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Potency of Plant-based Fungicides against a Rot Pathogen of White Yam (*Dioscorea rotundata* Poir)

Markson, Aniedi-Abasi Akpan[1], Omosun, Garuba[2*], Umana, Etim Johnson[1], Amadioha, Anderson Chidi[3] and Udo, Samuel Effiong[4]

[1]Department of Botany, University of Calabar, Calabar, Nigeria.
[2]Department of Plant Science and Biotechnology, Michael Okpara University of Agriculture, Umudike, Nigeria.
[3]Department of Plant Health Management, Michael Okpara University of Agriculture, Umudike, Nigeria.
[4]Department of Biological Sciences, Cross River State University of Technology, Calabar, Nigeria.

Authors' contributions

This work was carried out in collaboration between all authors. Author MAAA designed the study, did the literature search and wrote first draft of the manuscript. Author AAC contributed in the isolation and identification of isolates. Author OG identified and contributed in the preparation of plant extract. Authors MAAA, USE and UEJ did the collection of plant materials, the pathogenicity test and statistical analyses of data. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study assessed the *in vitro* antifungal potential of crude extracts of ten plants on the mycelial dry weight and spore germination of *Botryodiplodia theobromae* using hyphal extension bioassay.
Study Design: The experiment was a 10 x 2 x 6 factorial laid out in a Completely Randomized Design (CRD) and replicated three times. Means were separated using LSD (P<0.05).
Place and Duration of Study: Department of Botany, University of Calabar, Calabar and National Roots Crop Research Institute, Umudike both in Nigeria. The study was carried out between May 2015 and March, 2016.

**Corresponding author: E-mail: omosun.garuba@mouau.edu.ng, omosun@yahoo.co.uk;*

Methodology: The plant materials of *Afromomum melegueta, Zingiber officinale, Allium sativum*, *Cola attiensis, Ocimum gratissimum, Xylopia aethiopica, Piper guineense, Garcina cola, Monodora myristica* and *Senna occidentalis* were extracted in water and ethanol. Infected yams were collected and packaged in sterile polythene bags and taken to the laboratory for isolation of microorganisms and isolates identified. The pathogenicity of the isolates used was confirmed using axenic cultures.
Results: Solvent of extraction and concentration of the extracts exerted significant influence on the performance of the extracts. Water extract treatments of *Aframomum melegueta* and *Zingiber officinale* were the best and comparable ($P<0.05$) in reducing spore germination each recording a value of 97%. The most impressive result obtained for ethanol extracted plant material was a 100% spore reduction value recorded for *Z. officinale* at 100 g/l concentration. Seeds of *Aframomum melegueta* and rhizomes of *Zingiber officinale* extracted in both water and ethanol were superior in reducing the mycelial biomass of *B. theobromae* compared to others. Water extracts of *Aframomum melegueta* and *Zingiber officinale* reduced the mycelial biomass of the test pathogen by 84.7% and 85.7% at 100 g/l concentration respectively. Their performances were comparable ($P<0.05$). Extracts of *Allium sativum* and *Cola attiensis* recorded the least spore germination reduction and mycelial dry weight reduction values irrespective of the solvent of extraction. No growth reduction was evident in the control treatments throughout the course of the study.
Conclusion: All the plant extracts tested inhibited the growth and development of *B. theobromae* to varying degrees. Solvent of extraction and concentrationof the extractsexerted significant influence on the performance of the extracts.

Keywords: Plant based fungicides; rot pathogen; Dioscorea rotundata; white yam.

1. INTRODUCTION

Of the 600 known species of yam, ten are edible, and of the ten, six are cultivated and consumed in Nigeria [1,2]. Among these six species, white yam (*Dioscorea rotundata*) commands the highest market value owing to the superiority and preference of its tubers for food in Nigeria. The tubers have organoleptic qualities that make them the preferred carbohydrate food, hence contributing to 350 dietary calories per person per day for millions of people in the major producing countries [3]. As a valuable and staple food source for millions worldwide, yam is usually eaten directly with sauce after boiling, fried in oil or roasted. It is also boiled and pounded into a thick dough popularly called pounded yam, and eaten with soup. In the Western part of Nigeria, a reconstituted form called 'amala' (another popular dough) is the people's favourite [4]. A few commercial products have been developed from yam flakes and flour in Nigeria. Yam peels are commonly used to feed pigs on family-run farms. Industrially, yam flour mixed with rice is used in making pastries [5]. The medicinal value of yam is a function of its diosgenin content. Diosgenin (a kind of sapogenin) is a prominent source of hemi synthesis of birth control pills (with progesterone and estrogen as well as similar hormones) and corticosteroids [6].

Although yam is credited with a high profile nutritional status, medicinal and industrial uses, its production and effective long term storage have continued to remain a mirage due to a myriad of constraints. One of such is the attack by storage rot pathogens such as fungi and bacteria. The fungal pathogens penetrate through wounds created on the tubers by insects, nematodes, rodents or poor handling during and after harvest and then infect the inner tissues [7].These pathogens have been reported as the cause of about 50% losses of yam tubers. Attacks by *Botryodiplodia spp.* alone on *D. rotundata* have resulted in losses within the range of 20-40% [8]. *Botryodiplodia theobromae* causes dry black rot in yams and many other tuber crops like potato, cocoyam as well as cassava, and is known to survive in infected tissues for over eight months [3,9]. The use of fungicides in pest management have been proven to provide stability of the crop production and increase yield, however, exponential use of these chemicals has resulted in the development of fungicide tolerant pathogen strains and accumulation of fungicide residues in the food chain above safe limits [10,11]. Plants contain secondary metabolites with proven biological activity and their extracts have potentials for effective management of pathogen-incited diseases in plants [12]. Over the years, some workers have explored the anti-microbial potentials of various plant products with great success [13,14,15,16,17]. In an experiment to investigate the fungitoxic activity of extracts from *Azadirachta indica* and *Xylopia aethiopica* on

Colletotrichum lindemuthianum causing anthracnose disease of cowpea, Amadioha and Obi [18] recorded 100% control of the disease with oil extracts of both plant materials and reported a growth inhibition level of above 50% against the test fungus with 50% hot water extract of both plant materials. Hot water extract of *Xylopia aethiopica* was more effective *in vivo* recording over 20% reduction in lesion size compared with 12.6% reduction recorded in the control experiment where a synthetic fungicide – Benomyl was used. The antifungal efficacy of extracts of *Carica papaya*, *Costus afar* and *Ocimum gratissimum*, has been demonstrated against *Aspergillus niger*, *Aspergillus flavus* and *Fusarium oxysporum* isolated from stem bark of *Gmelina arborea* [19]. Aqueous extracts of *Inula viscosa* were shown to exhibit antifungal activity *in vitro* against late blight of potato and tomato, and downy mildew of grape [20,21] and its organic solvent demonstrated antifungal potency against downy mildew in cucumber, powdery mildew in wheat and rust in sunflower [16]. In an earlier study on *B. acerina* isolated from rotting cassava tubers, Amadioha and Markson [12] reported complete inhibition of radial growth and spore germination by aqueous and ethanol extracts of *Piper guineense* and *Aframomum melegueta in vitro,* and extended the shelf life of the tubers by 16 days. Thus, the aim of this study was to screen aqueous and ethanol extracts of ten plants *in vitro* against *B. theobromae* causing dry rot in white yam (*D. rotundata*) in Calabar, Cross River State, Nigeria.

2. MATERIALS AND METHODS

2.1 Collection of Yam Materials

White yam (*D. rotundata*) was used in all the investigations. The yams used were apparently healthy tubers obtained from open market stalls in major yam markets in Calabar Urban, Cross River State,Nigeria. *Dioscorea rotundata* has tubers which are normally cylindrical, with rounded pointed ends, brown skin, white flesh, and woody and inedible head end, and are usually produced singly during tuberization which is initiated after 16 weeks of planting [6].

2.2 Isolation and Identification of Fungi Associated with the Tubers

Rotting yams were collected packaged in sterile polythene bags and taken to the laboratory for isolation. Isolations were made from the infected yam tubers (*Dioscorea rotundata).* The samples were surfaced sterilized with 70% ethanol and cut open. Four pieces (5mm diameter) of the infected tissues were picked with flame-sterilized scalpel and inoculated on the solidified potato dextrose agar (PDA) medium in different plates. The inoculated plates were stored at room temperature (28℃) and observations were made daily for emergence of colonies. Sub-culturing was done to obtain pure cultures of the isolates. The isolates were stored on slants of potato dextrose agar (PDA) in McCartney bottles. Sub cultures were made in Petri dishes using PDA when the need arose. The structures of the isolates were observed under the light microscope (X40 and X100 magnification) and the isolates were identified by making reference to similar structures in atlas of imperfect fungi by Barnett and Hunter [22] and with literature on identification of pathogenic fungi by Rossman et al. [23]. Confirmation was made by comparing with a culture identified by International Mycological Institute, Egham, UK.

2.3 Koch's Postulates and Pathogenicity Test

The pathogenicity of the isolates from the potato tubers was confirmed. Axenic cultures of the isolate were used to inoculate the tubers of white yam with 5- mm-diameter mycelial agar plugs from a 4-day-old culture of the test pathogen. On appearance of symptoms, the tissues at the margins of the healthy and diseased parts were surface-sterilized, excised and plated onto PDA for incubation at 28℃ for four days. At the end of this period, morphological characteristics and growth patterns observed in the plates were compared with the ones of the original isolates [24].

2.4 Preparation of Plant Extracts

Two solvents, ethanol (98%) and distilled water were used in the extraction process. Mature seeds of *A. melegueta, S. occidentalis, G. cola, M. myristica seeds* and *P. guineense* were first sterilized in 70% ethanol for 15 minutes and then washed thoroughly under a running tap water and finally rinsed with distilled water. The seeds were air – dried for 48 hours then separately ground using a sterile mortar and pestle to obtain 1 kg of dry powder. Fresh uninfected leaves of *O. gratissimum* were harvested, surfaced – sterilized with 70% ethanol for 5 minutes and washed with distilled water before air – drying for 1 hour. One kilogram of each sample was ground separately using sterile mortar and pestle to

obtain a paste [12]. Water extracts of seed samples were prepared by adding 250 g of the sample materials (wt./v.) to 250 ml of distilled water in a 500 ml beaker at room temperature (28℃). This was vigorously stirred and allowed to stand for 48 hours. The solution was filtered through four fold of cheese cloth [25]. Water extracts of the leaves of *O. gratissimum* and cloves of *A. sativum* were prepared by adding 250 g of each paste to 250ml (wt./v.) of distilled water in a 500 ml beaker at room temperature (28^oC). The mixture in each case was stirred vigorously and allowed to stand for 24 hours and then filtered through four folds of cheesecloth [25,26]. The seeds of *S. occidentalis* and fruits of *X. aethiopica* were surfaced – sterilized, washed, air – dried for an hour and extracted as above. The filtrate was used as water extract of test plants in the experiments. Ethanol; extracts were similarly prepared except that the extraction solvent was ethanol.

2.5 Effect of Plant Extracts on Mycelial Dry Weight of *B. theobromae in vitro*

The method described by Markson et al. [14] was adopted in this study. Nutrient Broth was the medium used. Twenty-five ml of the medium was dispensed into each of the 100ml conical flasks. Two ml of water or ethanol extracts of the test plants were added to the medium in the conical flask except in the control experiments where only water or ethanol was used in place of the respective extracts. The flasks were inoculated with a disc of *B. theobromae*. The cultures were incubated for ten days at room temperature (28℃) after which the content of each flask was filtered through already weighed sterile filter paper. The filter paper containing the mycelial biomass was dried in an oven set at a temperature of 80℃ for 24 hours. The mycelial biomass of each pathogen was determined by subtracting the weight of the dry filter paper from the weight of the filter paper and mycelial biomass [14]. This experiment was a 10 x 2 x 6 factorial laid out in a Completely Randomized Design (CRD) and replicated three times. Means were separated using LSD ($P<0.05$).

2.6 Effect of Plant Extracts on Spore Germination of *B. theobromae in vitro*

A 2 ml suspension ($5x10^4$ spores per ml distilled water) of an 8 – day old culture of *B. theobromae* was filtered through a sterile cheesecloth and mixed with 2 ml of each extract and dropped on a film of molten medium on a glass slide and incubated in a micro-humidity chamber at room temperature (28℃) for 6 hours. In the control experiment, water or ethanol was mixed with the spore suspension. The number of spores germinated was counted under the light microscope (low power X10 magnifications) after 6 hours [26]. The percentage inhibition of spore germination was calculated thus:

$$\% \text{ inhibition} = \frac{NC - NT}{NC} x \frac{100}{1}$$

Where:

NC = Number of spores germinated in the control.
NT = Number of spores germinated in the treatment.

3. RESULTS AND DISCUSSION

3.1 Identification of Isolates

Following the isolations, two isolates of fungal colonies were identified. On the PDA, colonies of *B. theobromae* were initially white, fluffy and feathery, becoming grey and eventually black. The growth was radial in pattern from the centre of the plate outwards. *R. stolonifer* exhibited profused growth with their whitish thread-like mycelia dotted with grey coloured sporangiospores after three days of growth. Literature on identification of pathogenic fungi [23] and illustrations by Barnett and Hunter [22] corroborate these observations and the appearance of *B. theobromae* fitted the description of *Botryodiplodia* Pat. (=*Lasiodiplodi theobromae* (Pat.) Griff and Maubl.) as presented by Marley [27]. The true identity of each of these fungi was confirmed by comparing their cultures with those identified by International Mycological Institute, Egham, UK.

3.2 Koch's Postulates and Pathogenicity Test

The *B. theobromae* and *R. stolonifer* were pathogenic on the white yam tubers used for each pathogen for the test. Symptoms of decay (rot) caused by *B. theobromae* was seen as dry black rot. *R. stolonifer* produced soft rot symptoms. On re-isolation, the two isolates exhibited similar pattern of growth as observed in the original isolates. However, based on the severity of infection during pathogenicity test, *B. theobromae* was selected and used as the test pathogen throughout the experiment.

3.3 Effect of Plant Extracts on Spore Germination of *Botryodiplodia theobromae*

The result obtained for the effect of water and ethanol extracts of the test plant materials on the spore germination of *B. theobromae* is shown in Figs. 1 and 2 respectively. All the plant extracts used reduced to varying degrees the level of spore germination of *B. theobromae*. Concentration of the extracts and solvent of extraction exerted significant influence on the performance of the extracts. Effect of the extracts (water and ethanol) was concentration-dependent with higher concentrations recording lower level of spore germination. Water extracts of *Garcinia cola* recorded the highest percentage spore reduction (75.5%) at 20 g/l and was significantly different (P<0.05) from all other water extracts tested. This was followed by extracts of *Z. officinale* (71%), extracts of *Piper guineense* (68.5%) and *Aframomum melegueta* (68%) which were not significantly different (P<0.05) in performance. The reduction in spore germination increased with increasing concentration of the extracts, with *A. melegueta* and *Z. officinale* recording as high as 97% and *G. cola* 88% at 100 g/l. Water extracts of *C. attiensis, O. gratissimum, S. occidentalis* and *A. sativum* were not as effective. The least effective was extracts of *C. attiensis* which recorded only 22% reduction in spore germination at 100 g/l. The difference in the capacity of the various plant extracts to reduce spore germination is likely due to the difference in the amount and type of phytochemical compounds contained in them [28].

Some plant materials recorded higher activity when extracted with ethanol. Ethanol extracts of *X. aethiopica, M. myristica, S. occidentalis, A. sativum* and *C. attiensis* were more effective than their respective water extracts. At 20 g/l concentration level, *Z. officinale* recorded the highest reduction in spore germination (83.9%) compared with other plant extracts at this level. This was however not significantly different (P<0.05) from the spore germination inhibition activity shown by *A. melegueta*. This performance was followed by *P. guineense*, *X. aethiopica* and *G. cola*. The efficacy of extracts of *M. myristica* and *A. sativum* were comparable (P<0.05) at this level of concentration. The spore germination reduction capacity of *O. gratissimum* (42.7%), *M. myristica* (41.7%) and *A. sativum* (45.8%) were statistically similar (P<0.05). Extracts of *C. attiensis* was the least effective. At 100 g/l, *Z. officinale* still led in inhibitory efficacy. Extracts of *G. cola* and *X. aethiopica* were not significantly different (P<0.05) in activity. The results above indicate a better performance with ethanol extract treatments compared with water extracts. The superiority of ethanol extracts over that of water is probably a function of the ease of solubility of the active principles in the organic solvent (ethanol) as compared to water as extracting solvent [29]. The superiority of ethanol extracts over water extracts has earlier been reported by Udo, et al. [30] on a study on garlic. They attributed the superiority of ethanol extracts over water extracts to the higher levels of secondary metabolites found in the ethanol extracts following phytochemical screening of both extracts. The exceptional level of efficacy exhibited by extracts of *Z. officinale* and *A. melegueta* may stem from the vast number and amounts of biologically active compounds reported in the essential oils of these plants [31,32,33].

3.4 Effect of Plant Extracts on Mycelial Dry Weight of *Botryodiplodia theobromae*

The effect of water and ethanol extracts from test plants on the mycelial dry weight of *B. theobromae* is shown in Figs. 3 and 4 respectively. Water extract of *A. melegueta* recorded the highest reduction of mycelial dry weight followed by *Z. officinale* and *P. guineense* (Fig. 3). Extracts of *C. attiensis* and *A. sativum* were the least in reducing the growth of the pathogen *in vitro*.

The use of ethanol as a solvent of extraction improved the efficacy of some of the plant materials that could not record impressive performance when they were extracted with water (Fig. 4). Assessment of the mycelial dry weight reduction capacity of the individual plant materials indicated that *A. melegueta* and *Z. officinale* were the best and were comparable. This was followed by *P. guineense* (69.9%). *S. occidentalis* was comparable with *X. aethiopica* in growth reduction of the test fungus. The activity of *O. gratissimum* was significantly different (P<0.05) from that recorded by *X. aethiopica.* The efficacy of other plant materials against the growth of the pathogens was in the descending order; *G. cola, A. sativum* and *C. attiensis*. In all cases, there was no mycelial dry weight reduction recorded in the control experiments.

The efficacy of *A. melegueta, Z. officinale, P. guineense* and *M. myristica* extracts was maintained possibly because these four plant materials are rich in oil compared with others [29]. There is a possibility that the oil component of these materials may have interfered with the normal exchange of substances across the cell membrane of the mycelia of *B. theobromae*. The differences observed may also have been due to the variation in amounts of the active principles in

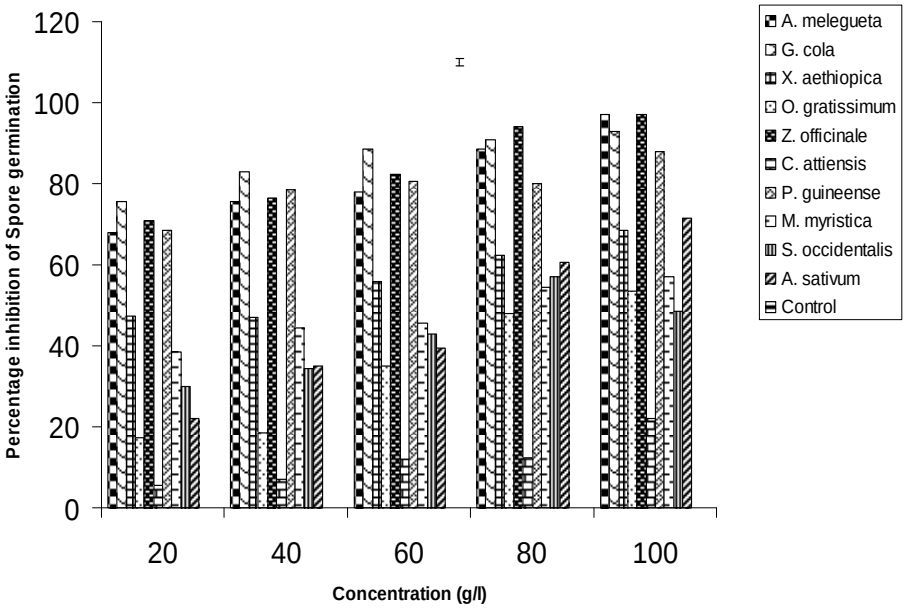

Fig. 1. Effect of water extracts on spore (conidia) germination of *Botryodiplodia theobromae* in culture

Bar represents LSD (P<0.05)

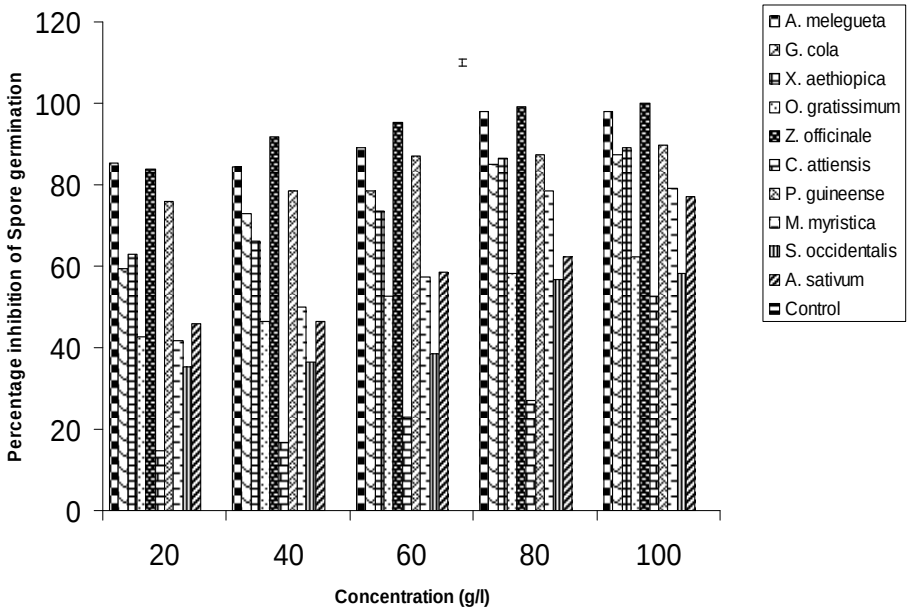

Fig. 2. Effect of ethanol extracts on spore (conidia) germination of *Botryodiplodia theobromae* in culture

Bar represents LSD (P< 0.05)

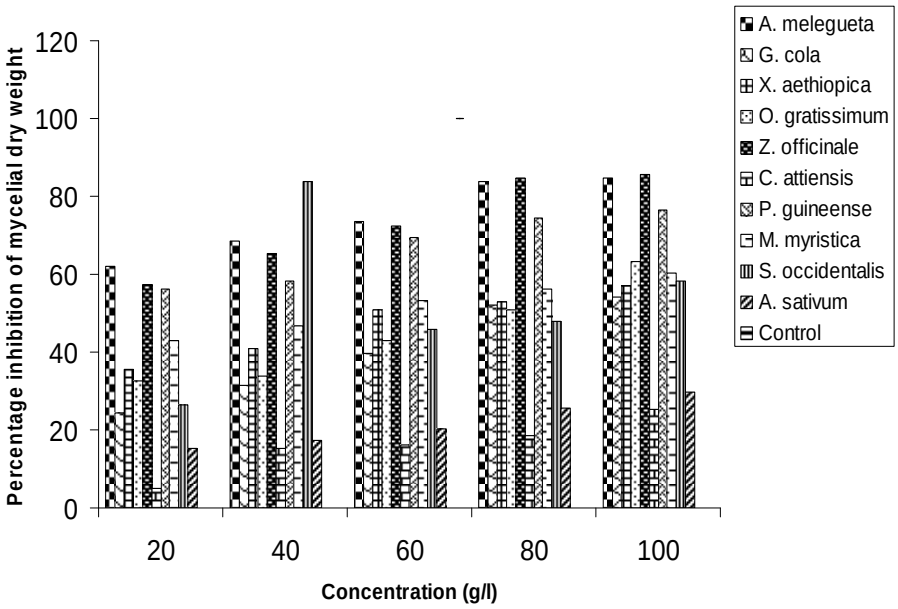

Fig. 3. Effect of water extracts on mycelial dry weight of *Botryodiplodia theobromae*
Bar represents LSD (P< 0.05)

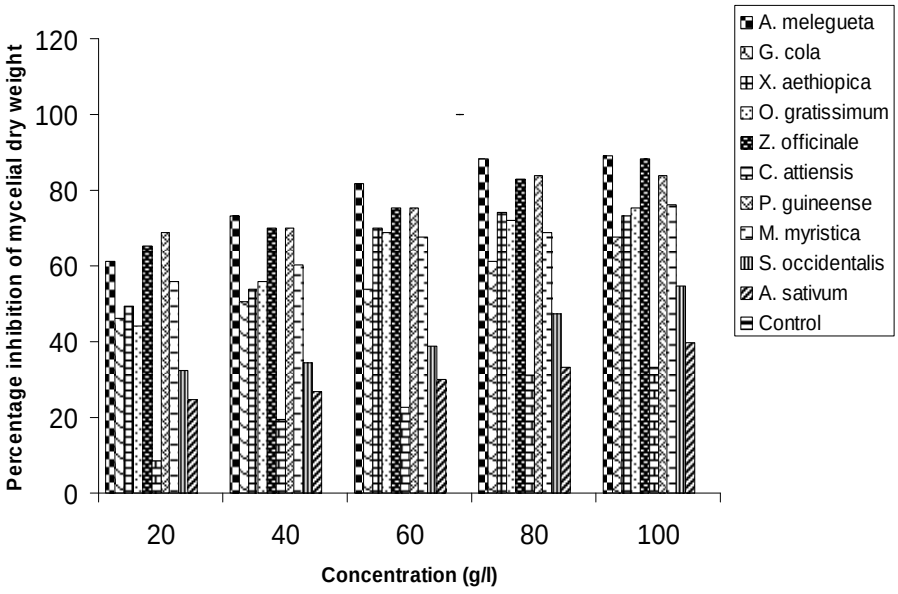

Fig. 4. Effect of ethanol extracts on mycelial dry weight of *Botryodiplodia theobromae*
Bar represents LSD (P< 0.05)

the extracts of these plant materials [30]. The efficacy of the extracts and essential oils of some of these plant materials have severally been demonstrated in many *in vitro* and *in vivo* experiments against fungi [12,18] and insects [31,32]. The result of this study is intended to encourage the use of plant products as safe and environmentally-friendly option for the preservation of yams and other stored products especially, tubers that are prone to attacks by fungal organisms particularly, *Botryodiplodia theobromae* and related stored products pathogens.

4. CONCLUSION

All the plant extracts tested inhibited the growth and development of B. theobromae to varying degrees. Solvent of extraction and concentrationof the extractsexerted significant influence on the performance of the extracts.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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