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Effect of *Moringa oleifera and Vernonia amygdalina* Leaf Extracts against *Aspergillus flavus and Botryodiplodia theobromae* Causing Rot of Cowpea (*Vigna unguiculata* (L.) *Walp*) Seeds

DP Mamkaa and VI Gwa*

Department of Crop Production and Protection, Federal University, Dutsin-Ma, PMB 5001, Katsina State, Nigeria

***Corresponding author:** Gwa VI

igwa@fudutsinma.edu.ng

Department of Crop Production and Protection, Faculty of Agriculture and Agricultural Technology, Federal University, Dutsin-Ma, PMB 5001, Katsina State, Nigeria.

Tel: +234(0)8039357109

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Abstract

Potency of some plant extracts (*Moringa oleifera* and *Vernonia amygdalina*) using three concentrations of hot aqueous plant extracts (5 g/250 mL, 10 g/250 mL and 15 g/250 mL) in culture for inhibition of *Aspergillus flavus* and *Botryodiplodia theobromae* mycelial growth were carried out at the Biological Science Laboratory, Federal University, Dutsin-Ma, Katsina State, Nigeria. Cowpea seeds showing various symptoms of rot were collected from Dutsin-Ma, Darawa and Makera markets and taken to the laboratory for isolation and identification of fungal pathogens. *A. flavus*, *B. theobromae*, *F. oxysporum*, *A. niger* and *Colletotrichum sp.* were isolated, identified and characterized from the pure culture of the fungi. Pathogenicity tests carried out confirmed that the isolates were pathogenic on the healthy cowpea seeds. 5 mL of *M. oleifera* and *V. amygdalina* were each mixed with 15 mL of potato dextrose agar separately on petri dishes and the pathogens were inoculated in the plates and incubated for 4 days. Measurements of mycelial radial growths were undertaken at 1 day interval throughout the period of incubation. The results obtained showed that *M. oleifera* and *V. amygdalina* all posses antifungal compounds that inhibited the growth of *A. flavus* and *B. theobromae* at all the level of concentrations and throughout the period of incubation. There was a significant difference ($P \leq 0.05$) among concentrations when *V. amygdalina* was used. There was, however, no significant difference ($P \leq 0.05$) among concentrations when *M. oleifera* was used. Extract of *V. amygdalina* was considered more effective in managing *A. flavus* compared to *M. oleifera*. Both *M. oleifera* and *V. amygdalina* significantly differed ($P \leq 0.05$) at all levels of concentrations in inhibiting the mycelial growth of *B. theobromae*. It is therefore concluded that both *M. oleifera* and *V. amygdalina* can be formulated at different concentrations and use in the management of *A. flavus* and *B. theobromae* of cowpea seeds.

Keywords: *Moringa oleifera*; *Vernonia amygdalina*; *Aspergillus flavus; Botryodiplodia theobromae; Vigna unguiculata*; Pathogenicity; Inhibition; Antifungal; Efficacy; Seeds

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Introduction

Cowpea (*Vigna unguicualata L. Walp*) belongs to the family Leguminosae and to the genus *Vigna*. The crop originated from Africa and is commonly cultivated in the semi-arid and forest margin tropics and sub-tropics where it is well adapted and probably the most popular grain legume crop in Nigeria. Cowpeas are the second most important food grain legume crop in tropical Africa, the most important being *Phaseolus vulgaris*, the common bean. Nigeria, Niger, Burkina Faso, Uganda and Senegal grow

cowpea for market, but they are widely grown as a subsistence crop for home use in nearly all African countries south of the Sahara. Cowpea is an important food for human beings due to their high amounts of the essential amino acid contained in its protein such as lysine and tryptophan. They also provide feed, forage, hay and silage for livestock and green manure and cover crop for maintaining the productivity of soils. Cowpeas are susceptible to serious damage by insect pest during storage. They are generally not as susceptible to epidemics of diseases as many other grain legumes. The most important diseases of cowpea are: brown blotch caused by *Colletotrichum capsici*, Cowpea wilt caused by *Fusarium oxysporum*, other pathogens of cowpea include; *Aspergillus flavus*, *Aspergillus niger, Brotryodiplodia theobromae, Rhizopus spp.*, scab (*sphuceloma spp.*) and septotial leaf spot (*Septoria spp.*) [1]. The control of seed-borne pathogens through the application of chemicals for seed dressing has been effective in reducing seed-borne pathogens and even improved the germination ability of seeds [2]. However, there is fear about the safety of chemical residues and the possibility of human toxicity and environmental pollution [3]. Chemical pesticides are also non-biodegradable and extremely toxic [4,5]. On the other hand, pesticides of plant origin are specific biodegradable, cheap, readily available and environmentally safe than synthetic chemicals. Hence, plant extracts could be an alternative to toxic fungicides for controlling plant pathogens [6-8]. The research focused on the management of cowpea seed pathogens isolated from different locations in Dutsin-Ma local government area of Katsina State, Nigeria, using *Moringa oleifera* and *Vernonia amygdalina* leaf extracts.

Materials and Methods

The study area

The study area lies on latitude 12° 27' 18'' N and longitude 07° 29' 29'' E. The experiment was conducted at the Biological Science Laboratory, Federal University Dutsin-Ma, Katsina state, Nigeria.

Collection of rotted and healthy cowpea seeds

Cowpea (*Vigna unguiculata*) seeds showing various degrees of rot symptoms of fungi organisms were collected from three locations of Darawa, Dutsin-Ma and Makera. The fungi organisms in the cowpea seeds were isolated and subsequently identified in the Biological Science Laboratory, Federal University Dutsin-Ma, Katsina state, Nigeria. The healthy cowpea seeds were used for pathogenicity test of the isolated fungi organisms.

Preparation of potato dextrose agar (PDA)

Potato dextrose agar (PDA) was prepared by measuring 10 g PDA and dissolving into 250 ml of distilled water using measuring cylinder and was autoclaved at 121°C for 15 minutes, the media was allowed to cool at room temperature to 45°C. 0.16 g/L streptomycin sulphate powder was added to suppress bacterial contaminations [9,10]. 15 ml of the molten PDA was poured into sterile glass petri dishes of 9 mm diameter and were allowed to cool at room temperature before inoculation of the test fungi.

Isolation of test fungi organisms

Rotted and diseased cowpea seeds were collected and firstly sterilized by dipping completely in a concentration of 5% sodium hypochlorite solution for 1 min. The seeds were then removed and rinsed in four successive changes of sterile distilled water. 0.16 g of streptomycin was added to suppress the growth of bacteria. The medium was thereafter poured in 9 cm sterile glass petri dishes before inoculation of the rotted cowpea seeds.

Inoculation: The infected cowpea seeds were transferred onto solidified potato dextrose agar (PDA) medium in petri dishes. Up to four seeds of cowpea seed were placed on each PDA plates and incubated at ambient room temperature for 7 days. The plates were examined daily for the development of fungal growth.

Identification of fungal growth: The mycelial growths of the fungal organisms that grew on PDA were used to sub-culture in order to obtain pure cultures. Sterilized inoculation needle was used to transfer the mycelial growth into sterile PDA plates. The inoculated plates were then incubated at ambient room temperature (30 ± 5°C) for 5 days. The pure isolates were kept in slants and stored for characterization and pathogenicity test. Macroscopic examination as well as microscopic and morphological characteristics and identification of fungi organisms were made and compared with other authorities [11].

Pathogenicity test

Healthy cowpea seeds were collected and thoroughly washed in 5% sodium hypochlorite solution and rinsed in sterile distilled water for three consecutive times [12]. About 20 ml of distilled water was mixed separately with 5 mm disc from 5 days old culture of *A. niger, B. theobromae and A. flavus* that were isolated from rotted cowpea seeds. The solutions made of the fungi organisms were spread on filter paper contained in petri dishes and the healthy cowpea seeds were inoculated on the fungi blot paper in the petri dishes. The same procedure was used for the control except that the blot paper was spread with distilled water instead of discs of fungi organisms and cowpea seeds were inoculated accordingly. The plates were incubated for 5 days to give enough time for maturity and growth of the fungus. When growth was fully established, the growth characteristics were compared with the original rotted cowpea seeds and examined for infection and disease development.

Preparation of plant extracts

Plants extracts were prepared according to the method of Gwa and Akombo with little modifications [13]. Extracts were obtained by addition of powder of 5 g, 10 g, and 15 g of each plant extract to 250 ml of sterile distilled water separately in 1000 ml Pyrex flask. These were left for 24 hours and subsequently filtered through four fold of sterile filter cloth. The filtrates obtained were used as the plant extracts in the experiment. About 5 ml of the extracts of plant at each level of concentration was mixed in sterile petri dishes containing 15 ml of PDA solution and allowed to solidify before inoculation of the pathogens.

Measurement of mycelial radial growth: The efficacies of the aqueous plant extracts and chemical fungicide were tested *in vitro* for their fungicidal activity against the cowpea (*V. unguiculata*) fungi pathogens caused by *A. flavus, B. theobromae and A. niger*. Three plates were treated with extract of each plant at each concentration. The control experiments had 5 ml of distilled water added to PDA in place of plant extracts respectively. The treatments and control were incubated for four days at ambient room temperature and measurement of growth as radius of a growing fungal colony were undertaken at intervals of one day for four days using a transparent ruler. The absence of growth in any of the plates was indication of the potency of the extract and the chemical fungicide against the test fungi. Fungitoxicity was determined as percent growth inhibition (PGI) according to the method described by Korsten and De Jager [14].

$$PGI(\%) = \frac{R - R_1}{R} \times 100$$

Where,

PGI=Percent Growth Inhibition

R=The distance (measured in mm) from the point of inoculation to the colony margin in control plate,

R_1=The distance of fungal growth from the point of inoculation to the colony margin in treated plate.

Experimental design and data analysis

Data collected were subjected to Analysis of Variance (ANOVA) using GenStat Discovery Edition 12 for ANOVA and means separation, Minitab Release 17 for descriptive statistics and Graph Pad Prism 6 for trend graphs. Statistical F-tests were evaluated at $P \leq 0.05$. Differences among treatment means for each measured parameter were separated using Fisher's Least Significant Difference (FLSD).

Results

Isolation and identification of fungal organisms

The fungal organisms isolated and identified from the rotted cowpea seeds were *Aspergillus niger*, *Aspergillus flavus, Botryodiplodia theobromae, Fusarium oxysporum* and *Colletotrichum spp*. Results presented in **Figure 1** show the culture characteristics of some of the isolated and identified fungi organisms.

Frequency of occurrence of fungal pathogens isolated from cowpea seeds at different locations

Results of the isolation of fungi organisms in **Table 1** show the mean frequency of the fungi organisms in different locations. *F. oxysporum* occurred most in Darawa and Makera (1.66) but least in Dutsin-Ma (1.00). *B. theobromae* however showed more occurrences in Dutsin-Ma (3.66) compared with Darawa and Makera. The occurrence of *A. flavus* was highest in Darawa and Dutsin-Ma (3.00) and least in Makera (2.66). The frequency of occurrence of *A. niger* was high in all the locations but highest in Makera (3.67) and lowest in Darawa (3.00) and Dutsin-Ma (3.00) respectively. *Colletotricum spp* was only isolated in Dutsin-Ma (0.33) location. There were no significant differences ($P \leq 0.05$) between locations for each of the fungal organisms isolated.

Number of fungal pathogens occurring at different locations

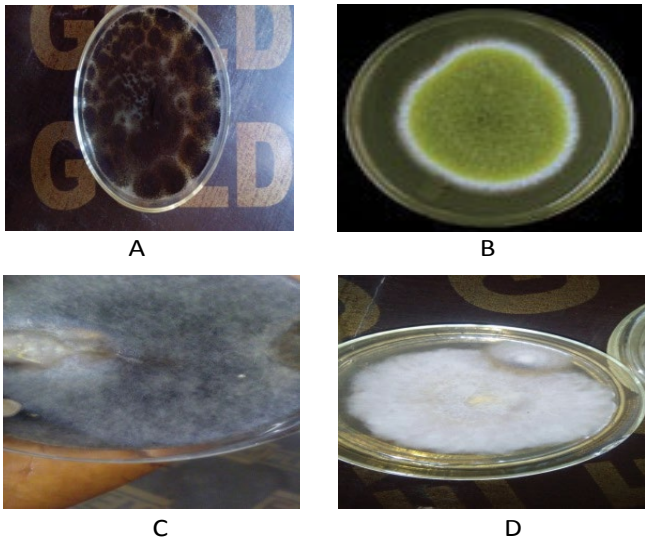
Result presented in **Figure 2** shows the different number of fungal organisms isolated from different locations. The result showed that the sum total of the fungi organisms isolated in three different locations were 13 (*F. oxysporum*), 22 *(B. theobromae)*, 26 (*A. flavus)*, 29 (*A. niger)* and 1 (*Colletotricum sp)*.

Pathogenicity test

Pathogenicity test revealed that healthy cowpea seeds showed symptoms of rot after 5 days of inoculation with fungi mycelial. Morphological and microscopic characteristics were compared with initial cultures after re-isolation and were found to be same. The control (healthy cowpea seeds inoculated without fungi mycelial) showed no symptoms of rot.

Effect of *Moringa oleifera* and *Vernonia amygdalina* extracts on mycelial growth inhibition of *A. flavus* and *B. theobromae*

Results obtained in **Table 2** shows that the different concentration



A B C D

Figure 1 Culture of (A) *Aspergillus niger* (B) *Aspergillus flavus* (C) *Botryodiplodia theobromea* and (D) *Fusarium oxysporum*.

Table 1 Mean frequency of occurrence of fungal pathogens from cowpea seeds at different locations.

Fungi	Locations			
	Darawa	Dutsin-Ma	Makera	P-Value
F. oxsporum	1.66 ± 0.58	1.00 ± 0.57	1.66 ± 0.33	0.70^{ns}
B. theobromae	2.00 ± 0.00	3.66 ± 0.33	1.67 ± 1.20	0.19^{ns}
A. flavus	3.00 ± 0.57	3.00 ± 1.53	2.66 ± 0.66	0.96^{ns}
C. gloesporiodes	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00	0.42^{ns}
A. niger	3.00 ± 1.00	3.00 ± 1.00	3.67 ± 1.20	0.88^{ns}
Colletotrichum sp.	0.00 ± 0.00	0.33 ± 0.33	0.00 ± 0.00	0.42^{ns}

Means on the same row with ns are not statistically significant ($P \leq 0.05$); ns=not significant

of *M. oleifera* and *V. amygdalina* show variation in effect on the fungi organisms tested. *Moringa* showed mycelial inhibition of 3.75 cm at 15 g/250 ml better than at 10 g/250 ml and 5 g/250 ml and there was no significant difference (P ≤ 0.05) between the concentrations tested. However, there were significant differences among concentrations when bitter leaf extract was used for the control of *A. flavus in vitro*. The result also showed that there was a significant difference (P ≤ 0.05) in activities of *M. oleifera* and *V. amygdalina* extracts in controlling *B. theobromae* at different concentrations with the highest level of inhibition recorded at 15 g/250 ml, followed by 10 g/250 ml and least in 5 g/250 ml respectively.

This work shows that the extract of *V. amygdalina* was more potent in growth inhibition of *A. flavus* compared with *M. oleifera.* On the other hand, the efficacy of *Moringa oleifera* was observed to inhibit the growth of *B. theobromae* more compared with *V. amygdalina.* Mean percentage growth inhibition of *M. oleifera and V. amygdalina* on *B. theobromae* at different concentrations showed a statistical significant (P ≤ 0.05). There was no significant difference (P ≤ 0.05) between concentrations and control (without Moringa extract) in controlling *A. flavus* when *M. oleifera* extract was used.

The results in **Table 3** compare the inhibition of *A. flavus* and *B. theobromae* using crude extracts of *M. oleifera, V. amygdalina*

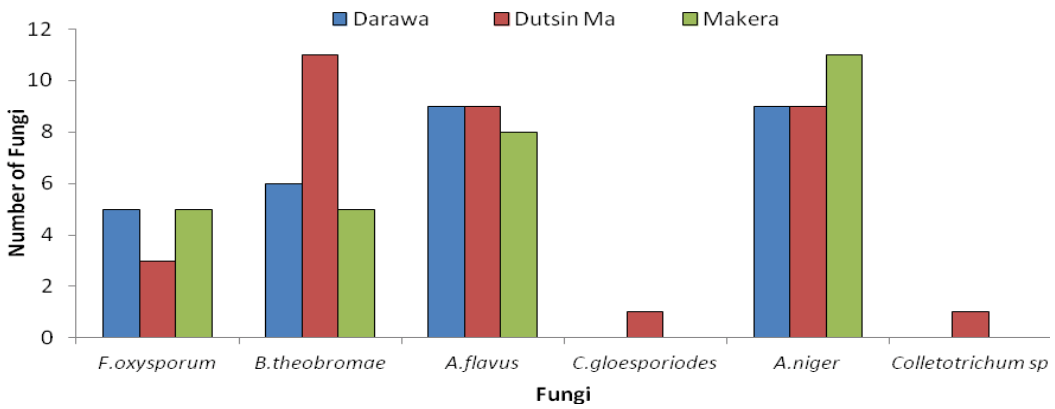


Figure 2 Number of fungal organisms isolated from different locations.

Table 2 *In vitro* mycelial growth inhibition of *A. flavus* and *B. theobromae* at different concentrations of plant extracts with control.

Pathogen/Plant extract	Treatments and mycelial growth inhibition				P-Value
	5 g/250 ml	10 g/250 ml	15 g/250 ml	Control	
A. flavus					
M. oleifera	5.37 ± 0.45	4.66 ± 0.47	3.75 ± 0.35	5.41 ± 0.95	0.18^{ns}
V. amygdalina	4.41 ± 0.36^{b}	3.41 ± 0.30^{bc}	2.70 ± 0.32^{c}	8.42 ± 1.00^{a}	0.00
B. theobromae					
M. oleifera	4.12 ± 0.50^{b}	2.95 ± 0.37^{b}	2.04 ± 0.39^{b}	7.38 ± 1.77^{a}	0.00
V. amygdalina	5.66 ± 0.72^{b}	3.66 ± 0.52^{b}	2.33 ± 0.37^{b}	8.55 ± 2.47^{a}	0.00
Means on the same row with different superscript are statistically significant (P ≤ 0.05); ns=not significant					

Table 3 *In vitro* comparative growth inhibition of *A. flavus* and *B. theobromae* using *M. oleifera* and *V. amygdalina* at different concentrations.

Treatment	Plant extract and growth inhibition		Df	T-Value	P-value
	M. oleifera	*V. amygdalina*			
A. flavus					
5 g	5.37 ± 0.45	4.41 ± 0.36	21	1.63	0.11
10 g	4.66 ± 0.47	3.41 ± 0.30	18	2.24	0.03*
15 g	3.75 ± 0.35	2.70 ± 0.32	21	2.19	0.04*
Control	5.41 ± 0.96	8.42 ± 1.00	21	-2.16	0.04*
B. theobromae					
5 g	4.12 ± 0.50	5.66 ± 0.73	19	-1.73	0.10
10 g	2.96 ± 0.37	3.66 ± 0.52	19	-1.10	0.28
15 g	2.04 ± 0.39	2.33 ± 0.37	21	-0.54	0.59
Control	7.38 ± 1.80	8.55 ± 2.50	19	-1.60	0.12
* indicates statistical significance at 0.05%					

at each concentration. The result revealed that there was no significant difference (P ≤ 0.05) between *Moringa* and bitter leaf extracts at 5 g/250 ml in controlling *A. flavus*. However, there were significant differences (P ≤ 0.05) in the activities of the extracts at 10 g/250 ml, 15 g/250 ml and control in inhibiting the mycelia growth of *A. flavus.* When *M. oleifera* and *V. amygdalina* were compared at each level of concentration as well as the control. There was no significant difference (P ≤ 0.05) in efficacy of the extracts in inhibiting the mycelia growth of *B. theobromae*. **Figure 3** shows the inhibitory effect of plant extracts on mycelia growth of *B. theobromae* for the period of four days.

Discussion

Fungal pathogens are commonly associated with rot of cowpea seeds similar to the work of Nyaka that isolated seven pathogenic fungi organisms responsible for cowpea seed rot disease. These pathogenic fungi included *Colletotrichum sp., Fusarium sp*., *Pestalotia sp*., *Geotrichum sp.*, *A. flavus, A. niger* and *B. theobromae.* Magdalena et al. observed damages caused by *Colletotrichum sp*. on cowpea similar to the work of Akinbode et al. who earlier observed the growth of *Colletotrichum sp.* on cowpea seeds.

The result obtained showed that all the leaf extracts at their different level of concentrations tested were effective in inhibiting the growth of seed-borne fungi pathogens of cowpea, however, the rate of growth was influenced by the type of the extracts used and the concentration. This result is in agreement with Domenico that described the antifungal activity of some plant extracts on the development of *Fusarium oxysporum* f. sp. *lycopersici* and selected the best extracts to be tested as phytofungicide to control crop diseases, with the ultimate goal of developing a green alternative to synthetic fungicides. Using the conidia germination assay, of the 24 plant extracts tested, 15 reduced conidia germination and 6 completely inhibited conidia germination. Extracts of *Rivina humulis, Brassica carinata, Brunfelsia calyicina, Salvia guaranitica* and *Punica granatum* showed good antifungal activity.

Gwa and Akombo observed that *Piper nigrum*, *Zingiber officinale, A. indica, C. papaya* and *N. tabacum* have significant effect (P ≤ 0.05) on mycelial growth of *A. flavus in vitro*. The authors showed that period of incubation and concentration influenced the efficacy of the extracts on mycelial growth of *A. flavus in vitro*. Sangoyomi et al. demonstrated the fungitoxic effect of extracts obtained from *Allium sativum, Ocimum gratissimum, Cassia alata, Azadiracta*

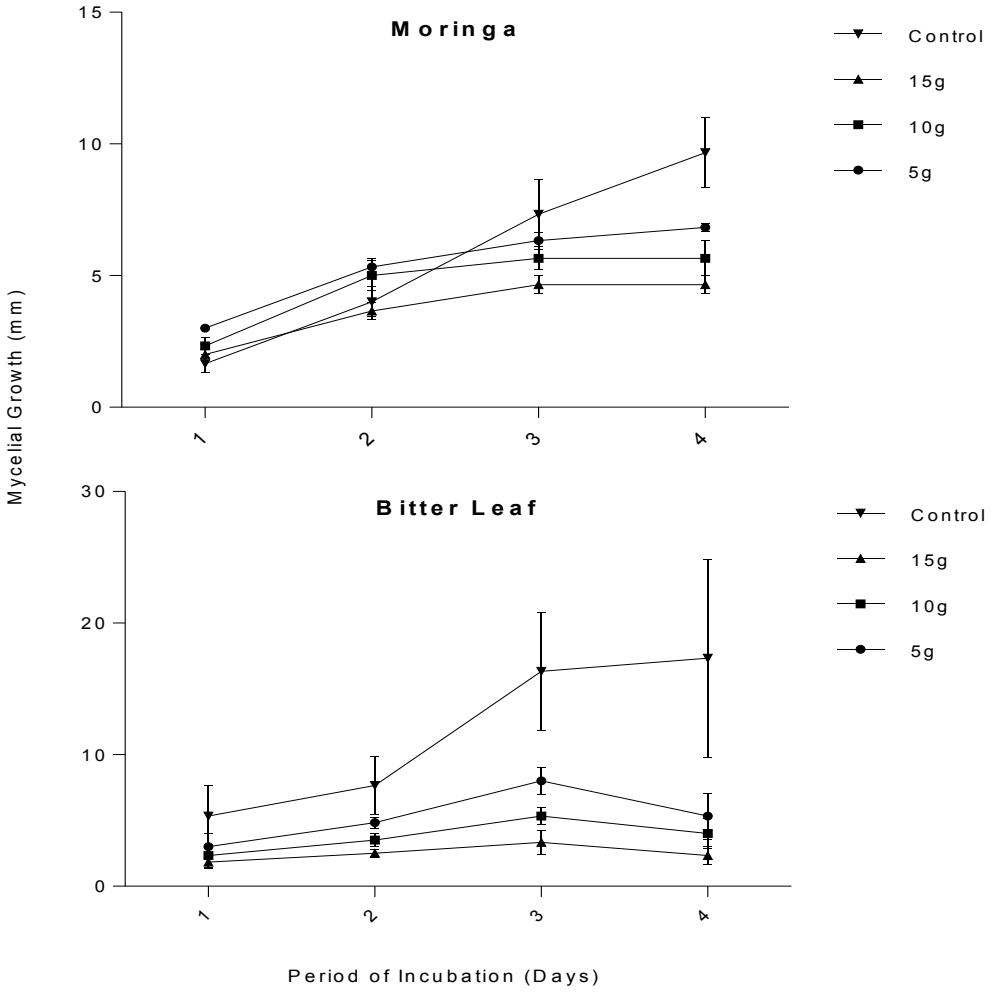


Figure 3 Growth inhibition of *B. theobromae* using moringa and bitter leaf extracts after four days of incubation.

indica, and *Hibiscus rosasinensis*. They showed that the extracts were able to inhibit mycelial growth and reduce production of conidia in the four major fungi associated with yam rot during storage. *M. oleifera* and *V. amygdalina* extracts when used at low concentrations were found to be effective against the growth of fungi organisms. There was a general increase in percentage growth inhibition with an increase in extract concentration. This finding agree with the work of Ghangaonkar, who observed that extracts of *Polyalthia longifolia, Annonas quamosa and Tridax procumbens* were found to be inhibitory for the growth of *Aspergillus flavus, Aspergillus niger, Fusarium oxysporum* and the mycelial growth of *Cladosporium allii* on cowpea seed. Cook and Baker revealed that the antifungal activities of the *V. amygdalina* inhibited the growth of *B. theobromae* and *A. flavus*. Sobowale et al. showed that *M. oleifera* retarded the maximum growth of *B. theobromae* 41% followed by *Aspergillus flavus* 29%, *Fusarium oxysporum 28%* and less effective against *A. niger* 11%. *Polyalthia longifolia* extract inhibited the fungi, *Penicillium digitatum* 50% followed by *Botrytis cinera 45%* over control [15-25].

Conclusion

Aspergillus niger, *Aspergillus flavus, Botryodiplodia theobromae, Fusarium oxysporum* and *Colletotrichum sp* are responsible for rot of cowpea seeds in Dutsin-Ma local government area of Katsina State. This reduced the seed quality and seed health of cowpea resulting in poor viability of seeds annually. Efforts should be made to increase the seed quality by using extracts of plant origin to control fungal pathogens of cowpea. The crude extracts of *M. oleifera* and *V. amygdalina* have the potency of controlling fungal pathogens of cowpea. Extract of *V. amygdalina* is the most effective extract in controlling *A. flavus* at all level of concentrations compared with *M. oleifera.* Both extracts at all level showed high efficiency in controlling *B. theobromae in vitro.* It is therefore concluded that both extracts could be formulated at the respective levels and used in the management of fungi pathogens of cowpea seeds.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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