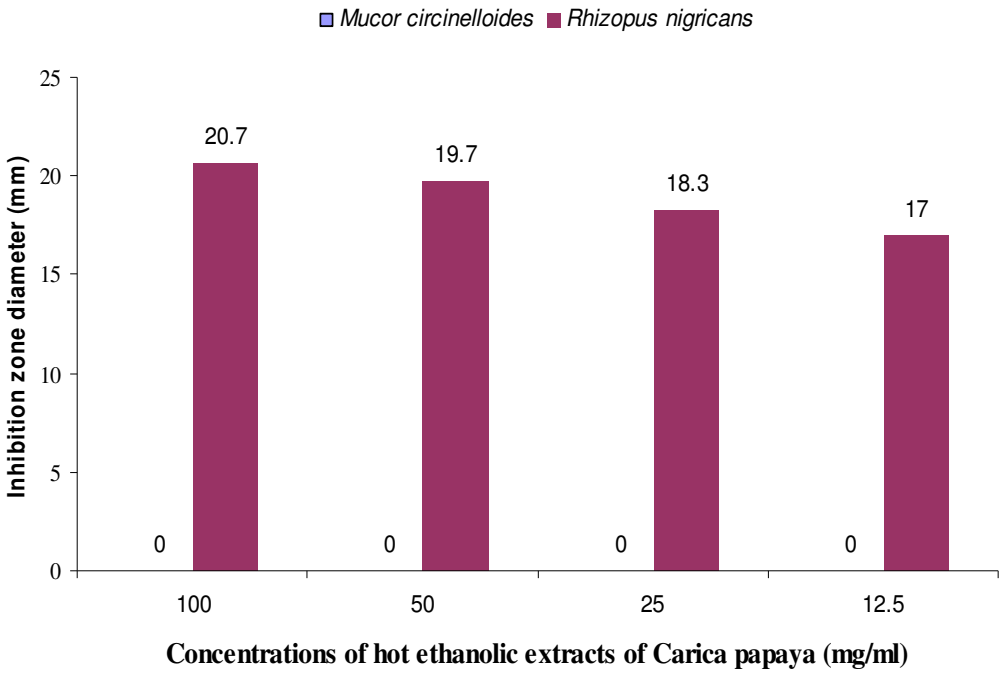

Figure 2 Relative effect of hot ethanolic leaf extracts of *Carica papaya* on *Mucor circinelloides* and *Rhizopus nigricans*.

The minimum inhibitory concentrations of the ethanolic extracts against fungal test isolates are shown in Table 1. Cold extract showed minimum inhibitory concentrations of 0.1mg/ml and 0.06mg/ml for *Mucor circinelloides* and *Rhizopus nigricans* respectively, while the minimum inhibitory concentration of hot extract for *Rhizopus nigricans* was 0.08mg/ml. Hot extract was not potent on *Mucor circinelloides*.

Phytochemical analysis of the leaf extracts of *Carica papaya* revealed the presence of tannins, glycosides, alkaloids, and flavonoids as shown in Table 2.

TABLE 1: MIC of ethanolic extracts on test organisms.

Extracts	Minimum Inhibitory Concentration (mg/ml)	
	Mucor circinelloides	*Rhizopus nigricans*
Carica papaya (C)	0.10	0.06
Carica papaya (H)	-	0.08

(H) = Hot Ethanolic Extract, (C) = Cold Ethanolic Extract.
Data are mean of three replicates in two experiments.

TABLE 2: Phytochemical analysis data of leaf extracts of *Carica papaya*.

Saponins	-
Tannins	+
Glycosides	+
Alkaloids	+
Flavonoids	+
Phenols	-

+ = positive (present)
- = negative (absent)

4. CONCLUSION

The *in vivo* experiments carried out in this study reveal the potential of leaf extracts of *Carica papaya* in controlling post harvest soft rot of yam. Hence this study may provide a basis for a user, cost, and environment friendlier alternative to existing chemical methods. Further experiments may be carried out in order to determine: in vitro effects of these extracts on yam tubers and other tuber crops (cassava, potato, etc), and the specific active principles involved.

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