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Research paper

Solvent-solvent fractionation can increase the antifungal activity of a *Melianthus comosus* (Melianthaceae) acetone extract to yield a potentially useful commercial antifungal product

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ABSTRACT

Fungal infections of plants cause major difficulties in agricultural food production and in storage of food especially due to the development of fungal resistance and the negative environmental impact of some chemical fungicides. The aim of this study was to develop a plant-based product that could be used to control plant fungal pathogens in agriculture. *Melianthus comosus* is a weedy shrub endemic to southern Africa. Its acetone leaf extract had good activity against plant fungal pathogens. We investigated the efficiency of ten extractants with varying polarities. Acetone was the best with a 52 times higher selectivity for antifungal activity against eight important fungal pathogens than methanol. The average minimum inhibitory concentration (MIC) was 100 µg/ml varying from 20 to 310 µg/ml against eight fungal pathogens. By using open column silica gel chromatography and bioautography the major antifungal compound was isolated and characterized as oleanolic acid. Two methods based on selective extraction and solvent-solvent extraction were used to potentize the activity of the extract by removing inactive polar and non-polar compounds. The product had an average MIC of 66 µg/ml varying between 20 and 160 µg/ml against 10 fungal pathogens. The product had low water solubility and was stable for a month at 55 °C. When used in a field trial on comfrey (*Symphytum officinale*) with a natural rust infection it was much more effective at a concentration of 0.2 mg/ml than the commercial product containing dicarboximide at a concentration of 1.5 mg/ml. The *Melianthus* extract treatment led to 50 infected leaves in the bed compared to 250 infected leaves of the dicarboximide treatment and extensive infection in the negative control. It appears that this product has the potential to be used in agricultural practice. This plant may become a new crop that may be useful for organic cultivation.

1. Introduction

Plant fungal pathogens cause enormous problems in the production of plants in agriculture (De Lucca, 2007). These losses can take place at different stages from the establishment of seedlings to affecting different parts during growth of the plant and finally to spoilage of silage, fruit and seed. This leads to very large annual losses of plant products and plays an important role in food security. Several fungi also produce fungal toxins when food is infected. This can lead to life-threatening human health problems (Riley et al., 1993). Without the use of chemical fungicides, food production in the world would be seriously curtailed. There is evidence of emerging fungal diseases threatening biodiversity as well as plant and animal health (Fisher et al., 2012). Unfortunately, due to the improper use of fungicides there is an increasing development of resistance of some fungal pathogens to existing chemical fungicides. Furthermore, some of these fungicides are toxic to humans or other organisms and may have a negative effect on soil and water ecosystems.

Many scientists have started investigating the possible use of plant extracts to control fungal pathogens (Mdee et al., 2009). In some cases remarkable success was obtained (Eloff et al., 2006).

The Phytomedicine Programme was approached to investigate the potential use of a *Melianthus comosus* extract to treat skin infections, based on the published traditional use of the plant, by the managing director of Healthtech Laboratories, Dr Johan Oosthuizen. We found that an acetone extract had mediocre antibacterial activity, but when

Abbreviations: BEA, benzene:ethanol:ammonium hydroxide (90:10:1 v/v/v); CEF, chloroform:ethyl acetate:formic acid (5:4:1 v/v/v); Chl, chloroform; Ctc, carbon tetrachloride; Dcm, dichloromethane; Dee, diethyl ether; DMSO, dimethyl sulphoxide; EMW, ethyl acetate:methanol:water (40:5.4:4 v/v/v); EtOH, ethanol; EtAc, ethyl acetate; Hex, hexane; INT, *p*-iodonitrotetrazolium violet;; MIC, minimum inhibitory concentration; MS, mass spectrometer; NMR, nuclear magnetic resonance; Rf, retardation factor; TLC, thin layer chromatography; Wat, water

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Fig. 1. Leaves, flowers and fruit of *Melianthus comosus*.

we examined the antifungal activity, the extract had excellent activity. We decided to continue working on the antifungal activity.

Melianthus comosus (Vahl) is a shrub with several branches that can grow up to 3 m high. Touching or bruising any part of the plant gives off a repulsive smell. This leads to the common Afrikaans name kruidjie-roer-my-nie (shrub-do-not-touch-me). It is an attractive plant that has been used as an ornamental plant (Fig. 1), with the more appealing name of honeyflower because the flowers produce large quantities of nectar in Europe. Leaf poultices and leaf decoctions have been used externally to treat septic wounds and sores among other applications (Watt and Breyer-Brandwijk, 1962). There are 8 species of *Melianthus* that are all endemic to South Africa. *M. major* with larger leaves and growing in the less arid areas of South Africa has been used traditionally for the same indications. Both species are very easy to grow and have become naturalized in parts of Africa, America, Australia, India and New Zealand (Wink and van Wyk, 2008).

Unfortunately, the plant contains several very toxic bufadienolides (Anderson and Koekemoer, 1969) and ingestion of the plant has led to human and animal deaths (van Wyk et al., 2009). Due to its toxicity products from this plant could not readily be considered for development as a human or animal medicine. Consequently we decided to investigate the antifungal activity and potential use against plant fungal pathogens especially in the horticultural industry.

2. Experimental

2.1. Plant material

Plant material was collected from the toxic plant garden of the Faculty of Veterinary Science, University of Pretoria and identity was confirmed by Prof CJ Botha, a toxicologist and head of the Department of Paraclinical Sciences. Plants that were planted in an orchard by Healthtech Laboratories were also collected and later transplanted to the toxic plant garden. An herbarium specimen was deposited in the toxic plant herbarium of the Department of Paraclinical Sciences. Healthy leaves of the plant were collected, dried in the shade at room temperature and ground to a fine powder.

2.2. Extraction

Finely ground material was extracted with ten different extractants with different polarities at an extractant to plant material ratio of 10:1, vigorously shaken and the extract was obtained by centrifugation. The process was repeated twice more on the marc (the plant material remaining after the previous extraction) (Kotze and Eloff, 2002). The solvent was removed by a cold stream of air for volatile solvents or by vacuum rotary evaporation for the rest. The extract was dissolved in acetone to a concentration of 10 mg/ml because fungal growth is not affected by acetone concentrations as high as 40% (Eloff et al., 2007).

2.3. Fungal pathogens used in the study

The origin of the plant fungal pathogens used in this study is presented in Table 1

The fungi were maintained on potato dextrose agar. The conidia of fungi were harvested from an agar plate culture using a sterile cotton swab and suspended uniformly in potato dextrose broth. The inoculum of each fungal species was quantified by counting the number of conidia using a haemocytometer and the number of conidia was adjusted to

Table 1
Origin of fungal pathogens used in this study obtained from the University of Pretoria Fungal Collection (UPFC).

UPFC no	Isolate name	Origin/Area	Person isolated	Substrate/host	Isolation date
21	*Fusarium oxysporum*	Delmas, Gauteng	C Cronje	Maize roots	Jun-99
40	*Penicillium janthinellum*	Letaba Estate, Tzaneen	C Cronje	Citrus soil	Jan-99
43	*Colletotrichum gloeosporioides*	Pretoria	M Truter	*Phormium* leaves	Jun-00
72	*Penicillium expansum*	Unrecorded	E de Jager	Litchi fruit	Unrecorded
89	*Trichoderma harzianum*	Pretoria	M Truter	*Acacia* roots	Apr-00
411	*Aspergillus niger*	Letaba Estate, Tzaneen	C Cronje	Citrus soil	Jan-99
454	*Aspergillus parasiticus*	Letaba Estate, Tzaneen	C Cronje	Citrus soil	Jan-99
RS2366	*Rhizoctonia solani*	KwaZulu-Natal	M Truter	Soil, potato	May-01
–	*Pythium ultimum*	Johannesburg	M Truter	*Lisianthus* roots	Mar-05
	Phytophthora nicotiana	Johannesburg	M. Truter	Unrecorded	Mar-05