Iranian Journal of Pharmaceutical Sciences
Spring 2006: 2(2): 103-108
www.ijps.ir

Original Article

Chemical and Antimicrobial Studies of *Cupressus sempervirens* L. and *C. horizentalis* Mill. Essential Oils

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Abstract

The essential oils of fresh fruits and terminal branches with adherent leaves of *Cupressus sempervirens* L. and *C. horizentalis* Mill., growing in Iran, were analyzed by gas chromatography-mass spectroscopy. The oils were also screened for bacteriostatic and fungistatic activities. Thirteen and ten components were identified in the essential oils of *C. sempervirens* and *C. horizentalis,* respectively. The main constituents of both fruits and leaves of *C. sempervirens* were a-pinene and Δ-3-carene, while they were α-pinene and Δ-2-carene for *C. horizentalis.* The essential oil of *C. horizentalis* leaves showed no antimicrobial activity against, *Bacillus subtilis, Candida albicans*, *Escherichia coli* and *Staphylococcus aureus,* while the essential oil of its fruits exhibited a weak effect on *Bacillus subtilis.* The essential oil of *C. sempervirens* leaves and fruits showed stronger antimicrobial activity against tested microorganisms compared to *C. horizentalis* essential oils. *C. sempervirens* fruits were relatively rich in tannins, and the leaves and fruits of *C. horizentalis* were quite rich in saponins and tannins. The amounts of flavonoids and alkaloids were not very high in both parts of these plants.

Keywords: Antimicrobial activity; Cupressaceae; *Cupressus horizentalis.* Mill.; *Cupressus sempervirens* L.; Essential oil.
Received: January 12, 2005; *Accepted:* July 25, 2005

1. Introduction

Cupressus sempervirens L. [*C. fastigiata* DC., *C. sempervirens* L. var. *fastigiata* Hansen], family Cupressaceae, is a monoecious and evergreen tree, up to 20-30 m high which grows in south and east of Europe, west part of Asia including various parts of Iran. Its Persian name is "Sarve Shirazi" or "Sarve Kashi" [1-8]. *C. horizentalis* Mill. [*C. sempervirens* L. var. *horizentalis*

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(Mill.) Loud.] family Cupressaceae, is a monoecious and evergreen aromatic tree, up to 25 m high, broad and pyramidal growing; branches horizontal. This plant is widely grown from Crete to Iran. Its Persian name is "Zarbin" [1, 6-8]. *C. sempervirens* L. is a medicinal plant and act as expectorant and used externally for treating coughs and bronchitis [9]. There is no previous report on phytochemical characteristics and biological effects of these plants in Iran. However, a few phytochemical and biological studies on *C. sempervirens* L. in other parts of the world have been reported [10 -14].

As a part of our systematic research on the chemical composition and antimicrobial properties of Iranian conifers, in this investigation, the essential oils content of fruits and terminal branches with adherent leaves (leaves) of both *C. sempervirens* and *C. horizentalis* was evaluated. In addition, the defatted ethanolic extracts of these organs were investigated qualitatively for the presence of alkaloids, flavonoids, saponins and tannins.

2. Materials and methods

2.1. Plant material

The fruits and leaves of *C. horizentalis* Mill. were collected from Sorkesh forest at the height of 1000 m from the sea. This place is in province of Golestan which is located in the north of Iran. The fruits and leaves of *C. sempervirens* L. were collected from the National Botanical Garden of Iran (Tehran). The collected materials were stored at -20 °C in order to prevent changes in the chemical components [15].

The plant material was identified by Dr. M. Assadi, Research Institute of Forest and Rangelands, Ministry of Jahad Keshavarzi, Iran. Voucher specimens have been deposited in the Herbarium of National Botanical Garden of Iran.

2.2. Isolation and analysis of non-volatile components

The fruits and leaves of each plant (500 g) were dried at 50 °C and powdered separately. Each powder was defatted with petroleum ether (b.p. 40-60 °C) using Soxhlet apparatus (6 h). The chemical components of defatted powders were extracted by maceration with 70% ethanol (×4). The ethanolic extracts were concentrated at reduced pressure and analyzed for the presence of alkaloids [16], flavonoids [17], saponins [18] and tannins [19].

2.3. Isolation and analysis of volatile components

Volatile oils were isolated from fresh plant material by wet steam distillation for 4 h [20]. The essential oils were separated from the aqueous layer and dried over anhydrous sodium sulfate and stored under nitrogen gas in a sealed vial and -20 °C until analyzed.

2.4. Gas chromatography-mass spectrometric analysis

The GC-MS apparatus consisted of a

Table 1. Major non-volatile components of fruits and leaves of *Cupressus sempervirens* L. and *C. horizentalis* Mill.

Chemical components	Average content*			
	C. sempervirens		*C. horizentalis*	
	Fruits	Leaves	Fruits	Leaves
Alkaloids	-	-	-	1+
Flavonoids	-	1+	1+	2+
Saponins	1+	1+	4+	4+
Tannins	2+	1+	4+	3+

* Average content was rated from - to 4+:1+, slightly positive; 2+, moderately positive; 3+, strongly positive; 4+, very strongly positive; -, not detected.

Table 2. Chemical composition of the volatile oil of *Cupressus sempervirens* L. fruits and leaves.

Components	Retention Index*	Leaves oil (%)	Fruits oil (%)
α-Pinene	940	21.4	46.0
Sabinene	974	2.8	2.7
β-Pinene	982	2.6	2.7
Myrcene	997	5.0	5.4
Δ -3-Carene	1018	16.0	27.0
Limonene	1035	3.3	2.2
Terpinolene	1092	5.9	6.4
α-Terpinyl acetate	1355	5.9	2.7
β-Caryophyllene	1416	4.2	t
α-Humulene	1454	4.2	t
Germacrene-D	1485	13.0	2.1
Δ-Cadinene	1530	1.6	t
Cedrol	1601	3.3	t
Grouped compounds:			
Monoterpene hydrocarbons		57.0	92.4
Oxygen-containing monoterpenes		5.9	2.7
Sesquiterpene hydrocarbons		23.0	2.1
Oxygen-containing sesquiterpenes		3.3	-

t = trace (< 0.1 %)

* The retention indices were determined on DB-5 capillary column.

Varian 3400 GC equipped with a fused-silica column (DB-5, 30 m × 0.25 mm i.d., film thickness 0.25 mm; J&W Scientific Inc), and interfaced with a quadruple mass spectrometric detector (Incos50, Finnigan). The operating conditions were: Oven temperature 60-280 °C with the rate of 3 °C /min., injector mode: split injection; with the carrier gas, He; flow rate 2 ml/min.; ion source, 70 eV; ionization current, 750 mA; scan range, 40-300 u.

The oil components were identified from their retention indices (RI) obtained with reference to n-alkane series (Sigma, UK) on DB-5 column, mass spectra with those of authentic samples, composition of their mass spectra and fragmentation patterns reported in literature [21], computer matching with MS-data bank (Saturn version 4). The peak area method was followed for quantitative determination of different constituents; the percentages were calculated relatively [21].

2.5. Antimicrobial activity determination

The antimicrobial activity of each essential oil obtained from different parts of the plant was determined using five strains of microorganisms from the Persian Type Culture Collection (PTCC) [22]. The following microbial strains were used: *Bacillus subtilis* (PTCC 1023), *Staphylococcus aureus* (PTCC 1112), *Escherichia coli* (PTCC 1038), *Pseudomonas aeroginosa* (PTCC 1074) and *Candida albicans* (PTCC 5027). Minimum inhibitory concentrations (MICs) were determined using the agar dilution method [23]. Muller Hinton Agar medium (Oxoid, France) was prepared and sterilized by autoclaving for 20 min. at 121 °C. The medium was cooled to 50 °C and two-fold serial dilutions of the parent solution (essential oil plus 30% DMSO v/v) in adequate melted (50 °C) agar medium (1 ml) were prepared and added to 24-well plates (Greiner, France). The medium was allowed to solidify and then inoculated with the previously prepared microorganism suspension using the quadran streak method. For each strain tested, the adequacy of growth conditions, the effect of positive controls

Table 3. Chemical composition of the volatile oil of *Cupressus horizentalis.* Mill. fruits and leaves.

Components	Retention Index*	Leaves oil (%)	Fruits oil (%)
α-Pinene	941	46.2	59.2
Camphene	955	1.73	-
Sabinene	980	2.2	2.0
β-Pinene	985	3.3	2.8
Myrcene	990	4.6	3.4
Δ -2-Carene	1005	22.7	14.9
Limonene	1034	2.8	2.4
Isoterpinolene	1080	3.7	3.2
α-Terpinyl acetate	1350	2.6	3.2
Germacrene-D	1482	6.3	2.1
Grouped compounds:			
Monoterpene hydrocarbons		87.2	87.9
Oxygen-containing monoterpenes		2.6	3.2
Sesquiterpene hydrocarbons		6.3	2.1

* The retention indices were determined on DB-5 capillary column.

(gentamycin for bacteria and clotrimazole for fungus) and the sterility of the medium were tested in two wells. Plates were incubated for 24 h at 37 °C for the bacteria and 48 h at 25 °C for *C. albicans.*

3. Results

The amount of non-volatile components in defatted ethanolic extracts of the fruits and leaves of *C. sempervirens* and *C. horizentalis* are shown in Table 1.

The essential oils isolated separately from fruits and leaves of *C. sempervirens* and *C. horizentalis* were colorless with a strong odor. The fruits and leaves of *C. sempervirens* yielded 0.1% and 0.3% v/w of volatile oil while the fruits and leaves of *C. horizentalis* yielded 0.4% and 0.5% (v/w) of volatile oil, respectively.

GC-MS analysis of leaves and fruits volatile oils of *C. sempervirens* led to the identification of 13 and 9 components, respectively (Table 2). However, for *C. horizentalis* leaves and fruits volatile oils 10 and 9 components were identified, respectively (Table 3).

The antimicrobial activity of essential oils of fruits and leaves of both plants were evaluated using the agar dilution method [23].

Only, the essential oils of fruits of *C. horizentalis* had a weak effect on *B. subtilis* (Table 4). The essential oil of leaves and fruits of *C. sempervirens* exhibited mild antimicrobial activity against all of the tested microorganisms (Table 5).

4. Discussion

The presence of non-volatile components in defatted ethanolic extracts of leaves and fruits of both *C. sempervirens* and *C. horizentalis* were investigated. These extracts were examined for alkaloids, flavonoids,

Table 4. Antimicrobial activity of the essential oil of the fruits and leaves of *Cupressus horizentalis.* Mill.

Microorganism	Minimum inhibitory concentration			
	Fruits oil (mg/ml)	Leaves oil (mg/ml)	Gentamycin (μg/ml)	Clotrimazole (μg/ml)
Bacillus subtilis	50	100	0.5	-
Staphylococcus aureus	100	>100	0.8	-
Escherichia coli	100	>100	0.5	-
Pseudomonas aeroginosa	100	>100	0.7	-
Candida albicans	100	>100	-	8.0

Table 5. Antimicrobial activity of the essential oil of the fruits and leaves of *Cupressus sempervirens* L.

Microorganism	Minimum inhibitory concentration			
	Fruits oil (mg/ml)	Leaves oil (mg/ml)	Gentamycin (μg/ml)	Clotrimazole (μg/ml)
Bacillus subtilis	25	50	0.5	-
Candida albicans	50	100	-	8.0
Escherichia coli	12.5	50	0.5	-
Pseudomonas aeroginosa	50	75	0.7	-
Staphylococcus aureus	6.25	25	0.8	-

saponins and tannins. As it can be seen from Table 1, the amounts of non-volatile compounds in leaves of *C. sempervirens* were not significant but the fruits of this plant were relatively rich in tannins. Leaves and fruits of *C. horizentalis* were quite rich in saponins and tannins while the amounts of flavonoids and alkaloids were not very high in both parts of this species.

In spite of the differences in the percentages of various components in the essential oils of fruits and leaves of these plants, the composition of the oils of leaves and fruits are fairly similar. Monoterpene hydrocarbons represented the most abundant constituents of the oil of the fruits and leaves of both plants (92.4% and 57.0% for fruits and leaves of *C. sempervirens* and 87.2% and 87.9% for leaves and fruits of *C. horizentalis,* respectively). The amounts of sesquiterpene hydrocarbons in leaves of these plants were higher than the amount in their fruits (23.0% and 2.1% for fruits and leaves of *C. sempervirens* and 6.3% and 2.1% for leaves and fruits of *C. horizentalis,* respectively). Oxygen-containing monoterpenes and sesquiterpenes were at relatively low levels in both fruits and leaves oils of *C. sempervirens*. No nonterpenic compounds were detected in these essential oils. Oxygen-containing monoterpenoids were low in leaves and fruits oils of *C. horizentalis* (2.6% for leaves and 3.2% for fruits), however, oxygenated sesquiterpenoids and nonterpenic constituents were not detected in the leaves and fruits of this species.

The main components of *C. horizentalis* leaves and fruits essential oils were α-pinene (46.2%, 59.2%) and Δ-2-carene (22.7%, 14.9%), respectively. On the other hand, the main components of both fruits and leaves essential oils of *C. sempervirens* were α-pinene, (46.0%, 21.4%) and Δ-3-carene (27.0%, 16.0%), respectively.

These findings are in general agreement with the previous reports [10, 11]. Although Δ-3-carene was not a major compound in the reported investigation for *C. sempervirens* [10], the amount of this compound in the essential oil of this plant was quite high (27.0% and 16.0%) in fruits and leaves, respectively.

The antimicrobial activity of the examined essential oils varied at different concentrations against bacteria and yeast. The essential oil of leaves of *C. horizentalis* had no antimicrobial activity against *B. subtilis, C. albicans*, *E. coli* and *S. aureus* while the essential oil of fruits of this species showed a weak antimicrobial activity against *B. subtilis. C. sempervirens* leaves essential oil had a weak antimicrobial activity while the essential oil of fruits of this species exhibited stronger antimicrobial activity against all tested microorganisms. This is in general agreement with the Egyptian report indicating that the leave oil of *C. sempervirens* was active against *P. aeroginosa* and *S. aureus,* while it was inactive against *B. subtilis* and *E. coil* [12].

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