International Journal of Microbiology Research and Reviews ISSN: 2329-9800 Vol. 2 (6), pp. 103-109, July, 2013. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Antifungal Activity of *Monathotaxis littoralis* essential oil against mycotoxigenic fungi isolated from maize

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Accepted 21 June, 2013

This study evaluated the antifungal activity of the essential oil which was isolated from *Monanthotaxis littoralis* (Annonaceae) against mycotoxigenic fungi from three genera (*Aspergillus, Fusarium and Penicillium)* isolated from maize samples. The oil of *M. littoralis* was obtained by hydro-distillation and analyzed by Gas Chromatography – Mass Spectrometry (GC-MS). Some of the major components of the oil were (+,-)-tetramisole, *Cis, trans*-2, 3-dimethylthiochroman-4-carbonitrile and 1-Adamantyl methyl ketone. Among the *Aspergillus* species the antifungal screening showed that the oil was more active against *Aspergillus flavus* with inhibition zone about 17.50 mm. From the *Fusarium* species *Fusarium proliferatum* had the highest inhibition zone of 20.17 mm and *Penicillium purporogenum* showed the lowest resistance to the oil from the *Penicillium* species with inhibition zone of 39.00 mm. The extent of inhibition of fungal growth was dependent on the concentration of the oil. The oil Minimum Inhibitory Concentration (MIC) values ranged from 26.00 to 410mg/ml. These results show that the essential oil from *M. littoralis* can be used to inhibit the growth of mycotoxigenic fungi that produce poisonous mycotoxins in foods.

Key words: *Aspergillus*, *Penicillium*, *Fusarium*, mycotoxigenic, antifungal, minimum inhibition concentration.

INTRODUCTION

Moulds cause extensive damage on food, feeds and other agricultural commodities in the field, during transportation, storage and processing which actually leads to postharvest losses. According to Agrios (1997) molds are known to destroy 10 to 30% of the total yield of crops and more than 30% for perishable crops by reducing their quality and quantity in developing countries. In addition moulds produce mycotoxins that can cause illness or even death to the consumer. Mycotoxins are toxic secondary metabolites produced by certain fungi in agricultural products that are susceptible to mould infestations (Wagacha and Muthomi, 2008). Mycotoxins contaminate 25% of agricultural commodities worldwide and are a source of morbidity and mortality throughout the world. Consumption of mycotoxin-contaminated foods has been associated with several cases of human poisoning or mycotoxicoses (Munimbazi and Bullerman, 1996). Acute liver damage, liver cirrhosis, induction of tumors and attack on central nervous system, skin disorders and hormonal effects are mostly caused by mycotoxicosis (Ibrahim et al., 2000; Oguz et al., 2003). Acute aflatoxicosis epidemics occurred in several parts of Africa and Asia leading to the death of several hundred people (Varga et al., 2009). Aflatoxicosis outbreak occurred in the eastern parts of Kenya in 2004 and 317 cases were reported with 125 deaths (Lewis et al., 2005).

Most mycotoxins are produced by *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins differ in their chemical formula, in the products and conditions which they are produced, effects on various animals and humans and degree of toxicity (Agrios, 1997). The most important in terms of economic importance are aflatoxins, deoxynivalenol, fumonisins, ochratoxins and zearalenones produced by *A.flavus*, *A. parasiticus*, *F. moniliforme*, *A. ochraceus* and *F. graminearum*, respectively (FAO, 1999). These mycotoxins are produced in infected cereal seeds, most le-

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gumes especially groundnuts, cotton seed, fishmeal, nuts etc (Kaaya et al., 2006). Most famous of these are the aflatoxins B_1, B_2, G_1 and G_2 which are produced by some strains of *A. flavus, A. parasiticus* and *A. nomius* with aflatoxin B_1 being the most common. Aflatoxins are known to be potent hepatocarcinogens in animals and humans (Dvorackova, 1990). Some of the toxins e.g. ochratoxins can persist in meat and milk of animals that fed on contaminated feed and can be transmitted to humans through the food chain (Lanyasunya *et al*., 2005).

The wide and indiscriminate use of chemical preservatives has been cause of appearance of resistant microorganisms, leading to occurrence of emerging food borne diseases (Akinpelu, 2001). As a result of this, there is an increasing interest to obtain alternative antimicrobial agents from natural sources for use in food preservation. Recent research has shown that some plants contain compounds like essential oils which are able to inhibit the microbial growth (Souza *et al*., 2005). According to Matasyoh *et al*., 2010, essential oil from *Cymbopogon citratus* was active against some *Aspergillus* species. Use of essential oils with antimould activities in food preservation would provide a technology to ensure that foods and feeds are free of moulds and mycotoxins. Such essential oils of recognized antimicrobial spectrum could appear in food preservation systems as main antimicrobial compounds or as adjuvant to improve the action of other antimicrobial compounds. *M*. *littoralis* belongs to the family Annonaceae and the genus *Monanthotaxis*. The Annonaceae family includes 130 genera and about 2300 species distributed in tropical and subtropical areas of America, Africa and Asia (Chang *et al.,* 1998). The aim of this study was, therefore, to evaluate the bioactivity of the secondary metabolites of *M. littoralis* against *Aspergillus*, *Fusarium and Penicillium* species isolated from maize, which is the staple food for most African and Latin American communities.

MATERIAL AND METHODS

Fresh leaves of *M. littoralis* were collected from Mabira equatorial forest in Uganda. A voucher specimen was deposited at the Department of Biological Sciences, Egerton University, Kenya.

Isolation of essential oils

Fresh leaves of *M. littoralis* were subjected to hydro-distillation in a modified Clevenger-type apparatus for a minimum of 4 hours. The extracted oil was dried using anhydrous Na_2SO_4

GC, GC-MS analysis

Samples of essential oils were diluted in methylertbuty- (MTBE) (1:100) and analyzed on an Agilent GC-MSD apparatus equipped with an Rtx-5SIL MS ('Restek') (30 m × 0.25 mm i.d., 0.25 µl film thickness) fused-silica capillary column. Helium (at 0.8 ml/min) was used as a carrier gas. Samples were injected in the split mode at a ratio of 1:10 – 1: 100. The injector was kept at 250°C and the transfer line at 280°C. The column was maintained at 50°C for 2 min and then programmed to 260°C at 5°C/min and held for 10 min at 260°C. The MS was operated in the EI mode at 70 eV, in m/z range 42-350. Identification of the compounds was performed by comparing their retention indices and mass spectra with those found in literature (Adams, 1995) and supplemented by Wiley and QuadLib 1607 GC-MS libraries. The relative proportions of the essential oil constituents are expressed as percentages obtained by peak area normalization, all relative response factors being taken as one.

Isolation of the fungi

Maize samples were collected from various households and market centers in Western Kenya. Moulds were isolated from these samples using the direct plating technique. For each sample, 20 seeds were picked randomly, surface-sterilized in 2.5% sodium hypochlorite for 30 s and rinsed in three changes of sterile distilled water. Five seeds per plate were blotted with sterile filter paper and plated on Czapek Dox and potato dextrose agar containing 7.5% sodium chloride and 133 mg streptomycin sulphate (for 1 L of media). The plates were incubated at 25°C and monitored daily for fungal growth for seven days.

Identification of the isolated fungi

The resulting cultures were identified based on cultural and morphological characteristics using taxonomic keys (Pitt, 1979; Nelson *et al*., 1983: Kozakiewiez, 1989; Klich, 2002). Morphological features of fungi were studied and the major and remarkable macroscopic features that were looked at are colony diameter, colony color on agar and reverse, exudates and colony texture. Microscopic characteristics that helped in identification are conidia heads, stipes, color and length, vesicles shape and seriation, metula covering, conidia size, shape and roughness (Diba *et al*., 2007). The number of seeds showing each type of mould growth were determined and compared for each sample.

Antifungal assays

Paper disc diffusion inhibition test was used to screen for antimicrobial activity of the essential oil as described by Souza *et al*. (2005). One hundred micro-liters of mould suspension (approximately 10^6 spores/ml) were uniformly

spread on sterile potato dextrose agar media containing 7.5% sodium chloride and 133 mg streptomycin sulphate (for 1 L of media) in Petri dishes. Sterile sensitivity discs were soaked with 10 µl of the essential oil and placed at the center of the inoculated culture plates. The plates were incubated at 25°C for 7 – 10 days. At the end of the incubation period, diameters of the inhibition zones were measured to the nearest millimeter (mm). Nystatin discs (100 µg) were used as positive control. Nystatin discs were placed on the centre of the inoculated culture plate and incubated for 7-10 days at 25°C. At the end of the incubation period, diameters of the inhibition zones were measured to the nearest millimeter (mm).

Determination of the Minimum Inhibition Concentration of the oil

One milliliter of mould suspension (approximately 10^6 spores) was uniformly spread on sterile potato dextrose agar media in petri dishes. Serial dilutions of the oil were done using pure dimethyl sulfoxide (DMSO) which was also used as the control. The oil was diluted to the following serial geometric dilutions: 50%, 25%, 12.5%, 6.25% and 3.13%. Sterile Whatman filter paper discs (No. 1, 6 mm in diameter) were soaked with 10 µl of the oil and placed at the center of the inoculated culture plates and then incubated for 7 to 10 days. The experiment was in three replicates. The resultant minimum inhibition zones were used to determine the range for MIC analyses.

Statistical analysis

Data on inhibition zones was analyzed using Microsoft Office Excel 2007 to derive means and standard deviations.

RESULTS

Chemical composition of *M. littoralis* essential oil

Fresh leaves from *M. littoralis* yielded oil on hydro-distillation and was analyzed by gas chromatography-mass spectrometry (GC-MS) and also evaluated for antifungal activity against 25 fungal species isolated from maize. Most of the oil components could not be identified using the available databases. Some of the oil constituents are summarized in table 1. The structures of the eight compounds are in figure 1.

Antifungal activity of oil against *Aspergillus* species

The oil was active against all the fungi screened except for *A. parasiticus* (Table 2). Among the *Aspergillus* species the highest inhibition activity of the oil was observed against *A. flavus* and *A. flavipe*s with inhibition zones of 18.33 mm and 17.50 mm respectively and similar MIC of 103.00 mg/ml. The concentrations of the oil were generally in the range of ten times more than the standard antifungal (Nystatin) and they showed marked antifungal activities as evidenced by their zones of inhibition. The oil activity against *A. flavus* was quite high compared to standard nystatin which was used as the positive control i.e. nysatin had inhibition zones of 12.50 mm. Nystatin activity against *A. flavipes* was higher than that of the oil extract though the difference was not quite high. Though *A. niger* and *A. ochraceus* showed lower inhibition zone than the two species above they showed lower MIC of 26.00 mg/ml and 51.00 mg/ml respectively implying the oil was more active against the two species. High activity of the oil was also recorded against *A. fumigatus* where inhibition zone of 13.33 mm was observed and MIC of 103.00 mg/ml recorded. The inhibition activity of the nystatin against *A. fumigatus* was quite high compared to the oil. The lowest activity of the oil was observed against *A. sparsus* and *A. versicolar*. These two species had the same inhibition zones of 7.50 mm and the highest MIC of 410m mg/ml. Inhibition zone of 20.33 mm was recorded for *A .versicolar* when nystatin was used implying that nystatin activity against this species was close to three times that of the oil. Inhibition zone of 12.00 mm was recorded when nystatin was used against *A. sparsus* implies that this species was also showing high resistance to nystatin.

Antifungal activity of oil against *Fusarium* species

Among the *Fusarium* species the highest inhibition activity of the oil was observed against *F. proliferatum*. This species had inhibition zone of 19.33 mm and an MIC of 51.00 mg/ml (Table 3). The standard activity against *F. proliferatum* was almost half that of the oil. Also high activity of the oil was recorded against *F. moniliforme, F. solani* and *F. subglutinans* since the three species had inhibition zones greater than 14.00 mm and MIC of 51.00 mg/ml for *F. moniliforme* and *F. subglutinans* and 103.00 mg/ml for *F. solani*. The nystatin activity against *F. moniliforme* and *F. subglutinans* was comparable to that of the oil but nystatin inhibition activity against *F. solani* was greater about two times than the oil. *F. culmorum* and *F. avenaceum* showed resistance to both oil and nystatin since inhibition zones slightly higher than 10.00 mm was recorded when nystatin and oil extract were used against the two species. The highest resistance to oil was observed against *F. nivale* and *F. oxysporum* where the highest MIC of 410 mg/ml being recorded. Nystatin inhibition activity against *F. oxysporum* was close to that of oil while inhibition activity against *F. nivale* was higher than threefold that of the oil.

Antifungal activity of oil against *Penicillium* species

The highest activity of the oil was observed against *P. purporogenum* and *P. islandicum* with inhibition zones of 39.17 mm and 29.60 mm respectively and the lowest MIC of 26.00 mg/ml (Table 4). The nyatatin activity against these

Table 1. Chemical composition of *M. littoralis* essential oil.

Compound Number	R.T (min)	Compound Name	%Concentration
1	12.96	(-)-*Beta*-necrodol	0.38
2	14.25	(1.α.(s*),5-β.)-1-(phenyl sulfinyl)-bicyclo-[3.3.1]nona-3-one	4.59
3	15.22	(2R,3R)-spiro-2,3-dimethyl-1,4-dioxolane-5,1',3'-cyanocyclopentane	0.40
4	11.005	1-Adamantyl methyl ketone	1.25
5	20.17	3,5-dimethoxybenzoic acid	0.90
6	22.12	(+,-)-tetramisole	5.22
7	25.65	*Cis*,trans-2,3-dimethylthiochroman-4-carbonitrile	1.92
8	38.55	1,14-dibromotetradecane	0.02

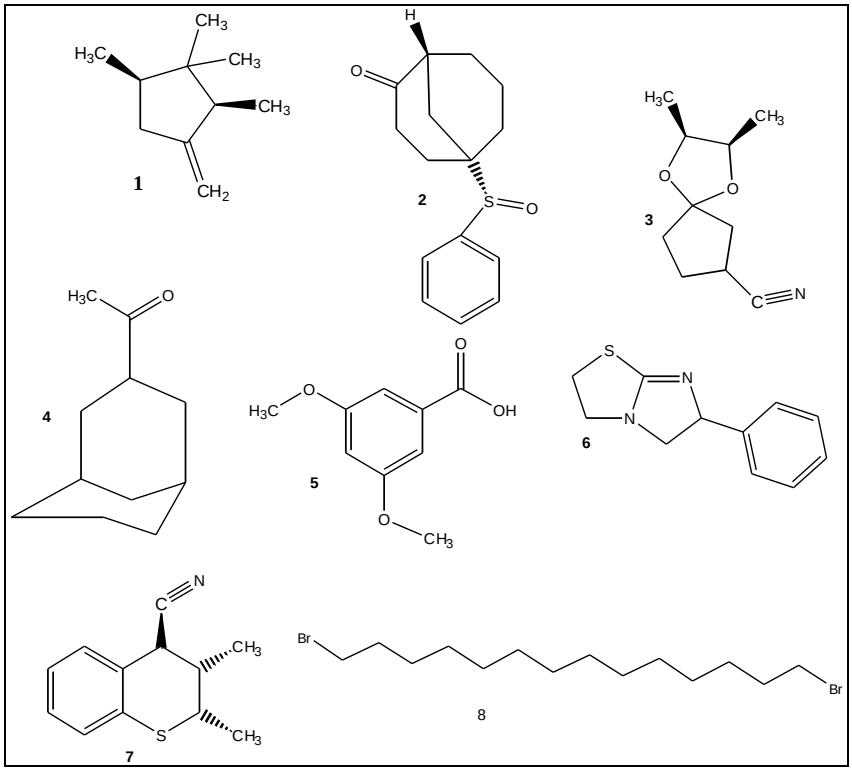

Figure 1. Structures of the compounds constituents of the oil

species was half of the oil. High activity of the oil was also recorded against *P. wortmani* with MIC of 51.00 mg/ml.

The nystatin inhibition activity against *P. wortmani* was similar to the oil. Though *P. rugulosum* and *P. viridicatum* had different inhibition zones similar MIC of 103 mg/ml was observed for the two species. Slightly lower inhibition zone than the oil was observed for *P. rugulosum* when nystatin was used but the nystatin inhibition activity against *P. viridicatum* was slightly higher than the oil. The highest resistance to the oil was observed in *P. rubrum, P .expansum, P. claviforme* and *P. digitatum* where MIC of 410.00 mg/ml though their inhibition zones were different.

Table 2. Inhibition zones (mm) of *M .littoralis* oil on *Aspergillus* species seven days after inoculation.

Fungi	Essential oil concentration (mg/ml) *10^2						Nystatin	Control	MIC (mg/ml)
	8.20	4.10	2.05	1.03	0.51	0.26			
A .niger	15.67 ± 1.25	13.33 ± 0.47	11.00 ± 0.82	8.33 ± 0.47	6.17 ± 0.24	0.00	19.67 ± 0.47	0.00	26.00
A .fumigatus	13.33 ± 0.47	10.00 ± 0.81	6.33 ± 0.24	0.00	0.00	0.00	20.33 ± 0.47	0.00	103.00
A .ochraceus	15.33 ± 0.47	12.00 ± 0.81	7.50 ± 0.41	6.17 ± 0.24	0.00	0.00	16.00 ± 0.82	0.00	51.00
A .flavipes	17.50 ± 0.50	11.33 ± 0.47	6.95 ± 0.24	0.00	0.00	0.00	20.67 ± 0.41	0.00	103.00
A .flavus	18.33 ± 0.47	12.33 ± 0.47	7.17 ± 0.62	0.00	0.00	0.00	13.00 ± 2.16	0.00	103.00
A .sparsus	7.50 ± 1.50	0.00	0.00	0.00	0.00	0.00	12.00 ± 0.00	0.00	410.00
A .versicolar	7.50 ± 0.50	0.00	0.00	0.00	0.00	0.00	20.33 ± 0.47	0.00	410.00
A .parasiticus	0.00	0.00	0.00	0.00	0.00	0.00	14.50 ± 0.41	0.00	-

Table 3. Inhibition zones (mm) of *M. littoralis* oil on *Fusarium* species seven days after inoculation.

Fungi	Essential oil concentration (mg/ml) *10^2						Nystatin	Control	MIC (mg/ml)
	8.20	4.10	2.05	1.03	0.51	0.26			
F. moniliforme	15.33 ± 1.11	13.33 ±0.44	10.00±0.67	6.83±0.67	0.00	0.00	18.67 ±1.25	0.00	51.00
F. proliferatum	19.33 ± 0.87	15.00 ±0.67	11.00 ±0.56	6.50±0.33	0.00	0.00	10.63±0.45	0.00	51.00
F .solani	14.67 ± 1.11	11.33 ±0.89	6.5 ± 0.33	0.00	0.00	0.00	28.33 ±1.70	0.00	103.00
F. subglutinans	15.67 ± 0.89	11.67±1.11	8.87 ± 0.47	6.33±0.22	0.00	0.00	19.33 ±0.47	0.00	51.00
F. culmorum	12.67 ± 0.94	8.63 ± 0.45	6.23 ± 0.33	0.00	0.00	0.00	12.50 ±0.41	0.00	103.00
F. avenaceum	12.33 ± 0.44	8.00 ± 0.67	6.20 ± 0.27	0.00	0.00	0.00	12.33 ±0.47	0.00	103.00
F. oxysporum	7.83 ± 0.85	0.00	0.00	0.00	0.00	0.00	11.33 ±0.47	0.00	410.00
F. nivale	9.67 ± 0.470	0.00	0.00	0.00	0.00	0.00	33.67 ±0.94	0.00	410.00

DISCUSSION

The analyzed essential oil of *M. littoralis* in this study showed antifungal activities against mycotoxigenic fungi from three different genera. The antifungal activity of the oil could have been contributed by the oil constituents. Presence of compound **(2)** in the oil (Figure 1) could have contributed to the antifungal activity of the oil due to the components like sulfinyl making up the molecule. This is because compounds containing sulfinyl have been reported

Table 4. Inhibition zones (mm) of *M. littoralis* oil on *Penicillium* species seven days after inoculation.

Fungi	Essential oil concentration (mg/ml) *10^2						Nystatin	Control	MIC (mg/ml)
	8.20	4.10	2.05	1.03	0.51	0.26			
P. purporogenum	39.17 ± 0.53	22.00 ± 0.29	10.33 ± 0.81	7.00 ± 0.33	6.00 ± 0.0	0.00	16.33 ± 0.94	0.00	26.00
P. islandicum	29.60 ± 0.47	18.17 ± 0.81	10.33 ± 0.58	6.96 ± 0.58	6.07± 0.12	0.00	15.33 ± 0.47	0.00	26.00
P. rugulosum	20.20 ± 0.26	9.97 ± 0.58	7.87 ± 0.81	0.00	0.00	0.00	15.50 ± 0.41	0.00	103.00
P. wortmani	17.33 ± 0.57	11.17 ± 0.76	6.67 ± 0.58	6.00 ± 0.00	0.00	0.00	17.83 ± 0.24	0.00	51.00
P. viriidicatum	10.33± 0.58	7.40 ± 0.96	6.17 ± 0.29	0.00	0.00	0.00	16.67 ± 0.47	0.00	103.00
P. rubrum	8.17 ± 0.24	0.00	0.00	0.00	0.00	0.00	18.33 ± 0.47	0.00	410.00
P .expansum	7.50 ± 1.08	0.00	0.00	0.00	0.00	0.00	17.67 ± 1.25	0.00	410.00
P. claviforme	9.33 ± 0.47	0.00	0.00	0.00	0.00	0.00	16.67 ± 0.94	0.00	410.00
P. digitatum	7.17 ± 0.62	0.00	0.00	0.00	0.00	0.00	18.33 ± 0.47	0.00	410.00

to have antifungal activity (Weiming *et al*., 2011, Prasanthy *et al*., 2011). Compound **(3)** could have also contributed to the activity of the oil extract against the various fungal species because of the dioxolane ring in the molecule. This is because compounds containing dioxolane have been reported to have high antifungal activity against plant pathogens *F. oxysporum* and *P. digitatum* (Adel *et al.,* 2005, Ganiyat *et al.,* 2011).

Adamantane derivatives to which compound **(4)** belong to have been reported to possess antibacterial and antifungal activities (Hemant *et al.,* 2011). Therefore the presence of this compound may have contributed to the fungal inhibition activity of the oil extract. Compound **(5)** which was identified as 3, 5-dimethoxybenzoic acid could have also contributed to the antifungal activity of the oil. It has been shown that natural compounds such as derivatives of benzoic acid can serve as potential alternative conventional antimicrobial agents (Beekrum *et al*., 2003). Benzoic acid has been shown to inhibit the growth of A*. flavus, A. fumigatus and A. terreus* and the activity of the acid increases with introduction of substituents like methoxy group. Also the activity of the benzoic acid derivatives depends on the position of the substituent i.e. the further the substituent is from the carboxyl group the higher the activity of the compound (Jong *et al*., 2010). Further, the oil antifungal activity can be attributed to compound **(6)** which is an imidazole. Imidazole is a planar five member hetero atomic molecule with pyrrole type and pyridine type annular. Imidazole derivatives are widely used in antifungal chemotherapy because of their broad spectrum and availability (Zahra *et al*., 2011). It has been recognized that imidazole acts with at least two distinct mechanisms. One is the inhibition of ergosterol biosynthesis at low concentration which is responsible for fungi static action. The other is physicochemical cell membrane damage exerted at high concentration which causes fungicidal effects (Beggs and Hughes, 1987). Carbonitriles compounds to which compound **(7)** belong to have been reported to have antifungal activity. Wilamowski *et al*., (2001) reported that compounds containing 2-carbonitriles had activity against plant pathogens *F.culmorum* and *P. expansum*. Further high activity was recorded for carbonitriles with hydrogen atoms and short alkyl groups like methyl group at position 3 or 4. Carbonitriles with a methyl group in position 2 have also been reported to have antifungal activity against the two pathogens above (Bortomiej et al., 2006). Therefore the presence of compound (7) in the oil could have contributed to the fungal inhibition activity of the oil. Compounds **(1)** and **(8)** are not known to show any antifungal activity.

The antifungal activity of the oil varied with its concentration and the kind of fungal species, indicating that its activity is proportional to its concentration. The highest activity of the oil was observed against *P. purporogenum, P. islandicum* and *A .niger* with an MIC of 26.00 mg/ml and the highest resistance was observed from all species with an MIC of 410.00 mg/ml. *A. flavus* has also been known to show resistance to basil and spearmint oils (Solimana and Badeaa, 2002). However, the inhibition level of the oil against this species in the current study was higher than that of the reference Nystatin indicating that, despite the resistance, the oil is relatively more effective.

CONCLUSIONS

The results obtained show that the essential oil isolated from *M littoralis* is a promising antifungal agent.

ACKNOWLEDGEMENTS

The authors are grateful to the Lake Victoria Basin Research Initiative (VicRes) for the financial support.

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