Chemical Composition and Fungitoxic Activity of Essential Oil of *Thuja orientalis* L. Grown in the North-Western Himalaya

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The essential oil from fresh leaves of *Thuja orientalis* L. grown in the north-western Himalaya was isolated by means of hydrodistillation and analyzed by GC and GC/MS. Twenty-two compounds representing 94.0% of the total oil were identified. The leaf oil contained α-pinene (29.2%), Δ-3-carene (20.1%), α-cedrol (9.8%), caryophyllene (7.5%), α-humulene (5.6%), limonene (5.4%), α-terpinolene (3.8%) and α-terpinyl acetate (3.5%) as major constituents. The essential oil showed antifungal activity against *Alternaria alternata* in a direct bioautography assay. Two main bioactive compounds named as b_1 (R_f = 0.54) and b_2 (R_f = 0.80) were observed and tested for antifungal activity; they produced an inhibition zone of 5 and 10 mm in diameter, respectively. The components b_1 and b_2 were further purified by preparative thin layer chromatography and their antifungal efficacy was re-tested. The minimum inhibitory amount (MIA) of b_1 and b_2 against *A. alternata* was determined as 30.5 and 4.5 μg, respectively, using a bioautography assay. The bioactive constituent corresponding to b_1 was determined as α-cedrol by using GC/MS analysis. The potential of essential oils as a source of natural biocides is discussed.

Key words: Thuja orientalis L., α-Cedrol, Antifungal Activity

Introduction

Thuja orientalis L. (Cupressaceae) is an evergreen species widely cultivated as a common ornamental plant (Assadi, 1998). During the last years most of the phytochemical studies on this plant species focused on the terpene composition of the essential oil, where α-pinene and α-cedrol have been reported as the major constituents of the leaf oil (Chen *et al.*, 1984; Li and Liu, 1997; Nickavar *et al.*, 2003). The fungitoxic activity of the essential oil of *T. orientalis* has not been the subject of much studies. Guleria and Kumar (2006) have reported the antifungal activity of a dichloromethane leaf extract of *T. orientalis* against *Alternaria alternata* and *Curvularia lunata* using direct bioautography.

In this work, the composition of the essential oil isolated by hydrodistillation from the leaves of *T. orientalis* grown in the north-western Himalaya is reported along with the fungitoxic activity of its bioactive compounds against *Alternaria alternata.*

Experimental

Plant material

Leaves from eastern thuya (*Thuja orientalis* L.) were collected on the campus of CSK Himachal Pradesh Agricultural University, Shivalik Agricultural Research and Extension Centre, Kangra (HP), India, during October 2006. They were identified at Herbal Garden and Herbarium Research Institute in ISM, Joginder Nagar, District Mandi (HP), India, and a voucher specimen (401) was deposited at the herbarium of the institute.

Isolation of the essential oil

Fresh leaves of *T. orientalis* were subjected to steam distillation. The distillate was then extracted with petroleum ether. The resulting extract was dried over anhydrous sodium sulfate. Petroleum ether was removed carefully under vacuum and the essential oil was obtained.

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Gas chromatography (GC)

GC analysis was carried out using a Shimadzu GC-2010 system (Shimadzu, Kyoto, Japan). A BP-1 column (30 m × 0.25 mm i. d., 0.25 μm film thickness) was used with nitrogen as carrier gas (flow rate 1.10 ml/min). The oven temperature programme used was holding at 70 °C for 4 min, heating to 220 °C at 5 °C/min and keeping the temperature constant at 220 °C for 5 min. Split flow was adjusted at 1:50. The injector and FID detector temperatures were at 250 °C. Relative percentage amounts of the separated compounds were calculated from FID chromatograms.

Gas chromatography/mass spectrometry (GC/MS)

Gas chromatography/mass spectrometry analysis was carried out at Institute of Himalayan Bioresource Technology (Council of Scientific and Industrial Research, India), Palampur (HP), India.

A Shimadzu GC/MS-QP2010 system with a BP-1 column (30 m × 0.25 mm i. d., 0.25 μm film thickness) was used with helium as carrier gas. The oven temperature programme used was holding at 70 °C for 4 min, heating to 220 °C at 5 °C/min and keeping the temperature constant at 220 °C for 5 min. Mass spectra were recorded at 70 eV and mass range was between *m/z* 40 to 600. Library search was carried out by using in-house Wiley 7 and NIST27 libraries of the instrument.

Isolation of Alternaria alternata and preparation of inoculum spray solution

The test fungus *Alternaria alternata* was isolated from a single spot on an infected leaf of sesame on potato dextrose agar (PDA), and the pure culture was maintained at (26 ± 2) °C. Conidia were collected from a 10-day-old culture of the pathogen by flooding the culture plates with 5 ml of sterile distilled water and dislodging the conidia by using a L-shaped glass rod. The conidial suspension was filtered through a sterile double layered muslin cloth to remove bits of mycelia. A spore suspension was then prepared in potato dextrose broth (200 g potato, 20 g dextrose and water to make a total volume of 1 l) to obtain a concentration of $3 \cdot 10^5$ conidia/ml.

Thin layer chromatography (TLC)

Silica gel $60F_{254}$-coated glass plates (20 × 20 cm, 250 μm thickness of layer) were activated for 1 h at 100 °C and stored in a desiccator to increase the reproducibility. *T. orientalis* essential oil was applied onto two TLC plates (5 μl each) using a micropipette and developed in *n*-hexane/ethyl acetate at a ratio of 9:1, v/v. On one plate separated compounds were visualized under UV light (365 and 254 nm) and by spraying with vanillin/sulfuric acid spray reagent. The other plate was used for the bioautography assay to determine the separated active compounds.

Bioautography

This technique was used to determine the active constituents of the botanical extracts/essential oils (Sridhar *et al.*, 2003; Guleria and Kumar, 2006). Aliquots of 25–50 ml of inoculum spray solution (ca. $3 \cdot 10^5$ conidia/ml) were used for bioautography. Using an 100 ml chromatographic sprayer, plates were sprayed lightly (to a damp appearance) three times with the spore suspension of *A. alternata* and incubated for 4 d in a dark moist chamber at 25 °C. Fungal growth inhibition appeared as clear zones against a dark background. The experiment was repeated twice and similar results were obtained.

Isolation of bioactive constituents

After identification of the active zones on the bioautographic TLC plate, preparative TLC was performed. The essential oil (approx. 100 mg) was applied onto a pre-activated Silica gel $60F_{254}$-coated glass plate (20 × 20 cm, 500 μm thickness of layer) which was developed in a *n*-hexane/ethyl acetate (9:1, v/v) solvent system. The separated compounds were visualized using UV light (365 and 254 nm) or vanillin/sulfuric acid spray reagent. The isolation was carried out by scraping off the detected zones corresponding to the bioactive constituents b_1 (R_f = 0.54) and b_2 (R_f = 0.80) with a spatula and transferring them into a percolator. The substances were then set free from silica gel by elution with dichloromethane.

Antifungal activity of active compounds isolated from T. orientalis

Different amounts of b_1 and b_2 were loaded onto the TLC plate and bioautography was performed as per the procedure described earlier using *A. alternata* as test pathogen. Antifungal activity was determined as minimum inhibitory amount

(MIA) of active compounds required for the inhibition of fungal growth on TLC plates.

Identification of bioactive constituent

The isolated bioactive compound corresponding to b_1 (R_f = 0.54) was determined using GC/MS analysis as described earlier.

Results and Discussion

Hydrodistillation of the leaves extract of eastern thuya (*Thuja orientalis* L.) grown in the northwestern Himalaya gave a yellowish oil with a yield of 0.20%. The oil was analyzed both by GC and GC/MS to determine its main constituents (Table I), and further investigated for its antifungal properties against *Alternaria alternata* (Tables II, III). As a result of GC and GC/MS analyses 22 components were identified representing 94.0% of the total oil. α-Pinene (29.2%), Δ-3-carene (20.1%), α-cedrol (9.8%), caryophyllene (7.5%), α-humulene (5.6%), limonene (5.4%), α-terpinolene (3.8%) and α-terpinyl acetate (3.5%) were the main constituents. The composition of the essential oil in this study was different from that previously reported from Iran by Nickavar *et al.* (2003). The major components of the essential oil from *T. orientalis* cultivated in Iran were α-pinene (21.9%), α-cedrol (20.3%), Δ-3-carene (10.5%) and limonene (7.2%) (Nickavar *et al.*, 2003). It is generally recognized that there are several chemotypes (marker compounds) in the same plant, such as those of *Thymus vulgaris*, and the composition of essential oils is affected by many factors, including the cultivation conditions of the plant and isolation technique (Janssen *et al.*, 1987).

A direct bioautographic procedure was used in the above study to examine the antifungal potential of *T. orientalis* essential oil using *Alternaria alternata*, a fungal pathogen of economic importance, as test organism. Bioautography is a very appropriate and simple technique for evaluating plant extracts and essential oils for their effect on human and plant pathogenic organisms (Marston *et al.*, 1997). This technique enables to trace active constituents. Consequently, it helps to visualize the inhibition zone after an incubation period. Application of the oil to the bioautographic assay system mentioned above showed the presence of two distinct inhibition zones (Fig. 1) on the TLC plate with diameters of 5 mm (R_f = 0.54) and 10 mm (R_f = 0.80) corresponding to b_1 and b_2 respectively. The antifungal activity observed above encouraged the application of preparative TLC on the

Table I. GC/MS analysis of *Thuja orientalis* L. essential oil.

Compound	RT [min]	Percentage
α-Thujene	5.70	0.2
α-Pinene	5.90	29.2 ± 0.3*
α-Fenchene	6.21	1.3
Sabinene	6.93	0.5
β-Pinene	7.06	0.9
β-Myrcene	7.46	1.6
α-Phellandrene	7.90	0.5
Δ-3-Carene	8.16	20.1 ± 0.3*
Limonene	8.72	5.4
γ-Terpinene	9.71	0.2
α-Terpinolene	10.76	3.8
Terpinene-4-ol	13.91	0.3
Bornyl acetate	17.79	0.5
α-Terpinyl acetate	20.02	3.5
β-Elemene	21.80	0.5
β-Cedrene	22.53	0.8
Caryophyllene	22.73	7.5
α-Humulene	23.81	5.6
Neoalloocimene	24.82	0.2
Δ-Cadinene	25.94	0.7
Elemol	26.63	0.6
α-Cedrol	28.20	9.8 ± 0.2*

RT, retention time.
* Mean ± s.d.

Table II. Antifungal activity of essential oil isolated from *Thuja orientalis* against *Alternaria alternata*.

	R_f value	Diameter of inhibition zone [mm][a]
b_1	0.54	5.00 ± 0.50*
b_2	0.80	10.00 ± 1.00*

[a] 5 µl of *T. orientalis* essential oil were loaded onto the TLC plate. The plate was developed with *n*-hexane/ethyl acetate in the ratio 9:1, v/v. Zones of fungal growth inhibition were produced after bioautography with *Alternaria alternata*.
* Mean ± s.d.

Table III. Antifungal activity of compounds isolated from *Thuja orientalis* essential oil against *Alternaria alternata*.

Compound	Antifungal activity [µg][a]
b_1, α-Cedrol	30.5
b_2	4.5

[a] Minimum inhibitory amount (MIA) of active constituents required for the inhibition of fungal growth on a TLC plate.

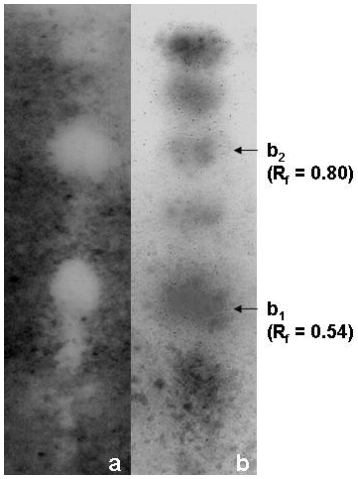

Fig. 1. Thin layer chromatogram of essential oil from *Thuja orientalis* leaves. (a) Bioautography with *Alternaria alternata* spores; (b) detection of fungitoxic compounds, namely b_1 (R_f = 0.54) and b_2 (R_f = 0.80), with vanillin/sulfuric acid reagent.

essential oil for the isolation of active constituents present in the inhibition zones. Preparative TLC of the essential oil yielded 7 mg of b_1 (R_f = 0.54) and 3 mg of b_2 (R_f = 0.80). Antifungal activity of the bioactive constituents b_1 and b_2 was their MIA required for the inhibition of the fungal growth on a TLC plate. The results of the above study revealed stronger inhibition of *A. alternata* spores by b_2 (MIA = 4.5 μg) than b_1 [MIA = 30.5 μg (Table III)]. Furthermore the bioactive compound corresponding to b_1 (R_f = 0.54) was identified as α-cedrol (Fig. 2) by GC/MS analysis. Further research is warranted to identify the fungitoxic compound corresponding to b_2.

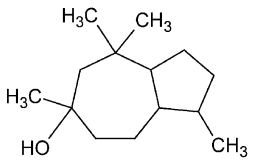

Fig. 2. Chemical structure of α-cedrol.

Pathogens are economically damaging in agriculture and food industry. Thus, an approach towards the discovery of lead compounds has been made using an essential oil derived from *T. orientalis* leaves as source. Sensitivity of *A. alternata* towards active constituents of *T. orientalis* has indicated the need for the application of natural biocides in the field and during post-harvest storage.

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