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Antifungal Acetylinic Thiophenes from *Tagetes minuta*: Potential Biopesticide

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Summary

Apart from thiophenes, which possess wide range of biocidal activity, aerial parts of *Tagetes* sp contain essential oil. Oil components were reported to have antifungal activity, thus making whole plant of Tagetes very useful for exploiting as natural fungistatic agent. In the present study, *Tagetes minuta* grown in north western Himalayan condition were evaluated for its potential for use as antifungal agent. Flower essential oil showed minimal antifungal activity. Whereas, leaf essential oil was found significant antifungal activity against three phytopathogenic fungi out of eight tested fungi. ED_{50} values were 165, 175 and 110 µg mL^{-1} against *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Sclerotium rolfsii*, respectively. Thiophene rich extract of *Tagetes minuta* was found comparatively lesser active (ED_{50}: 233-484 µg mL^{-1}) than leaf essential oil against the same fungi. The present study shows that essential oil from leaves and thiophene rich extracts from marigold roots have significantly good antifungal activity against a number of soil borne and foliar plant pathogens. The easy availability of these plants makes it an attractive potential candidate for development of natural fungicide.

Introduction

Plant secondary metabolites and their derivatives have been evaluated as viable alternatives to the persistent and less environmentally friendly synthetic fungicides (Dixon, 2001). Although a large number of phytochemicals are known for their insect control properties, only a few of them are known to impart antifungal activity. The use of pesticides of natural origin is becoming appealing because of the problem of environmental pollution arising from the use of persistent pesticides. To move forward in the discovery of natural plant metabolites, plant extracts were screened for potential candidates with antifungal activity (Crouse, 1998). Some of the most promising botanicals for use as pesticides have been extracted from species of plants in the families Meliaceae, Rutaceae, Asteraceae, Annonaceae, Labiatae, and Canellaceae (Jacobson, 1989). Although much of the literature on natural products in agricultural field concerns insect control, a smaller but emerging body of papers has reported the effectiveness of plant extracts and essential oils for controlling microbial pathogens of plants.

Marigold (*Tagetes* sp.), belonging to the Asteraceae family, is a commonly occurring plant all over the world and is well known for a wide range of biological properties. The plant has been credited with having anticancerous and antiageing effects (Block et al., 1992). It contains carotenoids which is used as food colorants and feed additives (Timberlake and Henry, 1986). Besides this the plant also has pharmacological properties as it contains flavonoids (Terschuk et al., 1997). The foliar parts of this plant possess essential oils, known for antibacterial and insecticidal properties (Piccaglia et al., 1997); and thiophenes which have a marked biocidal activity (Hulst et al., 1989). These polyacetylene derivatives, the activity of which depends on photodynamic activation (Hudson and Towers, 1991), act as antibiotics, insecticides, nematicides and fungicides (Gommers, 1981; Mares et al., 1990; Hudson, et al., 1983; Romagnoli et al., 1994, 1998). Further, constituents of essential oil, mainly (*Z*)- and (*E*)-ocimenones, along with piperitone, piperitenone, limonene, tagetone and caryophyllene, are the major terpenes present in leaves (Vasudevan et al., 1997). In addition, Hethelyi et al. (1986) assumed that the presence of linalool and linalyl acetate characterizes Indian *Tagetes patula* oil. Due to the high degree of chemodiversity observed within essential oils of *Tagetes* sp., biological activities also subjected to vary. However, even though the various properties of the *Tagetes* plant are well known, less attention has been focused on the studies of biological activity of essential oils from *Tagetes* species.

Therefore, the present work was aimed at developing an antifungal formulation from the total plant extract. Interestingly, synergestic effect on egg hatchability of *Globodera rostochinensis* was observed when treated with a combination of *T. erecta* leaf and root extracts (Sasanelli and Vito, 1991). Furthermore, juvenile of *M. incognita* populations were more suppressed by stem and whole plant rather than root portions (Siddiqui and Alam, 1988). Present study was aimed to evaluate the antifungal activity of the whole plant parts. Thus *in vitro* antifungal activity was evaluated against eight important plant pathogenic fungi. Antifungal formulation may be developed using essential oil from leaves and thiopene rich extract from roots and shoots.

Materials and methods

General experimental procedure

All chemicals and reagents were procured from Merck® India Ltd. Double-distilled water was used throughout the analysis. GC-MS analysis was carried out on a TRACE-GC (Thermo Finnigan) coupled with fison MD-800 quadrupole mass detector and DB-17 capillary column (30m × 0.32 mm i.d.; film thickness 0.25 mm). Temperature programming was done from 75-250 °C at 5 °C min^{-1}. Helium was used as the carrier gas at 1 mL min^{-1} flow rate. Mass spectra was recorded over 40-400 amu range at 1 scan s^{-1} with ionization energy of 70ev and ion source temperature was 250 °C. The split ratio was 1:20.

Plant material

The plant material (leaves, flower and roots) was collected during the month of September from experimental farm of Vivekananda Institute of Hill Agriculture located at Hawalbagh (Altitude: -1220 m a.s.l., Latitude: -29°38'4.8"N, Longitude: -79°37'48.6"E) in North Western Himalayan region. The identification of the plants was confirmed from Department of Botany, Almora Inter College, Kumaon University, Almora, India. Mancozeb and β-pinene, a constituent of essential oil was used as positive control (procured from Merck®).

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Test fungi

The essential oils / extracts were tested for their antifungal properties against eight plant pathogenic fungi. *Pyricularia grisea* was isolated from blast infected finger millet leaves; *Rhizoctonia solani* and *Fusarium solani* were isolated from infected roots of French bean, *Sclerotium rolfsii* from infected collar portion of French bean, *Fusarium oxysporum* f.sp. *pisi* from wilted pea plants, *Sclerotinia sclerotiorum* from white rot infected pea plants, *Fusarium oxysporum* f.sp. *lentis* from wilted lentil plants and *Alternaria solani* from tomato leaves.

Extraction of essential oil

Leaves (200 g) and flower (250 g) of *Tagetes* sp. were subjected to hydro-distillation separately for 3 h using a Clavenger apparatus to obtain essential oil. Yield of essential oil was 0.3-0.4% and 0.2-0.3% from leaves and flower respectively. The oil was dried with anhydrous sodium sulfate and preserved in a sealed vial at 4 °C until the moment of analysis.

Extraction and purification of thiophene

Extraction of thiophene from shoots and roots was done by following the modified method of Margl et al., 2002. Finely chopped air dried shoot and roots (600 g) from *Tagetes* sp., were extracted with methanol: water (3:1 v/v, 2500 mL) twice at room temperature with the help of mechanical stirrer and filtered. Volume of combined extract was reduced *in vacuo* at 45 °C. The concentrated extract was sequentially partitioned into hexane: chloroform (2:1 v/v). The partitioned organic solvent fractions were concentrated to dryness by rotary evaporation at 35 °C to get dark green coloured thiopene rich concentrate (625 mg). Dark green thiophene rich extract was dissolved in minimum quantity of hexane, filtered and concentrated to dryness. Then the residue was dissolved in Et_2O and passed through column, preconditioned with Et_2O and packed with 60-120 mesh silica gel. Purified extract was then subjected to GC-MS analysis.

Characterization

TIC of root and shoot extract was presented in Fig. 1. Extract contains essential oil component as well as acetylenic thiophenes. First compound in the TIC eluted as dihydrotagetone and it was confirmed by its mass fragmentation pattern. The compound was eluted at 2.267 minute. Molecular ion peak was detected as m/z 154 $[M]^+$ with the mass fragmentation of m/z 139, 125, 108, 93, 81, 71, 58 (Tab. 1). Second compound eluted in the TIC was confirmed by its molecular ion peak of m/z 152 and fragments of 137, 109, 95, 81,

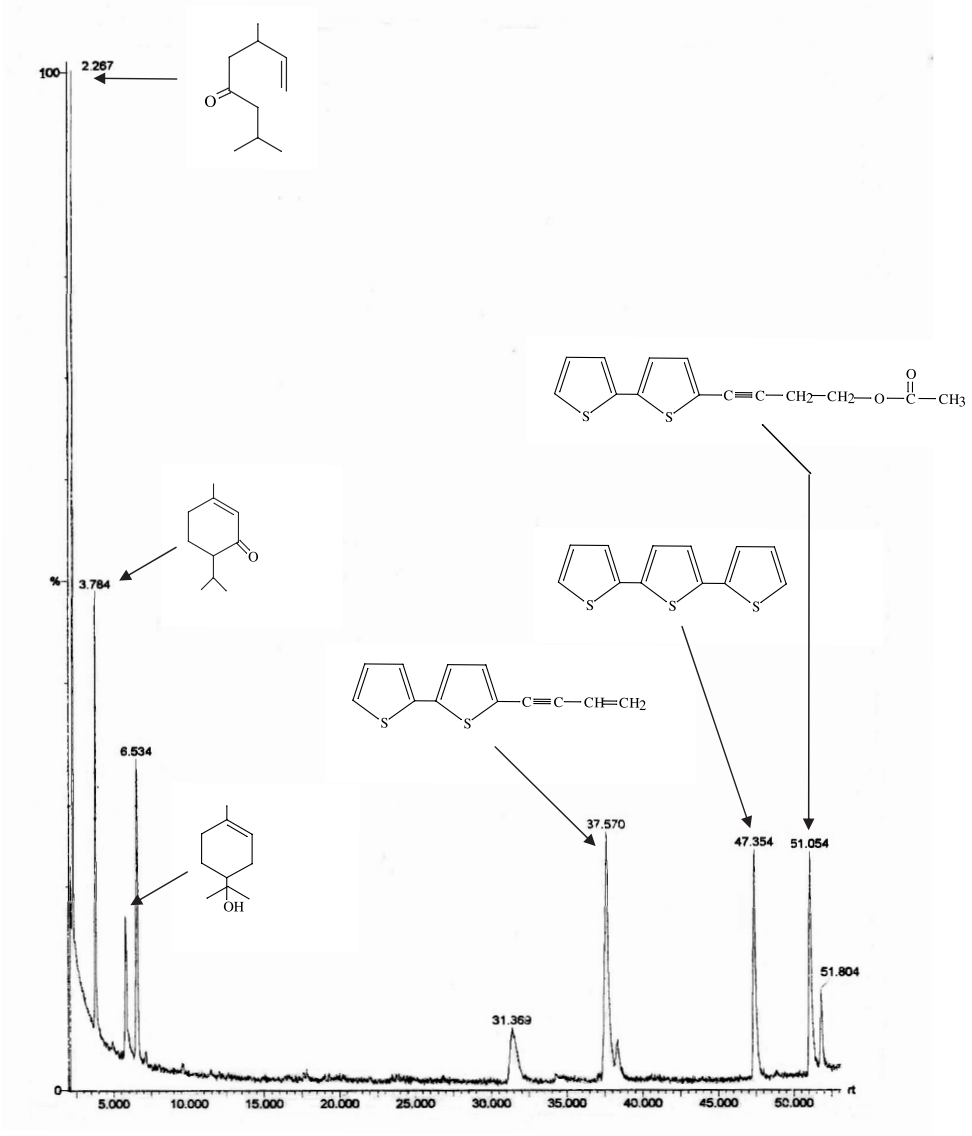

Fig. 1: TIC of thiophene rich extract.

Tab. 1: Mass spectra of compounds present in the purified root extract.

Compound	Rt [min]	m/z	Mass spectra (m/z, relative intensity)
Dihydrotagetone	2.267	154	58(100), 71(41), 81(54), 93(37), 108(54), 125(9), 139(32), 154(31)
Piperitone	3.784	152	58 (25), 69(49), 81(100), 95(5), 109(10), 137(6), 152(12)
α-terpineol	5.801	154	58(92), 71(100), 83(27), 93(78), 111(78), 121(15), 136(19), 154(18)
BBT	37.570	216	69(8), 95(29), 171(29), 216(100), 217(15), 218(12)
α-terthienyl	47.354	248	58(5), 69(10), 127(14), 171(9), 203(15), 248(100)
BBTOAc	51.054	276	95(6), 171(13), 203(16), 216(100)

69, 58 as piperitone. α-terpineol (m/z 154, 136, 121, 111, 93, 83, 71, 58) was eluted at 5.801 minute. Compound at Rt of 6.534 min was remain unidentified. Three thiophenes were identified as 5-(3-buten-1-ynyl)-2,2'-bithienyl (BBT) (37.57 min), α-terthienyl (47.354 min) and 5-(4-acetoxy-1-butynyl)-2,2'-bithienyl (BBTOAc). Molecular ion peak of these three compounds were m/z 218, 248 and 216 respectively with characteristic fragmentation pattern.

Antifungal bioassay

The cultures were maintained on PDA slants at 25 °C and were subcultured in petri dishes prior to testing. Weighed quantities of essential oil and extract (extracted under diffused sunlight) to be tested were dissolved in acetone (1 mL) and incorporated into the molten medium (at 45 °C) to reach the desired concentration. PDA containing only acetone served as control while β-pinene at 250 μg mL^{-1}, 500 μg mL^{-1} and 1000 μg mL^{-1} served as a positive control. Medium was poured into each sterile petri dish under aseptic conditions and left to settle. Circular discs (5 mm diameter) of the test mycelia mats (punched-in mycelia mats grown in sterile petri dishes) were inoculated centrally and left to grow. In all cases, triplicates were maintained. Radial growth inhibition of test fungi was evaluated. The diameter of fungal growth (mm) was noted at 72 h after inoculation and subsequently every 24 h for a total period of 240 h. These data were subjected to analysis of variance using completely randomised blocks. The percentage inhibition relative to the control, *I*, was converted to corrected percentage inhibition, *IC*, using the following formula:

$$IC = [(I - CF)/(100 - CF)] \times 100$$

$$CF = [(90 - C)/C] \times 100$$

where CF is the correction factor, 90 is the diameter of the petri dish (mm) and *C* is the diameter of the fungus in the control (mm).

Statistical analysis

Four replicates were used for each experimental treatment. The effective concentration for 50% inhibition of mycelial growth, ED_{50} (μg mL^{-1}), was calculated from the *IC* data using the Basic LD_{50} program v.1.1 as described by Trevors, 1986. Percentages were angular transformed (arc sine) for analysis and analysed by complete randomised design.

Results

Three monoterpenes and three thiophenes were identified from the shoot and root extract of *Tagetes minuta*. Terpenoids were identified as dihydrotagetone, piperitone and α-terpineol by their mass fragmentation pattern. Out of these three monoterpenes, one is acyclic i.e. dihydrotagetone and other two are cyclic monoterpenes. Romagnoli et al., 2005 reported these compounds to be present in flower essential oil along with other monoterpenes.

The antifungal activity of flower essential oil is not so encouraging (Tab. 2). Percent inhibition of flower essential oil at 1000 μg mL^{-1} was ranged between 8.9-35.1%. Highest activity was found against *Fusarium oxysporum pisi* and least activity was recorded against *Pyricularia grisea*. Antifungal activity of leaf essential oil against eight phytopathogenic fungi is presented in Tab. 3. Antifungal activity was comparatively superior in leaf essential oil than flower. ED_{50} value ranged between 110-1016 μg mL^{-1}. Leaf essential oil was most active against *Sclerotium rolfsii* and least active against *Fusarium oxysporum lentis*. The *in vitro* results were classified according to Aligiannis et al., 2001 and Duarte et al., 2005 who proposed that, when the minimum inhibitory concentration (MIC) is below 500 μg mL^{-1}, the antifungal activity is considered strong, and, if the extracts/compounds display an MIC between 600 and 1500 μg mL^{-1}, the antifungal activity is considered moderate. Compounds with an MIC above 1600 μg mL^{-1} are considered weak. As evident from the data, leaf essential oil exhibited strong activity against *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Sclerotium rolfsii*. Medium antifungal activity was recorded against other five phytopathogenic fungi. The commercial reference fungicide, mancozeb, was highly effective against all four test fungi. Whereas, essential constituent β-pinene showed ED_{50} value less than 180 μg mL^{-1}.

Antifungal activity of thiophene rich extract from shoot and root is presented in Tab. 4. ED_{50} value against eight phytopathogenic fungi ranged between 233 to 2227 μg mL^{-1}. The extract was most active against *R. solani* and least active against *P. grisea*.

Hyphal growth inhibition was maximum in *R. solani*, *S. sclerotiorum* and *S. rolfsii* and the activity was found to be strong. While, moderate antifungal activity was recorded in *F. solani, A. solani, F. oxysporum pisi* and *F. oxysporum lentis*. Weak antifungal activity was evidenced against *P. grisea*.

Tab. 2: Hyphal inhibition of eight major plant pathogens upon application of flower essential oil at 1000 μg mL^{-1} after 3 days.

Pathogen	Inhibition (%)
Rhizoctonia solani	5.7
Fusarium solani	13.8
Sclerotinia sclerotiorum	8.9
Fusarium oxysporum pisi	35.1
Scleroium rolfsii	19.5
Pyricularia grisea	26.4
Fusarium oxysporum lentis	22.9
Alternaria solani	21.9

Tab. 3: Antifungal activity of leaf essential oil against eight phytopathogenic fungi.

	ED_{50} [μg mL^{-1}]	χ^2_{exp} (3df, 95%)	Fiducial limit	Regression equation
Rhizoctonia solani	165	2.20	128-213	Y = 2.50+1.13x
Sclerotinia sclerotiorum	175	4.32	135-225	Y = 2.59+1.08x
Fusarium solani	1776	0.86	642-4915	Y = 3.14+0.57x
Alternaria solani	751	4.74	393-1436	Y = 3.29+0.60x
Pyricularia grisea	708	5.33	360-1393	Y = 3.10+0.68x
Fusarium oxysporum pisi	3716	1.23	890-15520	Y = 3.04+0.55x
Fusarium oxysporum lentis	6975	0.46	843-57700	Y = 3.24+0.46x
Sclerotium rolfsii	110	2.43	84-144	Y = 2.49+1.22x
Mancozeb	32	-	-	-
β-pinene	<180	-	-	-

Tab. 4: Antifungal activity thiophene rich extract against eight phytopathogenic fungi.

	ED_{50} [μg mL^{-1}]	χ^2_{exp} (3df, 95%)	Fiducial limit	Regression equation
Rhizoctonia solani	233	3.17	191-284	Y = 1.77+1.37x
Sclerotinia sclerotiorum	484	4.37	352-667	Y = 2.36+0.98x
Fusarium solani	3656	5.85	1195-11187	Y = 2.42+0.72x
Alternaria solani	1045	6.64	634-1722	Y = 2.18+0.93x
Pyricularia grisea	2227	2.00	819-6063	Y = 2.82+0.65x
Fusarium oxysporum pisi	1131	6.69	611-2094	Y = 2.63+0.78x
Fusarium oxysporum lentis	1244	5.56	680-2275	Y = 2.42+0.83x
Sclerotium rolfsii	285	4.31	232-350	Y = 1.78+1.31x
Mancozeb	32	-	-	-
β-pinene	<180	-	-	-

Discussion

The marigold plant has been credited with a number of properties including antimicrobial/insecticidal/nematicidal activity (Natarajan et al., 2006; Piccaglia et al., 1997; Hulst et al., 1989; Romagnoli et al., 1994). In the present work, a comparative study of the antifungal activity of essential oils/extracts from different parts of the *Tagetes* plant against eight important plant pathogenic fungi was done.

Out of six identified compounds, three were monoterpenic derivative and are component of essential oil. Three compounds namely, dihydrotagetone, piperitone and α-terpineol mostly came from shoot portion of the *Tagetes minuta* plant. Other three identified compounds were thiophenes and root portion contributed its lion share (Downum, 1983).

Antifungal activity of *Tagetes* sp. published so far is related both to the presence of thiophenic compounds alone and with UV-A irradiation (Mares et al., 1990; Romagnoli et al., 1994). Number of species also reported for different activity including antifungal activity. Reports on exploration of total plant extract of *T. minuta* for fungistatic activity is not so well documented.

Though Uhlenbroek and Bijloo (1958) found that nematicidal or nematostatic effects of Tagetes were due to α-terthienyl, Sasanelli and Vito (1991) suggested that there was a synergistic effect of leaf and root extract on egg hatchability of *G. rostochinensis*. Siilar effect was observed by Siddiqui and Alam, 1988, where it was concluded that stem and whole plants was more effective in suppressing juveniles of nematode than root extract.

All the different compounds (essential oils and extract) from *Tagetes* plant as well β-pinene exhibited antifungal activity against the eight pathogens. However, there were significant differences in the antifungal activity of the compounds from different parts of the plant. It was observed that leaf essential oil and root extract at 1000 μg mL^{-1} showed the highest inhibitory activity against all the eight pathogens which persisted for more than 6 days. Leaf essential oil showed hyphal growth inhibition of 34.1 to 85.6% while root extract treatment showed inhibition of 21.6 to 77.8% after 6 day against various pathogens. Even at lower concentrations of 500 μg mL^{-1} and 250 μg mL^{-1} leaf essential oil resulted in significant reduction in hyphal growth of five of the pathogens both 3 and 6 days after treatment.

However, the essential oil from flowers was not highly potent and exhibited minimal antifungal activity (<35%) at all three concentrations against the 8 pathogens. Its antifungal activity was particularly low against the fast growing pathogens *R. solani* and *S. sclerotiorum*.

Marigold (*Tagetes* sp.) plant is a commonly available plant in all regions of the world. The present study shows that essential oil from leaves and thiophene rich extracts from marigold roots have high antifungal activity against a number of soil borne and foliar plant pathogens. The easy availability of these plants makes them an attractive potential candidate for development of plant based biopesticide.

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