Evaluation of Natural Products as Possible Alternatives to Methyl Bromide in Soil Fumigation

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Abstract

Methyl bromide (CH_3Br) is a widely used fumigant in crop production and commodity preservation worldwide. It escapes to the stratosphere and releases bromine atom (Br), which contributes to significant destruction of ozone (O_3) layer. It is therefore necessary to explore alternatives to CH_3Br that are environmentally safe and economically viable. We present the results of the inhibitory activity of crude extracts from some Kenyan medicinal plants against the soil pathogens, *Fusarium oxysporum, Alternaria passiflorae* and *Aspergillus niger.* Crude organic extracts from *Warburgia ugandensis* Sprague, *Azadirachta indica* A. Juss and *Tagetes minuta* were active against the test soil pathogens while those from *Urtica massaica* were not. Chromatographic purification of the crude extract from *W. ugandensis* provided two pure compounds, muzigadial and muzigadiolide. The minimum inhibitory concentration (MIC) for muzigadial ranged from 5 to 100μg/ml for the different soil pathogens. Muzigadiolide was not active at concentrations tested. Greenhouse tests of *W. ugandensis* extracts against *F. oxysporum* pathogen showed the most effective inhibitory concentration to be at least 5 mg/ml.

Key words: Methyl bromide, ozone layer, soil fumigation, plant extracts, soil pathogens, and inhibitory concentration.

1.0 Introduction

Methyl Bromide (CH_3Br) has been used as a fumigant for over 60 years. An important valuable property of methyl bromide is the broad spectrum of activity against several pests. The largest single global use is as a soil fumigant (Wang et al., 1997). The ease of application of CH_3Br along with its reliability and speed of action have led to its widespread use in agricultural systems that produce economically important crops. Methyl bromide has a number of technical and legislative limitations that have led to restrictions on its use.

Methyl bromide can have adverse effects on a number of commodities; it is phytotoxic and causes taint and odors. Repeated fumigation with methyl bromide may result in the production of bromide ions (Br^-) residues that rapidly accumulate in the atmosphere. This is why some European countries are concerned about its toxicity on ground water and its ozone depleting potential. In November 1992, methyl bromide was listed as ozone depleting substance by the fourth meeting of the parties to the Montreal protocol on substances that deplete the Ozone Layer, in Compenhagen (Albritton and Watson, 1992). Since then, plant health services throughout the world have been advocating for the phasing out of methyl bromide. The Methyl Bromide Technical Option Committee (MBTOC) (Yuen et al., 1991) has proposed the technical availability of chemical and non-chemical alternatives of methyl bromide. Non-chemical alternatives include cultural practices, biological control, organic alternatives and physical methods (Watson et al., 1992). Chemical alternatives can either be fumigants or non-fumigants. The chemical alternatives have major setbacks: are phytotoxic, skin and eye irritants, sensitizers, genotoxic, and carcinogenic. Non-fumigants, such as organophosphates, are neurotoxins and do not exhibit broad range disinfestations properties typical of CH_3Br. Encouraged by recent use of crude plant extracts in combating *Striga* weeds in Nigeria soils, (Rugutt and Berner, 1998), the present study examines the anti-fungal activity of extracts from *Warburgia ugandensis* (*Conellaceae*), *Azadirachta indica* (neem tree, *Meliaceae*), *Tagetes minuta* (Mexican marigold, *Asteraceae*) and *Urtica massaica* (stinging nettle, *Urticaceae*). In the present study, the test microorganisms that were used belong to the genus *Fusarium*, *Alternaria* and *Aspergillus*.

The tomato, *Lycopersicon esculentu,* (used for greenhouse trials) belongs to a large family of plants called the *Solanaceae*, which contains many important food crops including potatoes and aubergine (egg plant). Tomatoes are one of the most widely grown vegetables in almost four million hectares worldwide (FAO, 2003). Tomatoes can be grown either in the field or under greenhouse conditions. In tropical and subtropical climates, there is a great deal of soilborne diseases and weed pressure, mainly due to high temperatures which normally cause massive weed germination and sprouting, and soil pathogen growth and reproduction. *Fusarium oxysporum* causes vascular wilt in a variety of crops and is a serious disease in tomato. It also survives in the soil for long periods. In the last decades, this situation has been

controlled with in-bed injections of methyl bromide. Suitable alternatives to methyl bromide need to be developed (Gilreath, et al., 2004).

2.0 MATERIALS AND METHODS

2.1 Plant Collection

All plant materials were collected in Kenya and identified at the Department of Botany, Moi University; voucher specimens were deposited at the Herbarium.

2.2 Plant Extraction and Isolation

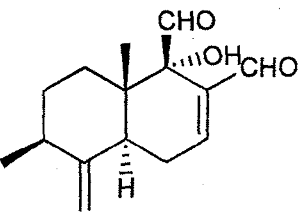
Wet stem bark (3 kg) of *Warburgia ugandensis* was extracted with methanol. The resulting crude extract was concentrated and partitioned into water and chloroform fractions. A portion (15 g) of the concentrated chloroform fraction was filtered (under vacuum) through a column packed with Thin Layer Chromatography (TLC), grade Merck silica gel using 150 ml ethyl acetate (EtOAc). The filtrate was concentrated, packed in a pre-packed Merck silica gel column (Φ=40mm) and then subjected to flash chromatography. The column was eluted with n-hexane/EtOAc mixtures in order of increasing polarity. This separation process afforded 1.03 g of compound (1) and 0.25 g of compound (4), structures as in figure 1.

Seeds and leaves from *Azadirachta indica*, aerial parts of *Tagetes minuta* and *Urtica massaica* leaves were soaked in methanol separately. The resulting crude extracts were concentrated under reduced pressure to yield dark pastes, which were subjected to anti-fungal assay.

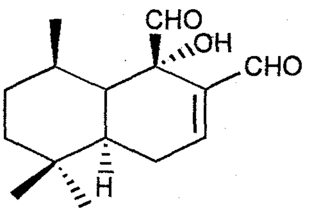
2.3 Structural Elucidation

The IR data of the two compounds were obtained from their spectra run using Shimadzu-IR408 spectrophotometer. Melting points were obtained by use of Reichet Thermovar apparatus. All 1D (^{1}H) NMR spectra were recorded at 298K using a Bruker AMX300 MHz spectrometer. Chemical shifts are expressed in δ (ppm) scale down field from TMS (internal reference standard).

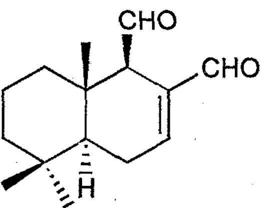
Figure 1: Structures 1 to 5



Muzigadial (1)

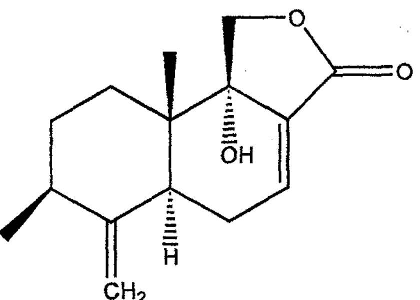


Warbuganal (2)

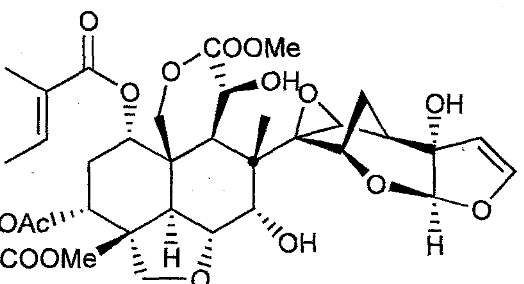


Polygodial (3)

Source Kioy et al., 1990



Muzigadiolide (4)



Azadirachtin (5)

Source Van der Nat et al., 1991

Table 1: Antifungal Activity of Crude Extracts

Tests microorganism	Disc crude extract content (mg)	Diameter of inhibition (mm)						
		W. ugandensis			*A. indica*		*T. minuta* Aerial part (MeOH) extract	*U. massaica* Leaves (MeOH) extract
		Stem bark ($CHCl_3$) fraction	Leaves (MeOH) extract	Leaves (hexane) extract	Leaves (MeOH) extract	Seeds (MeOH) extract		
Fusarium oxysporum	100	24.0	16.0	15.5	14.5	16.0	15.0	No activity
	50	21.5	15.5	13.0	13.0	13.5	13.0	No activity
	20	18.0	14.0	12.5	11.0	12.0	12.5	No activity
	10	15.5	12.0	11.5	10.0	9.0	10.0	No activity
	1	11.0	10.0	10.0	7.5	8.5	9.5	No activity
Alternaria passiflorae		Stem bark ($CHCl_3$) fraction		Leaves (MeOH) extract				
	100	23.4		18.0	14.0	15.0	15.0	No activity
	50	21.5		15.0	12.5	13.5	13.0	No activity
	20	19.0		14.5	11.0	12.5	12.5	No activity
	10	15.0		10.5	10.0	9.0	9.5	No activity
	1	11.5		9.5	7.0	7.5	6.5	No activity

2.4 Inhibitory Activities of Crude Extracts

Diffusion method (Rosaanairo and Ratsimamanga-Urveg, 1993) was employed in assessing the antifungal activity of crude extracts against *Fusarium oxysporum, Alternaria passiflorae* and *Aspergillus niger*. The clear zone of growth inhibition around the disk was measured and expressed as inhibition diameter.

2.5 Minimum Inhibitory Concentrations (MICs)

MICs of pure compounds were determined using the agar macro-dilution method (Rosaanairo and Ratsimamanga-Urveg, 1993). The lowest concentration at which no growth was observed visually was determined and indicated as MIC.

2.6 Greenhouse Tests

The pathogen, *Fusarium oxysporum*, was cultured in the laboratory two weeks prior to field experimentation using standard culturing methods (Rosaanairo and Ratsimamanga-Urveg, 1993).

Soils from a fertile field (virgin soils from forest cover, with high organic matter content) were pasteurised with aerated steam for sterilisation before use. The pots were sterilized by cleaning with distilled water, rinsed with sodium hypochlorite and air-dried. No nutrients were added to the soils. For soil infestation, the pathogen (*F. oxysporum*) spores, already cultured, were dissolved into 36 ml of sterile distilled water, to make the *Fusarium* inoculums stock solution. A 1 ml portion of this *Fusarium* inoculum stock solution was then diluted to 10 ml to make a total of 36 such dilutions. The 36 *Fusarium* inoculums dilutions were to cater for 9 out of 10 treatments, each in four replicates, totaling 36 pots. This was poured over the surface of the soil in each pot and allowed to infiltrate the soil. Four pots did not receive this treatment. The soils in the pots were allowed to stand in the green house for two weeks. To allow adequate conditioning of pathogen in the soil, distilled water was occasionally added before introducing crude extracts. A total of ten treatments were prepared. Seven treatments (in four replicates) of different concentrations of crude extract (100 ml each) were prepared: 1, 2, 5, 7, 10, 12 and 15 mg/ml respectively in 20% ethanol – water mixture and poured to soil surface. One treatment (four pots) had 100 ml of the solvent (20% ethanol- water) with no crude extract (negative controls). A single treatment (four pots) was prepared with the pathogen but

without either the crude extract or the solvent (negative controls). One treatment (four pots) with no pathogen inoculums, did not receive the crude extract and the solvent treatment (positive controls). The soils were allowed a period of two weeks with occasional watering before planting.

2.7 Determination of Antifungal Infection

Seeds of *lycopersicon esculentum* (a certified moneymaker tomato) that is prone to *Fusarium wilt* were planted 2 cm deep in the soil. Five seeds were planted in each pot for ten different treatments. Thinning was done to have two plants per pot after germination. The completely randomized block design was replicated four times. The pots were arranged randomly at a distance of 60 cm from each other and at a distance of 30cm from each other within the block. The indicators for fungal leaf infection (*Fusarium wilt*) that were monitored were wilted leaves, vascular discoloration of the hypocotyl tissues, branches and leaves exhibiting wilting and chlorosis, necrosis, premature defoliation and eventual plant death. Data indicating the severity of infection were taken for 21, 23, 25, 27, 30, 35, 40, and 50th day after planting and recorded on a one 1-9 scale as described in literature (Schoonhoven and Pastor, 1987).

3.0 RESULTS AND DISCUSSION

3.1 Characterization of Muzigadial and Muzigadiolide

The spectral data (NMR and IR) and melting point data obtained for the two compounds was in agreement with the literature data (Kioy et al., 1990). The compounds were identified as mizigadial and muzigadiolide (structure 1 and 4, fig.1).

Muzigadial (1): Crystals from EtOAc, hexane, melting point, 125^0C. ^{1}H NMR (300 MHz, $CDCl_3$): δ(ppm): 9.64 (1H, s, H-12), 9.44 (1H, H-11), 7.26(1H, t, H-7), 4.93, 4.76 (2x1H, 2x br s, CH_2), 4.08 (1H, s, 9-OH), 2.62 (1H, m, H-5), 1.08 (3H, d, 3-Me) and 0.89 (3H, s, 10-Me). IR $\ddot{\upsilon}_{max}$ cm^{-1} 3460, 2950, 2850, 1715, 1670, and 1630.

Muzigadiolide (4): Needles from EtOAc-hexane, melting point 142 ^{0}C. ^{1}H NMR (300MHz, $CDCl_3$): δ(ppm): 7.19 (1H, br, H-7), 4.93, 4.75 (2x1H, 2 x Br s, $=CH_2$), 4.31, 4.26 (2H, 11-CH_2), 2.62 (1H, m, H-5), 1.10(3H, d, 3Me) and 0.74 (3H, s, 10-Me). IR $\ddot{\upsilon}_{max}$ cm^{-1} 3400-3450, 2800, 1750, 1725, 1680 and 1635.

3.2 Antifungal Activity of Crude Extracts

Crude extracts from *W. ugandensis, A. indica,* and *T. minuta* inhibited the growth of *Fusarium oxysporum. Warburgia ugandensis* displayed inhibition diameters of 10.0mm for both MeOH and hexane leaves extracts, and 11.0mm for the stem bark extract at 1mg disc content. *Azadirachta indica* showed inhibition diameters of 7.5mm and 8.5mm for the MeOH leaves and MeOH seeds extracts for 1mg disc content. *Tagetes minuta* displayed an inhibition diameter of 9.5mm against *F. oxysporum* for 1mg disc content.

Warburgia ugandensis stem bark extract inhibited the growth of *Alternaria passiflorae* with an inhibition diameter of 11.5mm for 1mg disc content. Its MeOH leaves extract had an inhibition diameter of 9.5mm for 1mg disc content against *A. passiflorae. Azadirachta indica* leaves and seeds methanol extracts showed inhibition diameters of 7.0mm and 7.5mm respectively against *Alternaria passiflorae* for 1mg disc content. However, crude extract from *U. massaica* leaves showed no activity against *Fusarium oxysporum and Alternaria passiflorae.* As deduced from the diameters of inhibition by the crude extracts from *W. ugandensis* displayed higher activity than those from *A. indica* and *T. minuta. Azadiracha. indica* and *T. minuta* exhibited similar activity patterns against *Fusarium oxysporum. W. ugandensis* MeOH leaves extract displayed higher activity than the hexane extract. The data is presented in table 1. Inhibition diameters higher than 8mm are generally considered as positive results (Rosaanairo and Ratsimamanga-Urveg, 1993).

Fractions from crude (leaves) extracts of *W. ugandensis* showed significant activity against *Fusarium oxysporum* with inhibition diameters raging from 8.0 to 10.3mm for 1mg disc content to 13.0mm for 10mg disc content (Table 2). The antifungal activity by various fractions from *W. ugandensis* crude extract indicates the presence of more than one active component in the extract.

The Minimum Inhibitory Concentrations (MIC) for muzigadial was 50μg/ml for *F. oxysporum* and 5μg/ml for *A. niger.* The MIC for muzigadial against *A. passiflorae* was greater than 100μg/ml. Muzigadiolide showed no activity against the test pathogens. The data is presented in table 3. The difference in activity of compounds 1 and 4 can be attributed to the differences in the dialdehyde functional group (Jansen et al, 1989). A series of unique sesquiterpene 1,4-dialdehydes isolated from these plants exhibit broad antibacterial and

antifungal activities. Muzigadial (1), warbuganal (2), and polygodial (3) (fig.1) obtained from these plants show similar antibacterial spectra (Kubo and Taniguchi).

Table 2: Activity of Methanol Extract from leaves of *W. ugandensis against F. oxysporum*

Test Microorganism	Fractions	Disc content (mg)	Inhibition Diameter (mm)
Fusarium oxysporum	F_1	10	No growth inhibition
		1	No growth inhibition
	F_2	10	10.5
		1	8.0
	F_3	10	13.0
		1	10.0
	F_4	10	12.0
		1	10.3
	F_5	10	No growth inhibition and other fractions below F_5 had similar results.

F= FRACTION

F_1 to F_5 represents column fractions from *W. ugandensis* (methanol) leaves crude extract

Table 3: MICs for muzigadial (1) and muzigadiolide (4) against *F. oxysporum, A. Passiflorae* and *A. niger*

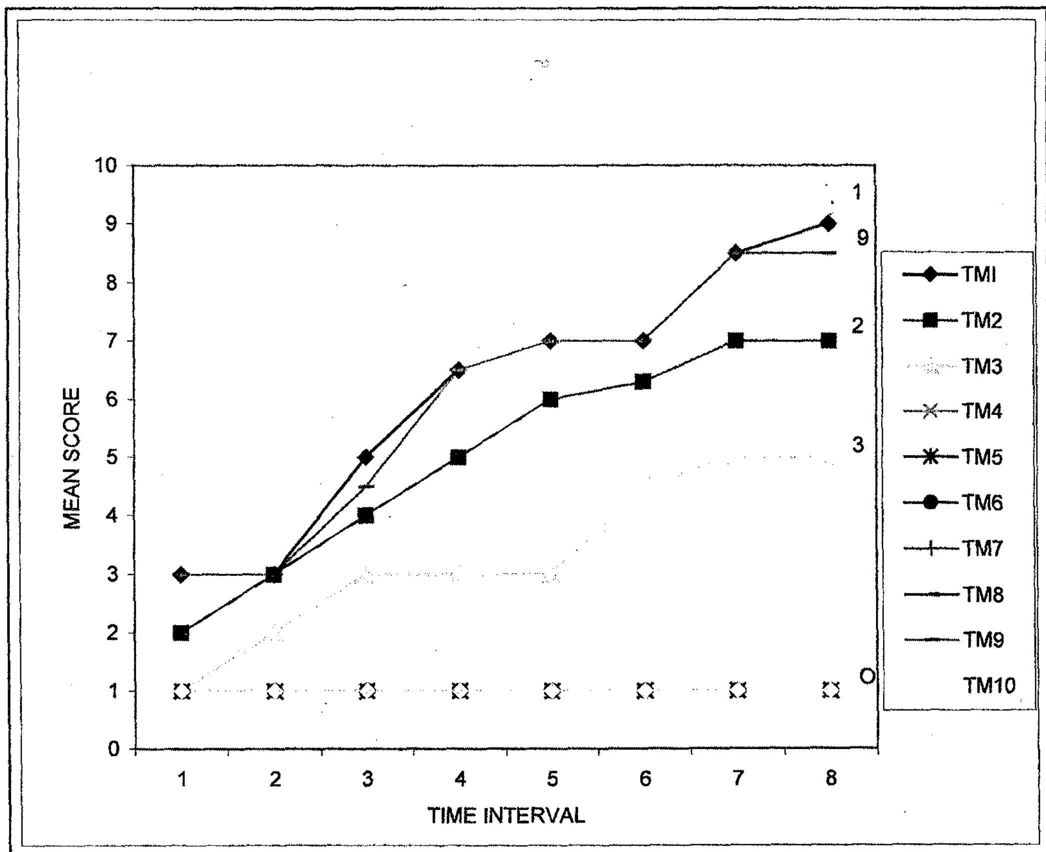
Soil Pathogens	MIC (µg/ml)	
	Muzigadial (1)	Muzigadiolide (4)
Fusarium oxysporum	50	No Activity
Alternaria passiflorae	> 100	No activity
Aspergilus niger	5	No Activity

Azadirachtin (5) is the most common interesting constituent of neem seeds (*Azadirachta indica* A juss, family *meliaaceae*). In principle, azadirachtin is notable for chemical complexity and biological activity; it exhibits a wide range of biological activities (van der Nat et al., 1991).

3.3 Greenhouse Experiments

The greenhouse data were subjected to analysis of variance, DMRT, and LSD tests. The difference among the treatments for all the days scored were significant at 1 and 5% level of significance. Application of 1mg/ml and 3mg/ml crude extracts was not effective in controlling the soil pathogen. Application of crude extract of 5 mg/ml and above effectively controlled the pathogen. The solvent (20% ethanol - water) had no effect on the pathogen. The severity of fungal infection for the days observed, expressed as a function of the mean score is as shown in figure 2.

Figure 2: Graph of severity of fungal infection for the days observed, expressed as a function of the mean score



TM=TREATMENT

SERIES=TREATMENT

Legends: Treatment 1: Pathogen alone; Treatment 2: 1 mg/ml of the crude extract; Treatment 3: 2 mg/ml; Treatment 4: 5 mg/ml; Treatment 5:7 mg/ml; Treatment 6:10 mg/ml; Treatment 7: 12 mg/ml; Treatment 8: 15 mg/ml; Treatment 9: pathogen plus solvent (20% ethanol - water); Treatment 10: No pathogen and no solvent; only sterilized soil.

The series-O in the graph represents an overlap of treatments 4, 5, 6, 7, 8, and 10.

ACKNOWLEDGEMENT

We are grateful for the partial scholarship offered to Anastasiah Njoki Ngigi by Moi University. We acknowledge the assistance given by Prof. M. S. Rajab in isolation of the compounds. Special thanks to the technicians and staff in the Departments of Chemistry, Forestry and Botany (Moi University) for their assistance.

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