Investigation of Physico-Chemical Composition and Antimicrobial Activity of Essential Oil Extracted from Lignin-Containing *Cupressus sempervirens*

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New, cost-effective source materials are being sought to enable the development of essential oils for potential use in pharmaceutical and commercial applications. The present study was aimed at investigating such features of *Cupressus sempervirens*, which despite its exotic legendary attributes has been almost ignored up until now. Gas liquid chromatography (GLC) was used to determine the various physico-chemical composition parameters (specific gravity, refractive index, acid and ester values profile) of the selected lignin-containing plant. The essential oil was extracted by a steam distillation technique using needles and twigs of *C. sempervirens*. The antimicrobial properties of the oil were investigated against a wide spectrum of microorganisms by flask culture and diffusion methods. In the flask culture method, only three strains, viz. *A. niger*, *A. flavous*, and *A. fumigates,* while in diffusion method seven strains, viz. *A. niger*, *A. flavous*, *A. fumigatous*, *F. solani*, *F. oxysporum*, *Penecillium digitatum,* and *Candida uterus,* and three strains of bacteria, viz. *E. coli*, *M. leutius*, *B. lacto,* exhibited 100% effectiveness in the presence of newly extracted *C. sempervirens* oil.

Keywords: Cupressus sempervirens; Steam distillation; Essential oil; Specific gravity; Refractive index; GLC; Antimicrobial activity

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INTRODUCTION

Essential oils can be used for the treatment of microbial infections and as food preservatives to inhibit the growth of microorganisms (Isman 2000). Synthetic preservatives are extensively used against microorganisms. However, due to the excessive and unsystematic use of antibiotics, microbes have built up high resistance against many antibiotics (Mukherjee *et al.* 2002). In this regard, the use of essential oils is less harmful to human health because they have fewer side effects and are generally less toxic (Isman 2000; Misra and Pavlovstathis 1997). Chemical preservatives are used in the food industry to prevent the growth of foodborne and spoiling microorganisms. There is great pressure from consumers to reduce or eliminate synthetic additives from foods to minimize health hazards. There is an increasing interest of the scientific community and the food industry in essential oils of plants due to their antibacterial and antifungal properties (Nascimento *et al.* 2000; Toroglu 2007).

Cupressus sempervirens belongs to the family Cupressaceae and is an evergreen tree with potential capabilities to meet the challenges of the modern world. *C. sempervirens* contains antispasmodic, astringent, aroma therapeutic antiseptic, deodorant, balsamic, and anti-inflammatory activities (Rawat *et al.* 2010). *Cupressus sempervirens*

is commonly known as the pencil pine with a conic crown and variably, loosely hanging branches, as shown in the Fig. 1.

Fig. 1 *Cupressus sempervirens*

The aim of the present research work was to assay the main constituents of the essential oil obtained from *Cupressus sempervirens* cultivated in Pakistan. The chemical composition results of *C. sempervirens* oil extract were compared with those extracted from the same species from different geographical origins. A comparative evaluation of the physiochemical and antimicrobial properties of an essential oil extracted from the leaves of *C. sempervirens* was also the main focus of the work.

EXPERIMENTAL

Collection of Plant Material

The needles and twigs of *C. sempervirens* were collected from a local area, and its identification was confirmed by the taxonomy lab, in collaboration with Dr. Mansoor Hameed, Assistant Professor of the Department of Botany, University of Agriculture Faisalabad, Pakistan.

Extraction of Essential Oil

The essential oil from the leaves of *C. sempervirens* was extracted by the steam distillation method as described previously by Mazari *et al.* (2010). Steam containing the volatile oil was first allowed to condense and then dried by anhydrous sodium sulfate and stored at a low temperature until further analysis.

Physiochemical Investigation

Specific gravity

Specific gravity of the essential oil was measured with a specific gravity bottle of 10 mL capacity. Following the acetone cleanse, the acetone fumes were removed by air blasts, and the specific gravity bottle was dried thoroughly. The specific gravity bottle was then filled with reference liquid, and its weight was measured on an analytical balance. Then, the specific gravity bottle was emptied, dried, filled with essential oil, and the weight was recorded accurately.

Refractive index

The refractive index of each essential oil was determined with use of an Abbe's Refractrometer. The prism was opened, washed with acetone, and dried. A few drops of the essential oil were placed on the prism. The field of vision was divided into light and dark portions. The refractive index of essential oil was noted on the display.

Acid value

The acid value is the number of mg of KOH required to neutralize one gram of oil. Acid value of was determined by adding 2.50 g of oil and 10 mL of alcohol in a titration flask and titrating 0.1 M KOH using phenolphthalein as indicator. A blank test was carried out similarly. The difference in the volume of two readings was used to determine the acid value.

Ester value

Oil (2.5 g) was weighed into a 200 mL flask followed by the addition of 5 mL of alcohol, 3 drops of 1% alcoholic phenolphthalein, and 20 mL of 0.5 M alcoholic KOH to the flask. The contents of the flask were refluxed for 2 h on the steam bath. The flask was then allowed to cool at 25^{o}C for 15 min and the excess of alkali was titrated against 0.5 M HCl. A blank titration was also carried out to determine the amount of alkali consumed. The difference in the values gave the amount of alkali used for saponification of the ester.

Compositional analysis by GLC

The compositional analysis of the essential oil was carried out by gas liquid chromatography. One microliter of the extracted oil solution prepared in acetone was injected into the GC apparatus. Chromatography was performed using a glass capillary column (60 m long, 0.25 mm width). The injector and the detector were maintained at 250 °C while helium was used as a carrier gas with the split ratio of 1:70. The peaks of different chemical compounds present in the essential oil were obtained after the analysis, and the percentages of these chemical compounds were determined.

Antimicrobial Activity

The effect of essential oil to suppress the growth of bacteria and fungi was also assessed using different fungal and bacterial strains.

Microbial strains

The essential oil of *C. sempervirens* was tested against three bacteria (*Escherichia coli, Micrococcus luteus,* and *Bifidobacterium lactis*) and seven fungi (*A. niger, A. flavous, A. fumigatus, F. solani, F. oxysporium, P. digitatum,* and *C. uterus*). These species of microorganisms were obtained from the Department of Plant Pathology, University of Agriculture Faisalabad, Pakistan.

Antibacterial Screening

Diffusion method

The well plate technique was used to check the antibacterial activity of essential oil of *C. sempervirens*. The bacterial cultures were spread on the surface of Mueller-Hinton agar present in petri dishes. The wells were made by stainless steel borer in each plate. Fifteen microliters of the essential oil of *C. sempervirens* was poured in each well.

Petri dishes were then incubated at 37°C. The inhibition zones were measured after 24, 48, 72, and 96 h to assess the antibacterial activity (Emami *et al.* 2006)

Antifungal Screening

Flask culture method

The antifungal activity of essential oil of *C. sempervirens* by flask culture method was tested by adding a known volume of essential oil to the culture of three types of selected species of fungi, and its effect on the growth of fungi was recorded. A malt extract medium was prepared, and chloromycetin was added during the preparation of malt extract medium to avoid bacterial contamination. The selected fungal species were inoculated onto malt extract medium, and 0.25, 0.50, and 0.75 mL of essential oil was added into the different flasks. Each flask was numbered to identify the amount of essential oil added. The control flask without essential oil was also run in parallel to the test culture. After 7 and 14 days of incubation at 28°C, the growth pattern in the flasks was recorded in terms of weight of fungus. For this purpose, the fungus was filtered from each flask on a pre-weighed filter paper and kept in oven at 80°C for 24 h to dry the fungus. The mean weight of each fungus was then calculated. The percent inhibition of fungal growth was calculated by the following formula,

$$\text{Percent inhibition} = C - T / C \times 100 \quad (1)$$

where, C is the control fungus weight with no essential oil and T is the fungal weight in the presence of essential oil (Bansod and Rai 2008).

Well method

Antifungal activity of essential oil of *C. sempervirens* was also assessed by the well method. The fungal cultures were spread on the surface of potato dextrose agar (PDA) present in petri dishes. A well was made in each petri dish and 15 uL of essential oil was poured in this well. All petri dishes were incubated at 28°C. The measurement of inhibition zones was made after every 24, 48, 72, and 96 h (Bansod and Rai 2008).

Statistical Analysis

All the experiments were conducted in triplicate, and statistical analysis of the data was performed by calculating mean and standard deviation (SD). STATISTICA 5.5 software was employed. All data are presented as mean values ± standard deviation (SD) of triplicated determinations.

RESULTS AND DISCUSSION

Physiochemical Parameters of the Essential Oil

The essential oil of *C. sempervirens* was extracted by steam distillation with a yield of 0.50%. The specific gravity, refractive index, acid value, and ester value of the essential oil of *C. sempervirens* were 0.825, 1.341, 0.22, and 24.60, respectively. Mazari *et al.* (2010) obtained a yield of 0.26% of *C. sempervirens* while, according to Tapondjou *et al*. (2005), Cameroonian *C. sempervirens* had a yield (1%) at least three times greater than the Algerian *C. sempervirens*.

The results obtained by gas chromatography analysis of the essential oil of *C. sempervirens* revealed 13 compounds, which are identified and quantified in Table 1. The GLC result profile revealed the oil was predominantly composed of α-pinene (55.86%) while the second major compound was Δ-carene (24.53%), followed by limonine (4.31%), sequiterpene (3.91%), α-terpinene (3.28%), and sabinene (3.18%). The results obtained in the present research work are in agreement with the results obtained by Mazari *et al.* (2010) with α-pinene as a major component (60.5%).

In contrast to the present investigation, the cedrol (8.3%) was found to be the second major component of the *C. sempervirens* oil. Tognolini *et al*. (2006) found the α-pinene as the major component of *C. sempervirens* oil; however, it was present in an amount of 26.4%. According to Sacchetti *et al*. (2005) and Tapondjou *et al*. (2005), α-pinene is the second and third major component, respectively.

The difference in the chemical composition of the essential oil of *C. sempervirens* may be due to the effect of different geographical areas and climates on the physiology of *C. sempervirens*. Previously, Hosseini Hashemi and Kord (2011) have successfully investigated various physical properties of *Cupressus sempervirens* L.

Table 1. Chemical Composition of Essential Oil of *C. sempervirens*

Compound Name	Percentage (%)
α-pinene	55.86
Δ-carene	24.53
Limonine	4.31
sequiterpene	3.91
α-terpinene	3.27
Sabinene	3.18
Carvone	1.50
4-terpinol	0.68
β-cymene	0.64
Carveol	0.58
Cedrol	0.31
α-thugene	0.02
Santene	0.015
Total identified (%)	98.805

Antimicrobial Activity

Antimicrobial activity of essential oil of *C. sempervirens* was assessed by antibacterial and antifungal activities.

Antibacterial Screening

The essential oil extracted from *C. sempervirens* was evaluated for antibacterial activity by a diffusion method. The results showed that the essential oil inhibited the growth of selected bacterial species (Table 2). The essential oil was most effective against *B. lactis*, moderately effective against *E. coli*, and least effective against *M. luteus*. This indicates that essential oil of *C. sempervirens* is bacteriostatic.

Table 2. Antibacterial Activity of Essential Oil of *C. sempervirens*

Test Organism	Inhibition zone (mm)				Inhibition zones (mm)		Results
	24 h	48 h	72 h	96 h	Vancomycin	Erythromycin	
Escherichia coli	None	13.00 ± 0.36	13.10 ± 0.42	16.11 ± 0.24	10 ± 0.25	12 ± 0.18	Positive antibacterial
Micrococcus luteus	None	10.00 ± 0.56	10.20 ± 0.44	11.90 ± 0.48	11 ± 0.17	13 ± 0.11	Positive antibacterial
Bifidobacterium lactis	None	20.60 ± 0.67	21.50 ± 0.71	24.05 ± 0.76	24 ± 0.36	31 ± 0.14	Positive antibacterial

Antifungal Screening

Antifungal activity of essential oil of *C. sempervirens* was assessed by flask culture method and diffusion method.

Flask culture method

In the flask culture method, three fungal species (*Aspergillus niger, Aspergillus flavous, and Aspergillus fumigates*) were tested to check the antifungal activity of essential oil of *C. sempervirens*. The results showed that the growth of selected fungal species was inhibited with increasing concentration of essential oil (Table 3). The essential oil was most effective against *Aspergillus niger* with inhibition of 63 % after 7 days.

Well method

The essential oil of *C. sempervirens* was tested against seven fungal species to check its antifungal activity by well method. The obtained results (Table 4) showed that the essential oil was effective against five fungal species (*Aspergillus fumigatus, Fusarium solani, Fusarium oxysporium, Penecillium digitatum,* and *Candida uterus*), while it was unable to inhibit the growth of two fungal species (*Aspergillus niger* and *Aspergillus flavous*). The essential oil was most effective against *Candida uterus* while the least effectiveness was shown against *Fusarium solani*.

Table 3. Antifungal Activity of Essential Oil extracted from *C. sempervirens* by Flask Culture Method

Percentage inhibition	Fungal species tested	Volume of oil (mL)			Control fungus weight (g/mL)	Results
		0.25	0.50	0.75		
7 days	*Aspergillus niger*	37% ± 0.17	52% ± 0.32	63% ± 0.24	0.46 ± 0.01	Positive antifungal
	Aspergillus flavous	9% ± 0.23	35% ± 0.12	47% ± 0.37	0.31 ± 0.02	Positive antifungal
	Aspergillus fumigatus	47% ± 0.15	50% ± 0.34	57% ± 0.26	0.34 ± 0.02	Positive antifungal
14 days	*Aspergillus niger*	13% ± 0.11	17% ± 0.43	29% ± 0.35	0.48 ± 0.01	Positive antifungal
	Aspergillus flavous	3% ± 0.18	11% ± 0.25	23% ± 0.43	0.32 ± 0.02	Positive antifungal
	Aspergillus fumigatus	37% ± 0.27	45% ± 0.14	47% ± 0.33	0.37 ± 0.02	Positive antifungal

Table 4. Antifungal Activity of Essential Oil of *C. sempervirens* by Well Method

Test Organism	Inhibition zone (mm)				Nystatin inhibition zone (mm)	Results
	24 h	48 h	72 h	96 h		
Aspergillus niger	None	5.50 ± 0.25	7.00 ± 0.61	8.00 ± 0.43	9.50 ± 0.17	Positive antifungal
Aspergillus flavous	None	6.00 ± 0.31	7.20 ± 0.53	8.50 ± 0.47	9.70 ± 0.21	Positive antifungal
Aspergillus fumigatus	None	5.20 ± 0.26	5.20 ± 0.63	7.05 ± 0.35	8.25 ± 0.14	Positive antifungal
Fusarium solani	None	4.00 ± 0.17	4.10 ± 0.69	5.70 ± 0.46	6.75 ± 0.26	Positive antifungal
Fusarium oxysporium	None	5.00 ± 0.23	6.00 ± 0.56	7.10 ± 0.39	8.30 ± 0.22	Positive antifungal
Penecillium digitatum	None	27.00 ± 0.19	27.00 ± 0.54	29.00 ± 0.37	31.15 ± 0.13	Positive antifungal
Candida uterus	None	19.00 ± 0.15	19.00 ± 0.58	21.00 ± 0.45	22.85 ± 0.24	Positive antifungal

The results obtained from antimicrobial activity of essential oil of *C. sempervirens* showed a trend in agreement with the results obtained by Mazari *et al*. (2010). The cited authors also reported the antibacterial and antifungal activity of essential oil of *C. sempervirens*. Similar results were obtained by Ross *et al*. (1980). In their study, the essential oil of *C. sempervirens* showed antimicrobial activity against *Escherichia coli* and *Candida albicans*. Emami *et al*. (2006) found that essential oil extracted from leaves displayed a weak antimicrobial activity, while the essential oil extracted from fruits had stronger antimicrobial activity. Pawar and Thaker (2006) reported the inhibitory effect of essential oils on hyphal growth and spore formation of *Aspergillus niger*.

The major constituents of the essential oils of *C. sempervirens* such as α- pinene, β-phellandrene, α-Terpinyl acetate, and cedrol have been reported to display antimicrobial effects. Therefore, the antimicrobial activity of the essential oils of *C. sempervirens* may be due to these constituents (Cosentino *et al.* 1999; Oliveira *et al.* 2006; Yang *et al.* 2007; Demirci *et al.* 2007). The antimicrobial activity of essential oils containing terpenes has also been reported (Dorman and Deans 2000). Moreover, the synergistic effects of minor components of the essential oil may also contribute to the antimicrobial activity of essential oil (Marino *et al.* 2001).

CONCLUSIONS

1. In summary, the essential oil of *C. sempervirens* was predominantly composed of α-pinene, Δ-carene limonine, sequiterpene, α-terpinene, and sabinene.
2. The essential oil of *C. sempervirens* also exhibited antimicrobial properties, which implies that the oil might be effectively used in food preservation, aroma therapy, and as a natural antimicrobial agent for infectious diseases of human.
3. In this article and for the first time, antimicrobial features of locally extracted *C. sempervirens* oil were investigated in detail with a wide spectrum of locally isolated fungal and bacterial species.

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