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Chemical Composition and *in Vitro* Antifungal Activity Screening of Seed and Leaf Extracts from *Aframomum meleguata* and *Monodora myristica* against *Sclertium rolfsii* of Cowpea Plant (*Vigna unguiculata* L. Walp)

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ABSTRACT

Phytochemical studies of *Aframomum meleguata* and *Monodora myristria* seed and leaf extracts revealed the presence of bioactive compounds comprising alkaloids (0.29–5.64%), flavonoids (0.12–8.29%), saponins (0.02–1.24%), tannins (0.03–0.39%), and phenols (0.02–0.39%). The growth of *Sclerotium rolfsii*, which causes basal stem rot in cowpea (*Vigna unguiculata*) and other crops such as tomatoes and tobacco, was inhibited *in vitro* by the extracts of both plants. The extracts from *A. meleguata* seed, *M. myristica* and *A. meleguata* leaves and *M. myristica* seed showed 86.65, 53.19, 43.47 and 39.31% inhibition, respectively. Analysis of chemical composition through infrared spectroscopy showed that the most active phytoconstituents are aldehydes, ketones, amines and phenolic compounds contained in *A. meleguata* seeds. The fungitoxicity of the extracts from *A. meleguata* seed was higher (86.65%) than that of benomyl (86.20%), a synthetic fungicide.

Keywords: alkaloid, antifungal property, inhibition, natural fungicide, phenolic compound

INTRODUCTION

In Nigeria, many plant extracts have been used successfully to control plant diseases. Numerous natural products of plant origin are pesticidal and have the potential to prevent crop fungal diseases. Extracts of medicinal plants contain toxic phytoconstituents which have the potential for use in the development of natural fungicides (Saxena and Kidiavai 1997; Okwu *et al.* 2007). Although the use of synthetic fungicides has helped to increase yield, one of the major problems with the constant use of synthetic chemicals is their resistance to target organisms (Okigbo and Ikediuwa 2000; Okigbo 2003, 2004, 2005). Synthetic chemicals can cause death through poisoning, accumulate in organisms or concentrate in food chains (Taiga and Olufolaji 2008). There is a need for more environmentally safe and more selective, efficacious fungicides. Most commercially successful fungicides have been discovered by screening natural products of plant origin for fungicide properties (Duke 2000). Considerable efforts have been directed and devoted towards screening plants in order to develop new natural fungicides as alternatives to the existing synthetic ones which are associated with problems such as phytotoxicity, vertebrate toxicity, pest resistance and resurgence, widespread environmental hazards and high cost (Okwu 2003; Okwu *et al.* 2007).

Aframomum meleguata (Zingiberaceae, ginger family) is commonly known as guinea pepper, meleguata pepper, alligator pepper, guinea grains or grains of paradise. It is a herbaceous perennial plant, native to swampy habitats along the West African Coast (Iwu *et al.* 1999). Its trumpet-shaped purple flowers develop into 5 to 7 cm long pods containing reddish-brown seeds. The seeds have a pungent, peppery taste due to aromatic ketones such as gingerol and paradol (Iwu 1993).

A. meleguata is used as a fungicide. Antifungal effects of the leaf extract of *A. meleguata* on spore germination and mycelial reduction of the most occurring fungal pathogens causing soft rots of white yam (*Disoscorea rotundata*) tuber have been investigated (Okigbo and Ogbonnaya 2006). Fungi isolated from rotten yams were *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Rhizopus stolo-niger*, *Botryodiplodia theobromae* and *Penicillum chrysogenum.* The ethanolic leaf extract followed by cold water and hot water extracts was most effective on these pathogens (Okigbo and Ogbonnaya 2006).

A. meleguata is very effective against major disease-causing microorganisms such as *Esherichia coli*, *Candida albicans*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Vibrio cholera*, *Salmonella* spp., *Streptococcus* sp. and *Neisseria gonorrhea* (Iwu *et al.* 1999). The plant has been extensively used in herbal medicine not only for its oxytocic, analgesic, anti-inflammatory and antimicrobial properties but also provides relief in the treatment of human gastrointestinal, hypermobility and peptic ulceration (Gill 1992).

Monodora myristica (Jamaican nutmeg, African nutmeg, false nutmeg, calabash nutmeg; (Iwu 1993) is a tree that belongs to the Annonaceae family and that commonly grows in the rainforest zone of West Africa (Keay 1989). It grows up to 30 m high with dense forge and a spreading crown. It produces soft-ball sized fruits with edible seeds that have a nutmeg-like flavour. The pulp is white and contains numerous seeds of about 2.5 cm long. Fruiting occurs from August to November (Agoha 1974). The monoterpenoid content of *M. myristica* is mainly hydrocarbons comprising 0.11% phellandrene, 0.83% α-pinene, 0.84% limonene, 0.40% myrcene and thujene 0.22% (Okwu 2001). The essential oil is associated in the seeds with solid fat. The oil contains 0.83% α-pinene, 0.10% camphene, geraniol 0.34% and 0.42% eugenol (Okwu 2001). The presence of bioactive compounds in the plant makes it possible for the seeds to be used in traditional medicine as well as a spice in local foods

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(Okwu and Ibeawuchi 2005). The oil has antimicrobial activity against *Bacillus subtilis*, *Candida albicans* and *Staphyloccus aureus* (Odoh *et al.* 2004).

Cowpea (*Vigna unguiculata*) is a dicotyledonous plant which belongs to the family Fabacease. It originated from Africa and become an integral part of traditional cropping systems through out Africa (Singh *et al.* 1997). Cowpea is an important legume of the tropics, with its multipurpose utilization as grains in processed food, as vegetable (fresh leaves, peas and pods) and as dry haulms and fodder (Padmlosi and Ng 1991). It is an inexpensive source of vegetable protein and a hardy crop well adapted to relatively dry environment. However, cowpea is affected by several diseases. Every stage in the life cycle of cowpea has at least one major fungal disease that could cause serious damage and reduced yield. Cowpea diseases induced by various pathogenic groups (fungi, bacteria, viruses, nematodes and parasitic flower plants) constitute one of the most important constraints to cowpea production in all agro-ecological zones where the crop is grown (Fatokun *et al.* 2002). Some of the diseases of cowpea include black leaf spot or leaf smut caused by *Protomycopsis phaseoli*, Fusarium wilt caused by *Fusarium oxysporum*, Phytopthora stem rot caused by *Phytophythora vignal*, and Pythium soft stem rot caused by *Pythium aphania dermatum* and *Sclerotium rolfsii* (Fatokun *et al.* 2002). The latter is a fungus that causes basal stem rot in cowpea and other Solanaceous plants such as tomatoes (*Lycopersican esculentum*) and tobacco (*Nicotina tabacum*). The basal stem rot disease caused by this fungus is a major constraint on vegetable production in the agro-ecological savanna zone of Nigeria (Wokocha 1987). The affected plant turns yellow and the leaves die very easily. The stalks from the ground surface dry up with the development of white mycelial web over the diseased area. A dark-brown *Sclerotia* becomes noticeable around the affected areas (Wokocha 1987). The average grain yield in farmers' fields is generally reduced due to these diseases. Farmers especially those in the dry savanna area resort to harvesting the fodder in order to get some income (Fatokun 1991). There is no doubt that farmers' get more financial benefits from cowpea grains than from fodder. It is necessary to explore the possibility of minimizing the mortality of this crop through the utilization of plants' extracts.

In spite of the various uses of *A. meleguata* and *M. myristica* in herbal medicine in Nigeria and as spices in food and drug production, the phytoconstituents of these plants have not been fully documented. The present study was undertaken to evaluate the secondary metabolites constituents and consequently assess the extracts from these plants as low cost fungicide for peasant farmers.

MATERIALS AND METHODS

General experimental procedure

IR spectra were determined on a Thermo Nicollet Nexus 470 FT-IR spectrometer. Column chromatography was carried out with silica gel (200–300 mesh) and to monitor the preparative separations, analytical thin layer chromatography (TLC) was performed at room temperature on precoated 0.25 mm thick silica gel 60 F_{254} aluminum plates 20 × 20 cm Merck, Darmstadi, Germany.

Plant materials

The experiment was carried out in the Department of Chemistry and Plant Health Management Laboratories, Michael Okpara University of Agriculture Umudike, Nigeria in March 2007. Fresh leaves and seeds of *A. meleguata* and *M. myristica*, Voucher Nos. AF/3355 and MS/3356, respectively, were collected from Ibekuta Ibeku, Okwuato Aboh Mbaise Local Government of Imo State Nigeria. They were botanically identified by Dr. A. Nmeregini of the Taxanomy Unit of the Department of Forestry of this University.

Preparation of plant extracts

The leaves and seeds of *A. meleguata* and *M. myristica* were air-dried on the laboratory bench for 10 days and then ground into a uniform powder using Thomas Wiley Mill machine (Model Ed-5 USA). The powdered materials (950 g powder for each sample) were stored in air-tight bottles for chemical analysis. The powder plant samples (500 g) each were separately packed into a Soxhlet apparatus (2 L) and extracted exhaustively with 1000 ml chloroform for 24 h. The chloroform was evaporated using a water bath and then left overnight at laboratory temperature for evaporation of the remaining chloroform. The yield obtained were dark brown oil (10.9 g) for *A. meleguata* leaf and (13.50 g) for *A. meleguata* seeds while *M. myristica* seed afforded (15.6 g) yellow oil and the leaf produced (8.60 g) dark green oil. The column chromatography of the extracts was carried out using the chloroform fractions. The column was packed with silica gel and eluted with methanol: chloroform: petroleum ether (20: 30: 50) to get a light brown oil (0.86 mg), $R_f = 0.4667$ for *A. meleguata* leaf; orange oil (0.98 mg), $R_f = 0.5333$ for *A. meleguata* seed; light brown oil (0.76 mg), $R_f = 0.7586$ for *M. myristica* leaf; light yellow oil (1.20 mg), $R_f = 0.5667$ for *M. myristica* seed. The infrared result is shown in **Table 2**.

Extract for antifungal activity test

The chloroform extracts were collected and the test solution of each extract was prepared by dissolving 10 g of crude plant extract separately in 100 ml sterile distilled water in a 250 ml Erlenmeyer flask in a water bath at 80°C for 2 h. Extracts were subsequently filtered through four folds of cheese cloth.

Isolation of the inoculum

An infected cowpea plant (*V. unguiculata*) was collected from the research farm of Michael Okpara University of Agriculture Umudike, Nigeria. The petiole leaf and stem of the infected plant were cut in bits using a sharp blade and then placed in a Petri-dish and were disinfected with 50 ml of 70% ethanol and finally rinsed with three changes of 500 ml of sterile water, after which the tissues were placed in a Petri-dish containing a moist filter paper at room temperature of 27°C. After a week, a pronounced whitish growth was observed on the surface of the tissues. 300 g of potato were brought to the laboratory and were washed five times with tap water until adhered soil was completely removed. 200 g of the potato were peeled and boiled in 1 L of water and filtered. 20 g of agar and 20 g of dextrose were added to the filtrate and made up to 1 L with sterilized water and use to subculture the organism and obtained a pure culture, which was finally examined using a compound microscope. The organism was confirmed to be *S. rolfsii* with the aid of an identification manual by Barnett and Huntter (1972).

In vitro experiment

Each of the Petri-dishes contained potato (20 g), dextrose agar (20 g), (PDA) and a 10% concentration of 5 ml plant extract were mixed together and allowed to solidify. Dishes were inoculated with the fungus by cutting a 4 mm-diameter disc from a pure culture of *S. rolfsii* growing the PDA using a cork borer. This was done for each of the extracts as well as for two controls: a plate containing 5 ml of benomyl mixed with PDA and another without plant extracts. The cultures were incubated at 27°C in an incubation chamber for 9 days. Radial growth of the fungus for each treatment was measured at the 9th day of inoculation using a ruler and the percentage inhibition was calculated using the formula of Amadioha (2003) as shown below.

$$\% \text{ Growth inhibition} = \frac{(DC - DT) \times 100}{DT}$$

where: DC = Colony diameter of control and DT = Colony diameter of treated plates.

Phytochemical analysis

Alkaloids and phenols were determined according to the method of Harborne (1973), while tannins were determined using the method of Van Burden and Robinson (1981). Saponin was determined using the method of Obadoni and Ochuko (2001). Flavonoids were determined according to the method of Boham and Kocipai (1994).

Statistical analysis

All measurements were replicated three times and standard deviations determined. The student's *t*-test at $P < 0.05$ was applied to assess the difference between the means (Steel and Torrie 1980).

RESULTS AND DISCUSSION

The phytochemical contents of *A. meleguata* and *M. myristica* seeds and leaf extracts are shown in **Table 1**. The alkaloid content of *A. meleguata* seeds was very high (5.64%), followed by *M. myristica* leaf (4.28%) while the leaf of *A. meleguata* contained 0.29% of alkaloids. The high alkaloids content on *A. meleguata* seeds explains the reason for its high inhibitory activity on *Sclorofium rolfii.* Alkaloids are plant-derived compounds that are toxic or physiologically active and contain nitrogen in a heterocyclic ring. They are basic and have a complex structure and are of limited distribution in the plant kingdom (Okwu 2005). Methoxy indole (4-methyl 1′, 7, dihydroxy 1′ ethylene 1, 2′, 2′ trimethoxy indole) isolated from the leaf of *A. meleguata* is an alkaloid.

However, pure isolated plant alkaloids and their synthetic derivatives are used as basic medicinal agents for their analgesics, antimalarial, fungicidal and bactericidal properties (Okwu 2005). They exhibit marked physiological activity when administered to animals like cattle, goats and sheep. Again the high alkaloids content in *A. meleguata* explain their therapeutic and medicinal properties and the use of this plant in herbal medicine in Nigeria, Ghana and Cameroon. The presence of alkaloids and saponin in *A. meleguata* seed may be the reason for the hot and pungent taste of the seeds.

The flavonoid content was high in the leaf of *M. myristica* (8.29%) followed by the seeds of *A. meleguata* which contained 5.78% of flavonoids. The leaf of *A. meleguata* and the seeds of *M. myristica* contained 2.15% and 0.12% of flavonoids, respectively. Flavonoids represent the most common and widely distributed group of plant phenolics. The presence of phenolic compounds in *A. meleguata* and *M. myristica* indicates that the plants might be anti-microbial agents. Phenolic compounds are toxic to living cells such as fungi and bacteria. It acts on the cell by denaturing and coagulating the protein content of the cell (Baker and Breach 1990). *A. meleguata* and *M. myristica* contain substantial amounts of compounds: 0.84% limonene, 0.83% α-pinene, 0.40% myrcene and 0.11% phellandrene (Okwu 2001; Okwu and Ibeawuchi 2005). These phytoconstituents are monoterpenoids that not only posses anti-viral and antibacterial properties but also exhibit antifungal properties (Paticowska 2006; Okwu *et al.* 2007). These compounds, which occur in high concentrations in *A. meleguata* seeds, are responsible for the pungent, hot and peppery taste of the seeds. They may also be responsible for its high pesticidal properties, providing peasant farmers from rural communities with locally available, biodegradable and inexpensive materials for pest control.

A. meleguata contain hydroarylalkaloids on acetone extract (Okwu 2005). The seeds have a pungent peppery taste due to aromatic ketones (Iwu *et al.* 1999). The constituents are essential oils such as gingerol and paradol. The essential oil from the grains of *A. meleguata* is dominated by sesquiterpene hydrocarbons such as humulene and β-caryophyllene and their oxides (Iwu *et al.* 1999). The monoterpenoid content of *M. myristica* is mainly hydrocarbons comprising phellandrene, α-pinene, limonene, myrcene and thujene. They also contain eugenol and geraniol. These phytoconstituents inhibits the development of bacteria and fungi (Iwu *et al.* 1999; Okigbo and Ogbonnaya 2006).

Phenolics form a large group of naturally occurring, diverse and widespread compounds. They are characterized by the presence of an aromatic ring with one or more hydroxyl groups. These phenolic compounds in *A. meleguata* and *M. myristica* may be responsible for the antiseptic, antifungal or bactericides properties of the plants (Okwu and Morah 2007). The mechanism of inhibitory action of these alkaloids and phenolic compounds on micro organisms may be due to impairment of variety of enzyme systems, including those involved in energy production, interference with the integrity of the cell membranes and structural component synthesis (Huang and Chung 2003; Okwu *et al.* 2007). The antimicrobial activities of phenols are further evidenced by their active role in plant disease resistance and prevention (Matern and Kneusel 1988; Russel and Chopra 1990). Moreover, phenolic compounds from plant extracts act as anti-microbial agents (Okwu 2005). Phenolic compounds are also considered to be bacteriostatic and fungistatic. These compounds caused swelling of hyphae tips, plasma seeping around hyphae, leaking of plasma, cell wall distortion, abnormal branching or fusion of hyphae surface (Huang and Chung 2003; Okwu *et al.* 2007). Furthermore, the effects of *A. meleguata* and *M. myristica* seeds and leaves extracts on fungi may be due to inhibition in the formation of zoosporangia and germination of the pathogens zoospores thereby limiting the growth of mycelium (Orlikowski 2001; Okwu *et al.* 2007). *A. meleguata* and *M. myristica* extracts inhibited the growth of mycelium, the formation of conidial spores and chlamydospores of *S. rolfsii* thereby reducing the number of propagation units of this fungus in the medium (Patkowski 2006; Okwu *et al.* 2007).

A. meleguata seeds contained 1.24% of saponin and the leaves contained 0.14% of saponin while the *M. myristica* seed and leaf contained 0.87% and 0.02% of saponin, respectively. Saponins natural tendency to ward off microbes makes them good candidates for treating fungal and yeast infections. Plant saponins help humans to fight fungal infections, combat microbes and viruses and boost the effectiveness of certain vaccines (Okwu and Ezenagu 2008). The high content of saponins on the seeds of *A. meleguata* may be responsible for its high inhibition on the fungus *S. rolfsii* (**Table 3**) compared to the synthetic fungicide (benomyl).

Table 2 shows the infrared analysis of the extracts. The broad peak around 3401–3442 cm^{-1} is likely due to -OH stretching of the phenolic compounds. The amine functions appear at 3008–3108 cm^{-1} indicating the alkaloids. The peaks at 2854–2925 cm^{-1} denotes C-H stretching vibrations of hydrocarbons. C=O stretching of esters and ketones were represented by the peaks around 1634–1738 cm^{-1}. At 1376–1464 cm^{-1} C=C aromatic stretching vibrations occurs. These

Table 1 Phytochemical content of *Aframomum meleguata* and *Monodora myristica*.

Phytochemicals	*Aframomum meleguata*		*Monodora myristica*	
	Leaf	**Seed**	**Leaf**	**Seed**
Alkaloids	0.29 ± 0.20 b	5.64 ± 0.10 a	4.28 ± 0.11 a	0.41 ± 0.01 a
Flavonoids	2.15 ± 0.21 a	5.78 ± 0.10 a	8.29 ± 0.10 a	0.12 ± 0.01 b
Tannins	0.16 ± 0.30 c	0.39 ± 0.11 c	0.34 ± 0.11 b	0.03 ± 0.20 c
Saponins	0.14 ± 0.01 c	1.24 ± 0.30 b	0.02 ± 0.10 c	0.87 ± 0.02 a
Phenols	0.10 ± 0.01 c	0.11 ± 0.10 c	0.03 ± 0.01 c	0.02 ± 0.10 c

Data are means ± standard deviation of triplicate determination on dry weight bases, values with the same superscript in each column are not significantly different at $P < 0.05$

Table 2 Infrared analysis of Isolates from *Aframomum meleguata* and *Monodora myristica*.

Plant isolate	Frequency cm^{-1}	Functional group	Compound type
A. meleguata seed	3413.07	OH	Hydroxyl phenol
	3108.11	C-N	Amine
	2925.88	C-H	Aliphatic stretching
	2854.04	C-H	Aliphatic stretching
	1709.15	C = O	Carbonyl ester
	1654.99	C = O	Carbonyl ketone
	1604.88	C = C	Aromatic
	1121.00	C-O	Ether
A. meleguata leaf	3418.90	O-H	Hydroxyl phenol
	2924.00	C-H	Aliphatic stretching
	2852.41	C-H	Aliphatic stretching
	1738.83	C = O	Carbonyl ester
	1615.89	C = C	Aromatic
	1455.75	C = C	Aromatic
	1376.98	C = C	Aromatic substitution
	1164.45	C-O	Aromatic substitution
A. meleguata leaf	3401	O-H	Hydroxyl phenol
	3008	C-N	Amine
	2926	C-H	Aliphatic stretching
	2864	C-H	Aliphatic stretching
	1464	C = C	Aromatic
	1378	C-O	Ester
	1244	C-O	Ether
	1177	C-N	Amine
	1098	C-O	Ether
	1050	= C-H	Aromatic substitution
	801	= C-H	Aromatic substitution
	723	= C-H	Aromatic substitution
M. myristica seed	3442.25	O-H	Hydroxyl phenol
	2359.42	C-H	Aliphatic hydrocarbon
	1634.29	C = O	Carbonyl
	1539.26	C = C	Aromatic
	1455.90	C = C	Aromatic
	668.13	= C-H	Aromatic substitution
M. myristica leaf	3421.22	O-H	Hydroxyl
	2854.11	C-H	Aliphatic stretching
	2360.52	C-H	Aliphatic stretching
	1735.85	C = O	Carbonyl ester
	1653.09	C = O	Carbonyl ester
	1558.43	C = O	Carbonyl ketone
	1507.08	C = O	Carbonyl ketone
	1457.81	C = O	Carbonyl ketone
	1375.28	C-O	Ether flavonoid
	1260.39	C-O	Ether flavonoid
	1022.31	C-O	Ether flavonoid
	799.60	= CH	Aromatic substitution

peaks confirmed the aromatic phenolic compounds, aldehydes and ketones available in the *A. meleguata* and *M. myristica* extracts. The finger print regions (1400–400 cm^{-1}) are very important in identifying the functional groups in a particular compound. In this region the peaks at 1376–1336 are likely due to C-CH_2- stretching and have been noticed in the condensed tannins (Ukoha *et al.* 2005). C-O stretching of esters is centered at 1302–1034 cm^{-1}. The peaks at 799, 723, and 468 cm^{-1} are probably due to aromatic substitutions (Ukoha *et al.* 2005).

Table 3 shows the inhibitory effects of 10% concentration seed and leaf extracts of *A. meleguata*, *M. myristica* and benomyl on *in vitro* growth of *S. rolfsii.* Both *A. meleguata* seed and synthetic fungicide (benomyl) have 86.65 and 86.20% inhibition, respectively. This could be linked to the fact that *A. meleguata* seed has enormous deposit of alkaloids and phenolic flavonoid content to protect and preserve the seeds from microbial attack (Okwu and Emenike 2006). Greater inhibition of *S. rolfsii* was obtained with *A. meleguata* seeds than on the leaf extracts. This could also be attributed to the fact that more phytochemicals were deposited on the seeds particularly alkaloids and phenolic flavonoids. The extracts showed a remarkable effectiveness in controlling *S. rolfsii.* The inhibitory profile of *S. rolfsii* by *A. meleguata* and *M. myristica* seed and leaf extracts has been depicted in **Table 3**.

Plants store these antifungal, antibacterial and antiviral chemicals for protection against microbial attack. Phytochemicals are reserved in plants to protect the plants against the attack and inversion of microorganisms. The extracts and synthetic fungicide (benomyl) inhibited the growth of this organism *S. rolfsii.* In the *in vitro* experiment, all the extracts were highly effective in the inhibition of the organism. Extracts of *A. meleguata* and *M. myristica* contains antifungal compounds that can be used as alternative to synthetic fungicides, including fumigants and contact pesticides. The prospects of using *A. meleguata* and *M. myristica* for development of natural fungicides are appealing and acceptable. This is because these plants are readily available, environmentally safe, and less hazardous to non target organisms and pest resurgence, less adverse effect on plant growth, less harmful to seed viability and quality and above all less expensive (Prakesh and Rao 1997; Okwu *et al.* 2007).

Based on these findings, *A. meleguata* and *M. myristica* extracts are viable and can be possible alternative to synthetic pesticides for control of fungal diseases.

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Table 3 Inhibitory effects of 10% concentration of seed and leaf extracts of *Aframomium meleguata*, *Monodora myristica* and Benomyl on *in vitro* growth of *Sclerotium rolfsii*.

Inhibition	*Aframomium meleguata*		*Monodora myristica*		Benomyl	Control
	Seed	Leaf	Seed	Leaf		
% Inhibition	86.65 ± 0.20 a	43.47 ± 0.20 b	39.31 ± 0.11 b	53.19 ± 0.10 a	86.20 ± 0.11 a	0.00 c

Data are mean ± standard deviation of triplicate determination on dry weight basis. Values with superscript that are the same are not significant different at P< 0.05

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