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Morphological and molecular identification of pathogenic fungi of *Monodora myristica* Dunal kernels and their response to different phytoextracts

Joseph Djeugap Fovo[1,2]*, Cyril Akoula Nzong[1], Kyalo Martina[2], Patrick Njukeng Achiangia[3], Joseph Hubert Galani Yamdeu[4], Jules-Roger Kuiate[5] and Sita Ghimire[2]

[1]Phytopathology and Agricultural Zoology Research Unit, Department of Plant Protection, Faculty of Agronomy and Agricultural Science, P. O. Box 222 Dschang, University of Dschang, Cameroon.
[2]Biosciences Eastern and Central Africa-International Livestock Research Institute (BecA-ILRI) Hub, P. O. Box 30709-00100, Nairobi, Kenya.
[3]Applied Botany Research Unit, Department of Plant Biology, Faculty of Sciences, University of Dschang, P. O. Box 67 Dschang, Cameroon.
[4]Department of Agriculture and Veterinary Medicine, Université des Montagnes, P. O. Box 208, Bangangté, Cameroon.
[5]Microbiology and Antimicrobials Substances Research Unit, Department of Biochemistry, Faculty of Sciences, University of Dschang, P. O. Box 67 Dschang, Cameroon.

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ABSTRACT

Identification of fungi from calabash nutmeg kernels was based on their morphological characteristics and analysis of the internal transcribed spacer (ITS) sequences of their genomic DNA. Antifungal activity of aqueous, methanolic and ethanolic extracts of four plants species (*Azadirachta indica*, *Citrus sinensis*, *Moringa oleifera* and *Tithonia diversifolia*) was tested *in vitro* at 50, 75, 100 and 125 mg/ml for aqueous extracts and 40, 60, 80 and 100 mg/ml for methanolic and ethanolic extracts. Mancozeb (1 mg/ml) and distilled water were used as positive and negative controls, respectively. The most frequently isolated fungi were *Cercospora purpurea* (34.28%), *Fusarium oxysporum* (23.81%) and *Aspergillus flavus* (17.14%). *C. purpurea* and *F. oxysporum* isolates were more aggressive after inoculation on healthy kernels. All the extracts tested, inhibited the growth of the fungi compared to the negative control, except the aqueous extract of *T. diversifolia* against *C. purpurea* and *F. oxysporum* and the methanolic extract of *M. oleifera* against *F. oxysporum* at 75 mg/ml. The efficiency of aqueous extracts of *M. oleifera* and *C. sinensis* was significantly lower (P<0.05) as compared to the reference fungicide on growth of *A. niger* at 125 mg/ml. Antifungal activity of methanolic extracts of *A. indica*, *C. sinensis* and *T. diversifolia* as well as ethanolic extracts of *A. indica* and *M. oleifera* was significantly equal to mancozeb at 100 mg/ml on *A. flavus*. Aqueous extracts of *M. oleifera* and methanolic extracts of *A. indica* and *C. sinensis* could be used for protection of *Monodora myristica* kernels against post-harvest fungi.

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INTRODUCTION

Calabash nutmeg (*Monodora myristica* Dunal) is a perennial plant belonging to the family *Annonaceae*. It is common in the evergreen forests of West and Central Africa (Burabai et al., 2007). In Cameroon, its seeds and bark are used for food and medicinal properties. Seeds are the most important part of the tree as they have an odour and taste similar to nutmeg, and are therefore used as a popular spice in the West and Central African

cuisine. It is also used as substitute for nutmeg in soups, stews and cakes. In traditional medicine, the seeds are used as a stimulant and stomachic. They are also used as rosary beads and are considered by some to have magical properties (Faleyimu and Oluwalana, 2008). The seeds are rich in alkaloids and used in the treatment of headache and as an antiseptic (N'guessan et al., 2009). Aqueous extract of *M. myristica* dry fruits possess cholesterol lowering potentials and protective ability (Nwozo et al., 2015). The stem bark is used in the treatments of hemorrhoids, stomach ache, fever pains and eye diseases (Uwakwe and Nwaoguikpe, 2008).

Despite these tremendous nutritional and medicinal values of *M. myristica* kernels, no research has been carried out to study pathogenic fungi associated with kernels of this species. Observations made in the field reveal that *M. myristica* kernels are mostly colonized by molds that could be potentially harmful to consumers if they produce mycotoxins. Post-harvest diseases are usually controlled with chemicals, which despite their effectiveness, could be toxic to consumers and the environment. This is why the discovery of novel antimicrobial agents of plant origin, is important to preserve the environment and the health of consumers. The antifungal potential of plant extracts has long been investigated as they contain several bioactive compounds for plant disease control, such as flavonoids, polyphenols and saponins (Joshi et al., 2011; Patel et al., 2014; Riaz et al., 2015). Due to their low toxicity, there is growing interest in using plant products (extracts, essential oils and powders) as a source of bioactive phytochemicals for antifungal properties in controlling plant diseases (Nguefack et al., 2013; Zaker, 2014). To the best of our knowledge, no study has been carried out on the identification of post-harvest pathogenic fungi of *M. myristica* kernels and on the assessment of antifungal properties of extracts from selected ethno-medicinal plants. The results from this study are expected to reduce post-harvest losses, increase the income of farmers and traders and enhance consumer health.

MATERIALS AND METHODS

Collection of plant materials

Symptomatic kernels of *M. myrstica* were collected from Ebolowa, Yokadouma, Kumba, Dschang and Fontem markets of Cameroon, and stored at 4°C in the laboratory prior to isolation of the associated pathogens. Leaves of Mexican sunflower (*T. diversifolia*), neem (*A. indica*) and moringa (*M. oleifera*) and pericarp of orange fruits (*C. sinensis*) collected from Dschang were used to prepare phytoextracts. These plant species were choosen based on their proven antimicrobial properties against either plant or animal pathogens (Trabi et al., 2008; Nweke and Ibian, 2012; Abiamere et al., 2014).

Preparation of plant extracts

The plant parts collected were washed with tap water and rinsed thrice with distilled water. Subsequently, the leaves and barks were chopped into small pieces, dried in shade for a week, and powdered in an electronic blender. One hundred grams of fine powder of each sample was macerated in 500 ml of water, methanol or ethanol for 2 days then filtered through muslin cloth. The aqueous extract was oven-dried at 50°C for 7 days while ethanol and methanolic extracts were evaporated on a shaking water bath at 60 rpm at 60°C. Extracts were separately stored in small containers at room temperature for further experiments.

Isolation and morphological identification of fungi

Kernels of *M. myristica* showing disease symptoms were sliced into 2 mm² pieces and disinfected for 3 min in 5% sodium hypochlorite solution, the slices were rinsed in three successive changes of sterile distilled water and transfered on sterile potato dextrose agar medium (PDA) supplemented with chloramphenicol (1 g/l) as antibacterial, for fungal isolation and purification (Korsten et al., 1994). The pure fungal isolates obtained were identified based on morphological characteristics, that is mycelium structure and spore morphology using keys of fungi identification (Barnett and Hunter, 1972; Alexopoulos et al., 1996; Champignon, 1997). The frequency of isolation (F) of each fungus was calculated using the following formula, $F = (NF \div NT) \times 100$, where F represents the frequency of occurrence (%) of a fungus, NF is the total number of samples from which a particular fungus was isolated and NT is the total number of samples from which isolations were carried out (Iqbal and Saeed, 2012).

Molecular identification of fungi

Fungal identity was confirmed using molecular method. For this purpose, fungal genomic DNA was extracted using ZR Plant/Seed DNA MiniPrep ™ kit (Zymo Research) following manufacturer's instructions. The universal fungi species primers, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used (White et

*Corresponding author: joseph.djeugapfovo@univ-dschang.org.

al., 1990). DNA quality and quantity were checked on 1% agarose gel (w/v) and NanoDrop Spectrophotometer. The genomic DNA was adjusted to the final concentration of 20 ng/L and stored at 4°C for PCR amplification. Amplification was performed in 30 µL reaction volume containing 1 × Accu Power PCR Master Mix, 0.1 µM of each primer, and 40 ng of genomic DNA. The PCR program was as follows : initial denaturation at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 60s, extension at 72°C for 2 min, and final extension at 72°C for 10 min. Purified PCR products were sent for Sanger sequencing by Macrogen (Amsterdam, Netherlands). All reactions were run following the manufacturer's protocols. Nucleotide sequences were aligned using ClustalW program (Thompson et al., 1994), and sequences identified using NCBI-BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Pathogenicity test of fungi

For each fungus isolated and identified, a 10-day-old culture in the Petri dishes was used for inoculum preparation. Healthy kernels of *M. myristica* were disinfected with bleach solution (5%, 2 min), rinsed in sterile water for three times and soaked during 30 min in a conidial suspension calibrated at 2×10^3 conidia/ml using a hemacytometer. After inoculation, the kernels were removed and introduced into new Petri dishes containing moisten filter paper (Imathiu et al., 2014). All procedures were the same for the control plate but no inoculum was applied. After 7 days of incubation at 22°C, infected kernels were recorded as the presence or absence of fungal infection.

In vitro efficacy of plant extracts on fungal growth

Mycelia disks were obtained using a cookie cutter of 5 mm diameter and taken from the margin of 10 days-old culure of *A. flavus*, *A. niger*, *C. purpurea* and *F. oxysporum.* Mycelial discs were deposited in the center of each Petri dish containing PDA medium enriched with different extracts or fungicide Mancozeb (1 mg/ml) as positive control. A negative control, non-supplemented with extract but solvent control dilution were prepared. Each treatment was conducted in four replications. The plates were incubated at 25°C and daily measurements were taken for fungal growth from the second day of the experiment. The experiment was stopped when Petri dishes of negative control were completely covered by the fungus. The radial growth of the fungi was measured daily (48 h after inoculation) and at the same time, the two perpendicular diameters along the tracks on the back of the Petri dish. The average of two perpendicular measurements of the diameter minus the diameter of explants represents the measurement of the radial growth of the fungus (Djeugap et al., 2011). The inhibition percent (IP) was calculated by the formula given by Ul-Haq et al. (2014): IP = (GC-GT)÷GC×100; where GC=growth in the control, GT=growth in the treated groups. An average of four replications of each test was taken for calculations.

Data analyses

Data on radial growth and inhibition percentage were analysed by SAS software (version 9.1) (SAS Institute, Cary, NC). Data were submitted to a one-way analysis of variance (ANOVA). Where the ANOVA was significant at 5%, means were separated using a Duncan multiple range test.

RESULTS

Characteristics of plant extracts and extraction yields

The yields of aqueous extracts were higher than those of the methanolic and ethanolic extracts. The aqueous extract of *C. sinensis* gave the highest yield (32.91%) followed by *A. indica* (17.71%), *T. diversifolia* (16.40%) and *M. oleifera* (7.65%).

For methanolic and ethanolic extracts, the yield of *C. sinensis* was highest (16.61 and 9.9%) while *T. diversifolia* (2.46 and 2.44%), respectively was the lowest (Table 1).

Identification and pathogenicity of fungi isolated from *M. myristica* kernels

Based on morphological and molecular identification, fungi associated with *M. myristica* kernels were *A. flavus*, *A. niger*, *A. oryzae, C. purpurea*, *Chaetonium reflexum*, *Cunninghamella bainieri*, *F. oxysporum* and *Rhizopus nigricans*. The most frequent fungi were *C. purpurea* (60.66%), *F. oxysporum* (22.96%), *A. flavus* (17.14%) and *A. niger* (8.57%) (Table 2). The fungal species and sequence identity of selected fungi of calabash kernels used for pathogenicity test are presented in Table 2. Among fungal isolates tested, E_{012}_MM and K_{112}_MM were very aggressive while D_{032}_MM, Y_{046}_MM, E_{023}_MM and F_{181}_MM were non-pathogenic isolates (Table 2). These four most frequent fungi developed symptoms at 7 days after inoculation on healthy kernels, and symptoms ranged from whitish to dark mycelium and fruiting bodies; while other isolates did not develop symptom (Figure 1).

Antifungal activity of aqueous extracts

Antifungal activity of plant extracts was tested against *C.*

Table 1. Physical characteristic and extraction yield of plant extracts.

Plants species	Physical aspect	Color	Yield (% of crude extracts)
***A. indica* (leaves)**			
Aqueous extract	Powdery	Brown	17.71
Methanolic extract	Thick	Black	6.78
Ethanolic extract	Thick	Black	5.7
***M. oleifera* (leaves)**			
Aqueous extract	Dense	Greenish	7.65
Methanolic extract	Thick	Black	5.70
Ethanolic extract	Dense	Black	5.20
***C. sinensis* (pericarp)**			
Aqueous extract	Thick	Brown	32.91
Methanolic extract	Dense	Brown	16.61
Ethanolic extract	Dense	Brown	9.9
***T. diversifolia* (leaves)**			
Aqueous extract	Thick	Greenish	16.49
Methanolic extract	Thick	Black	2.46
Ethanolic extract	Thick	Black	2.44

Table 2. Fungal species isolated from *Monodora myristica* kernels with accession numbers, isolate code, maximum percent identity with Genbank sequences, isolation percentage and pathogenicity.

Fungal species and accession	Isolate code	Max % identity	Isolation percentage (%)	Pathogenicity test*
C. purpurea (JX143676)	E_{012}_MM	100	34.28	+++
F. oxysporum (JN230149)	K_{112}_MM	100	23.81	+++
A. flavus (HQ340108)	E_{015}_MM	100	17.14	++
A. niger (KU681408)	E_{028}_MM	100	8.57	++
R. nigricans (KT852980)	D_{032}_MM	98.6	7.62	-
A. oryzae (AP007173)	Y_{046}_MM	95.7	3.81	-
C. reflexum (U3461168)	E_{023}_MM	94.6	2.85	-
C. bainieri (KP024561)	F_{181}_MM	92.5	1.90	-

***+++,** very aggressive isolates, **++,** moderately aggressive isolates; **-,** non-pathogenic isolates.
D, E, F and K are the abbreviations for Dschang, Ebolowa, Fontem and Kumba, respectively, the name of the locality of isolate origin.

purpurea, *F. oxysporum*, *A. flavus* and *A. niger.* Based on the literature, they are among pathogenic fungi responsible for post-harvest losses in foodstuffs. All aqueous extracts significantly inhibited the growth of all the fungi tested, compared to the untreated control (Table 3). Treatment with extracts of *A. indica*, *M. oleifera* and *C. sinensis*, inhibited the radial growth of *C. purpurea* at 125 mg/ml and was significantly greater than the negative control. Similarly, the extract of *A. indica* significantly reduced the radial growth of *F. oxysporum* at 125 mg/ml, more than the control without fungicide. Antifungal activity of aqueous extracts of *M. oleifera* and *C. sinensis* was significantly lower as compared to Mancozeb against *A. niger* at 125 mg/ml. The extracts of *A. indica*, *M. oleifera* and *C. sinensis* significantly reduced the radial growth of *A. flavus* (Table 3).

Antifungal activity of methanolic and ethanolic extracts

All methanolic extracts significantly reduced the radial growth of the fungi compared to the negative control in all concentrations tested (Table 4). The methanolic extracts of *A. indica*, *C. sinensis* and *T. diversifolia* presented an antifungal activity against *A. flavus,* which were significantly lower to Mancozeb at 100 mg/ml.

Ethanol extracts of all the tested plants significantly reduced the radial growth of *C. purpurea* and *A. flavus*, as compared to the negative control, in all concentrations

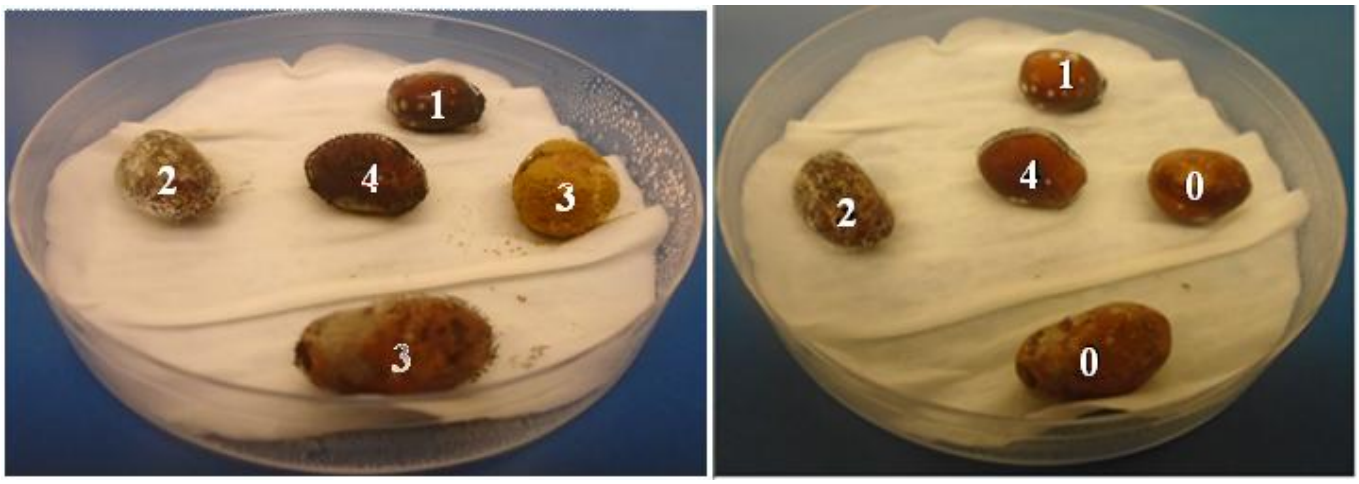

Figure 1. Pathogenicity test of some fungal species on kernels of *M. myristica*. Mycelium and fruiting bodies were visible on kernels inoculated with *C. purpurea* (1), *F. oxysporum* (2), *A. flavus* (3) and *A. niger* (4), and control kernel (0).

Table 3. Diameter of radial growth of fungi in PDA medium supplemented with aqueous extracts.

Concentration (mg/ml)	Radial growth of fungi (mm)*			
	A. indica	***C. sinensis***	***M. oleifera***	***T. diversifolia***
			A. flavus	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
50	26.3±2.7^{c}	22.3±1.4^{d}	23.2±1.2^{b}	29.6±2.5^{d}
75	24.7±2.5^{c}	20.4±3.5^{d}	21.3±1.1^{b}	27.9±1.4^{d}
100	24.5±3.1^{c}	17.8±0.2^{c}	19.1±2.3^{b}	31.2±1.1^{d}
125	21.8±1.3^{d}	16.7±1.4^{c}	17.7±1.2^{b}	34.8±3.7^{d}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{d}	3.0±0.9^{f}	3.7±1.6^{g}
			A. niger	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
50	21.7±2.6^{d}	16.5± 3.1^{e}	23.4±4.1^{d}	21.9 ±2.7^{e}
75	18.7±2.6^{d}	13.1± 1.2^{ef}	19.1± 3.1^{d}	15.5±2.3^{f}
100	13.8±2.8^{de}	11.1± 3.2^{ef}	13.3± 1.4^{de}	17.3± 1.8^{f}
125	8.3±1.0^{e}	5.3 ±1.0^{f}	6.2± 0.6^{e}	21.7± 2.5^{e}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{g}	3.0±0.9^{f}	3.7±1.6^{g}
			C. purpurea	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
50	61.3±7.2^{b}	57.6±6.2^{b}	55.5±5.7^{b}	57.3±5.5^{c}
75	55.7±6.3^{b}	54.6±8.3^{b}	53.8±7.4^{b}	63.2±9.7^{c}
100	46.3±2.1^{c}	52.3±2.7^{b}	39.5±4.3^{c}	68.7±5.4^{c}
125	29.8±3.9^{c}	43.1±2.0^{c}	31.2±4.9^{cd}	76.2±6.5^{b}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{g}	3.0±0.9^{f}	3.7±1.6^{g}
			F. oxysporum	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
50	30.7±3.9^{c}	12.9±1.8^{ef}	11.3±1.5^{de}	51.6±7.3^{c}
75	30.2±5.1^{c}	10.5±3.5^{ef}	27.7±4.0^{d}	21.9±5.2^{e}
100	29.8±6.4^{c}	10.3±2.1^{ef}	18.3±5.5^{d}	46.2±8.6^{cd}
125	21.1±8.3^{d}	31.2±8.1^{d}	42.5±7.3^{c}	48.1±9.9^{cd}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{g}	3.0±0.9^{f}	3.7±1.6^{g}

*Means follow by the same letter in the column are not significantly different based on Duncan multiple range test at 5%.

Table 4. Diameter of radial growth of fungi in methanolic extracts.

Concentration (mg/ml)	Radial growth of fungi (mm)*			
	Azadirachta indica	***C. sinensis***	***M. oleifera***	***T. diversifolia***
		A. flavus		
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	18.6±2.2^{e}	15.5±3.6^{f}	15.6±5.8^{d}	19.9±1.5^{e}
60	14.4±1.2^{f}	14.3±0.7^{f}	14.4±1.5^{d}	15.7±1.1^{f}
80	14.1±1.5^{f}	19.2±4.2^{e}	12.6±1.2^{de}	15.5±1.3^{f}
100	12.8±2.1^{f}	19.4±4.6^{e}	11.8±0.7^{de}	14.2±0.8^{f}
Mancozeb (1 mg/ml)	3.5±1.1^{g}	2.9±1.2^{g}	3.0±0.9^{f}	3.7±1.6^{g}
		A. niger		
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	20.2±1.2^{e}	22.4±1.5^{e}	19.9±2.7^{d}	22.3±2.8^{ef}
60	19.1±1.5^{e}	22.3±1.6^{e}	17.4±1.2^{d}	20.6±2.4^{ef}
80	17.4±2.9^{e}	20.8±2.7^{e}	16.8±2.1^{d}	20.3±2.4^{ef}
100	13.6±6,1^{f}	19.7±3.8^{d}	14.1±3.5^{d}	16.3±2.5^{g}
Mancozeb (1 mg/ml)	3.5±1.1^{g}	2.9±1.2^{g}	3.0±0.9^{e}	3.7±1.6^{h}
		C. purpurea		
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	58.7 ±4.5^{b}	60.6 ±5.4^{b}	61.3± 3.2^{b}	75.7± 6.9^{b}
60	57.2± 3.1^{b}	55.2 ±4.7^{c}	59.5 ±5.3^{b}	65.5±4.2^{c}
80	55.7± 4.9^{b}	49.3± 2.1^{c}	59.4 ±2.1^{b}	61.6±8.4^{c}
100	46.3± 4.8^{c}	46.8 ±3.5^{c}	57.3± 4.4^{b}	55.7± 1.2^{d}
Mancozeb (1 mg/ml)	3.5±1.1^{g}	2.9±1.2^{g}	3.0±0.9^{e}	3.7±1.6^{h}
		F. oxysporum		
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	33.3±6.3^{d}	41.4±5.9^{cd}	35.3±5.6^{c}	36,7±9,3^{e}
60	35.4±8.7^{d}	38.5±0.9^{cd}	38.5±6.3^{c}	31.5±4.6^{e}
80	36.5±3.1^{d}	34.2±5.9^{d}	34.2±4.5^{c}	30.3±3.2^{e}
100	42.3±4.1^{c}	22.3±1.8^{e}	37.7±3.9^{c}	23.6±2.2^{ef}
Mancozeb (1 mg/ml)	3.5±1.1^{g}	2.9±1.2^{g}	3.0±0.9^{e}	3.7±1.6^{h}

*Means follow by the same letter in the column are not significantly different based on Duncan multiple range test at 5%.

tested.

On the other hand, the extract of *M. oleifera* presented an antifungal activity significantly lower to the negative control at 40 mg/ml with respect to *F. oxysporum.* It was the same for the extract of *C. sinensis* at concentrations of 40, 60 and 80 mg/ml against *A. niger*. Extracts of *A. indica* and *M. oleifera* have shown antifungal activity against *A. flavus* comparable to mancozeb at the concentration of 100 mg/ml (Table 5).

DISCUSSION

Yields of plant extracts

The variability of extraction yields from one plant species to another can be explained by the difference in plant species used, and the stage of the vegetative cycle of the plant during harvest. Svoboda and Hampson (1999) and Smallfield (2001) also reported that environmental conditions, the harvest period and the age of the plant material, can influence the extraction yields. In addition, the yeild may depends on the botanical family to which the species belongs (Valnet, 1980; Djeugap et al., 2011).

Inventory of fungal species associated with *M. myristica* kernels

Several known post-harvest fungi were associated with *M. myristica* kernels. They included *A. flavus*, *A. oryzae, A. niger, C. purpurea, F. oxysporum* and *R. nigricans*.

Table 5. Diameter of radial growth of fungi in ethanolic extracts.

Concentration (mg/ml)	Radial growth of fungi (mm)*			
	A. indica	***C. sinensis***	***M. oleifera***	***T. diversifolia***
			A. flavus	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	22.3±3.6^{d}	27.5±3.2^{de}	21.4±2.8^{e}	24.8±1.3^{f}
60	19.1±2.4^{d}	25.5±1.3^{de}	16.5±1.1^{f}	20.6±3.7^{f}
80	17.4±1.5^{de}	22.5±4.1^{e}	15.2±2.6^{f}	18.2±1.4^{f}
100	14.5±2.1^{e}	22.7±4.5^{e}	11.3±2.8^{g}	17.6±2.3^{f}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{f}	3.0±0.9^{h}	3.7±1.6^{g}
			A. niger	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	21.5±2.1^{d}	30.5±2.7^{de}	24.7±3.1^{e}	25.3±3.1^{f}
60	20.5±1.8^{d}	28.5±2.1^{de}	19.8±1.5^{ef}	24.6±1.2^{f}
80	21.7±2.6^{d}	28.2±2.3^{de}	18.5±3.4^{ef}	22.1±1.5^{f}
100	19.3±2.2^{d}	23.5±1.2^{e}	16.3±2.1^{f}	21.8±2.6^{f}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{f}	3.0±0.9^{h}	3.7±1.6^{g}
			C. purpurea	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	60.1±1.4^{b}	55.3±4.6^{b}	60.4±2.3^{b}	61.5±2.8^{b}
60	59.3±0.7^{b}	50.4±3.5^{b}	60.1±3.7^{b}	54.8±2.5^{c}
80	55.1±3.2^{c}	45.8±3.4^{c}	55.5±3.9^{c}	46.4±3.4^{d}
100	53.6±1.1^{c}	42.3±3.3^{c}	53.7±4.8^{c}	43.7±2.1^{d}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{f}	3.0±0.9^{h}	3.7±1.6^{g}
			F. oxysporum	
0	87.6±2.7^{a}	88.6±1.7^{a}	85.7±4.5^{a}	85.8±4.1^{a}
40	52.6±3.4^{c}	34.6±2.6^{d}	56.3±2.5^{c}	45.8±3.1^{d}
60	48.1±4.9^{cd}	30.5±3.1^{de}	45.6±4.2^{d}	44.3±3.5^{d}
80	47.6±5.4^{cd}	30.4±3.2^{de}	44.5±8.6^{d}	37.7±3.6^{e}
100	45.4±7.1^{cd}	27.6±6.3^{de}	40.2±6.1^{d}	34.8±2.2^{e}
Mancozeb (1 mg/ml)	3.5±1.1^{f}	2.9±1.2^{f}	3.0±0.9^{h}	3.7±1.6^{g}

*Means follow by the same letter are not significantly different based on Duncan test at 5%.

Most of these fungi have been isolated from other edible non-timber forest products and other crop products. They are generally the cause of the post-harvest damage in certain fruits and foodstuffs, with high losses (El-Guilli et al., 2009; Enyiukwu et al., 2014; Onuorah and Orji, 2015). This is the case of *C. purpurea* which was also isolated on *Persea americana* fruit with a high occurrence frequency (Erute and Oyibo, 2008; Djeugap et al*.,* 2015). *F. oxysporum*, isolated from *M. myristica* kernels was also reported on *P. americana,* but at a low frequency (Erute and Oyibo, 2008). Gaikwad et al. (2014) showed that *F. oxysporum* was pathogenic on onion (*Allium cepa* L.) during storage. It was also found that *A. niger* was responsible for post-harvest losses on *Psidium guajava* and *P. americana* with a high isolation frequency (Erute and Oyibo, 2008; Amadi et al., 2014). Djeugap et al. (2009) showed that *R. nigricans* and other fungal species were associated with post-harvest pathology of mangoes. A higher isolation frequency of *F. oxysporum* was also reported by Ebele (2011) on papaya fruit (*Carica papaya* L.); however, that of *A. niger* was high in papaya compared to *M. myristica*. Marin et al. (1996), during studies carried out in Mexico, Costa Rica and Ecuador showed that *Fusarium* spp. and *Penicillium* spp. were identified as the main causes of bananas crown rot. The high occurrence frequencies of *C. purpurea*, *F. oxysporum* and *A. flavus* obtained from *M. myristica* kernels could be explained by the fact that these fungi are polyphagous and cosmopolitan. The poor storage conditions of the kernels (in air tight bags for a long period of time) could also favor their presence in the kernels. To the best of our knowledge, this is the first time

these fungi are reported in *M. myristica* kernels.

Evaluation of the antifungal efficiency of plant extracts

In vitro effectiveness of plant extracts on fungal growth differs from one fungus to another and from one plant extract to another. The plant extracts tested showed a higher suppressive effect on the radial growth of the four fungi tested, compared to the negative control. Aqueous extracts of *M. oleifera* and *C. sinensis* showed a suppressive effect which was comparable to the reference fungicide used. *Aspergillus niger* was the most sensitive fungus to the aqueous extracts, compared to *A. flavus, C. purpurea* and *F. oxysporum*. Onyeani et al. (2012) obtained similar results with the aqueous extracts of *Acalypha ciliata*, *Aloe vera* and *Vernonia amygdalina* on radial growth of *A. flavus* and *Penicillium expansum*. The efficacy of the aqueous extracts of *A. indica* is similar to results obtained by Nweke and Ibian (2012) and Tsopmbeng et al. (2014) against *Colletotrichum gloeoporioides* and *Phytophthora colocasiae*, respectively. However, Oluma and Elaigwe (2006) reported that the aqueous extracts of *A. indica* have no inhibitory effect on the growth of *Macrophomina phaseolina* and *Rhizoctonia solani*. The efficiency of the plants tested in this study against foodborne fungi was previously established against other plant and human pathogens. Maragathavalli et al. (2012) have demonstrated that maximum growth inhibition of *Bacillus pumilus*, *Pseudomonas aeruginosa* and *Staphylococcus* was obtained with methanol and ethanol extract of *A. indica* leaves, compared to antibiotics gentamycin (200 mg) and gentamycin (10 mg). Leaves and bark extract of the same plant was also efficient against *Escherichia coli* and *Staphylococcus aureus*. Madhuri et al. (2014) showed that the peel extract of *Citrus* fruits was efficient against *Colletotrichum capsici,* which causes anthracnose of chili. Antifungal properties of *T. diversifolia* extracts have been previously established against plant pathogenic fungal species, such as *Alternaria alternata, A. solani, P. expansum* and *P. italicum* and human pathogenic bacteria such as *Enterococcus faecalis, E. coli, P. aeruginosa* and *S. aureus* (Linthoingambi and Singh, 2013). Therefore, the plants tested have molecules with antifungal properties. Phytochemical screening of extracts of some of these plants had been carried out showing that they possess many organic compounds that could be responsible for their antimicrobial activities. It is the case of *T. diversifolia* leave extracts contain alkaloids, saponins, tannins, cardiac glycosides and volatile oils (Dewole and Oni, 2013); *M. oleifera* petroleum leaves extracts are rich in tannins, phenols, alkaloids, flavonoids, oxalates and saponins, which forms bioactive components against *Streptococcus* spp. growth (Ndhlala et al., 2014; Ajayi and Fadeyi, 2015). The peel of *C. sinensis* fruit contains limonene, linalool and α-pinene which have antifungal activities against foodborne pathogens such as *A. paraciticus* (Abdel-Fattah et al., 2015; Shalu et al., 2015). Nevertheless, in some cases there is an increase in radial growth of fungi with increase of plant extracts concentration. Bonzi (2007) showed that, depending on the fungus species and the maceration time, plant extracts could have opposite effects. For example, aqueous extracts of *Cymbopogon citratus* macerated during 6 and 12 h stimulate the radial growth of *Phoma sorghina,* whereas those macerated during 24 and 48 h completely inhibit the growth of that pathogen. In this study *T. diversifolia* aqueous extract increase radial growth of all the fungi tested (*A. flavus, A. niger, C. purpurea* and *F. oxysporum*) from 75 to 125 mg/ml. This could be due to the high nitrogen content of *T. diversifolia* leaves (Kaho et al., 2011; Kiye et al., 2013). Nitrogen is one of the key elements that enhance growth and increase microbial biomass (Wang et al., 2008; Ramirez et al., 2012).

Conclusion

The most frequent fungi associated with *M. myristica* kernels are *A. flavus*, *A. niger, C. purpurea* and *F. oxysporum*. *C. purpurea* and *F. oxysporum* isolates were more aggressive in inoculation studies. Aqueous extracts of *C. sinensis* and *M. oleifera* showed high antifungal activity at 125 mg/ml against *A. niger* which was comparable to Mancozeb. Methanolic extracts of *A. indica*, *C. sinensis* and *T. diversifolia* showed antifungal activity at 100 mg/ml against *A. flavus* which was comparable to the Mancozeb. However, the aqueous extract of *T. diversifolia* favored the growth of the tested fungi from the concentration of 75 mg/ml. *A. indica*, *C. sinensis*, *M. oleifera* and *T. diversifolia* extracts indeed possessed valuable products which could be exploited to control post-harvest diseases on *M. myristica* kernels. *Aspergillus* isolates should be tested for their ability to produce aflatoxin and *in vivo* tests should be carried out to confirm the efficiency of these plant extracts in managing fungi of *M. myristica* kernels.

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