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CONSTITUENTS AND BIOLOGICAL ACTIVITY OF THE CHLOROFORM EXTRACT AND ESSENTIAL OIL OF *Cupressus sempervirens*

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The essential oil of the leaves of Cupressus sempervirens *L. was isolated by hydrodistillation and tested against gram positive and gram negative bacteria, showing remarkable antimicrobial activity against* Bacillus subtilis *with minimum inhibitory concentration (MIC) 75%. The antiviral activity of the essential oil was tested against Herpes simplex virus type 1 (HSV-1), showing antiviral activity with virucidal percentages of 68.0% and 53.2% at concentrations of 1:32 and 1:64, respectively. We firstly reported the isolation of two* epi*-betulin esters of fatty acids from the $CHCl_3$ fraction of* Cupressus sempervirens *L. leaves, which were isolated and purified using HPLC, and identified using PMR and MS. The $CHCl_3$ fraction showed significant cytotoxicity against HeLa cells.*

Key words: *Cupressus sempervirens* L., *epi*-betulin esters, essential oil, antimicrobial, antiviral, antitumor activity.

Cupressus sempervirens L. is an ornamental tree belonging to the family Cupressaceae. It is native to Northern America, Africa, Southeastern Europe, and Western Asia. It was used to protect fields from wind [1]. *Cupressus sempervirens* L. is used in traditional medicine for the treatment of coughs, colds, parasitic infections, inflammation, hemorrhoids, and as a strong hair tonic; the fruit of the plant is used traditionally for curing diabetes and as an antiseptic [2–5].

In a continuous study of the chemical composition of the leaves of the *Cupressus sempervirens* L. we have reported the phenolic constituents of the MeOH extract of the leaves, which showed significant hepatoprotective and antioxidant activity [6]. The present study aims to investigate the essential oil and its antimicrobial and antiviral activities, as well as the hydrocarbons, sterols, and fatty acid components in the plant leaves, which have not been investigated before. We first reported the presence of *allo*-ocimene (24.00%) as one of the main constituents of the oil together with α-pinene and α-cedrol. The literature survey of the essential oil showed α-pinene as the major component of the isolated essential oils from the different kinds and species of *Cupressus sempervirens* L. [7]. This is the first investigation reporting the isolation of triterpene fatty acid esters, known as *epi*-betulin, from the $CHCl_3$ fraction, which showed significant anticancer activity.

The results of GC-MS analysis of the essential oil are given in Table 1, revealing the presence of 15 compounds found to represent 99.9% of the total oil constituents; the main constituents were *allo*-ocimene (24.00%), α-cedrol (23.68%), and α-pinene (21.15%).

The antimicrobial activity of the essential oil was evaluated by the inhibition zone method against different microorganisms, *Bacillus subtilis*, *Escherichia coli*, *Candida albicans, Staphylococcus aureus*, and *Aspergillus niger*; *Bacillus subtilis* was found to be the most sensitive one, while the oil had moderate activity against *Aspergillus niger*, *Candida albicans,* and *Staphylococcus aureus*; *Escherichia coli* was not inhibited at all. The minimum inhibitory concentration (MIC) values of the essential oil are given in Table 2, showing that the oil inhibited the growth at dilution 1:50–75 (v/v).

The antiviral activity of the essential oil tested against Herpes simplex virus type-1 (HSV-1) is given in Table 3. The obtained results revealed that the concentrations of 1:32 and 1:64 gave high antiviral activity with virucidal percentages of 68.0% and 53.2%, respectively, while at a concentration 1:128 no significant antiviral activity (18.6%) was recorded.

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TABLE 1. The Composition of the Essential Oil

Compound	Rt, min	%	Compound	Rt, min	%
α-Pinene	3.521	21.15	*allo*-Ocimene	6.097	24.00
cis-2,7-Dimethyl oct-5-yn-3-ene	3.712	4.37	4(15),6-Eudesmadiene	7.555	0.90
2-Pentyl-2-cyclopenten-1-one	3.862	1.55	Germacrene D	7.773	0.90
Terpinen-4-ol	4.134	6.98	δ-Cadinene	7.964	2.18
exo-Bornyl acetate	5.347	1.94	γ-Cadinene	8.237	1.29
iso-Bornyl acetate	5.361	0.90	α-Cedrol	9.286	23.68
Camphene	5.497	1.36	Dehydroabietane	14.015	8.02
α-Terpinolene	5.524	0.68			

Rt: retention time.

TABLE 2. The Minimum Inhibitory Concentration and the Sensitivities of Some Microorganisms to the Essential Oil

Microorganism	*Cupressus sempervirens* L. essential oil		Microorganism	*Cupressus sempervirens* L. essential oil	
	diameter of the inhibited zones (dilution 1:10), mm	MIC		diameter of the inhibited zones (dilution 1:10), mm	MIC
Staphylococcus aureus	1.1	50	*Candida albicans*	1.1	50
Bacillus subtilis	1.2	75	*Aspergillus niger*	1.2	50
Escherichia coli	–	50			

TABLE 3. Antiviral Activity of the Essential Oil

Dilution	HSV-1		
	Initial virus count, PFU/mL*	Virus count, PFU/mL	Percentage of virucidal effect
1:32	$4.3\times10^{6.5}$	$1.36\times10^{6.5}$	68.0
1:64	$4.3\times10^{6.5}$	$2.00\times10^{6.5}$	53.2
1:128	$4.3\times10^{6.5}$	$3.50\times10^{6.5}$	18.6

*Plaque forming unit/mL.

TABLE 4. Composition of USM

Compound	Rt	%	Compound	Rt	%
Hexadecane	11.06	5.58	Hexacosane	22.74	4.23
Heptadecane	11.96	2.41	Heptacosane	23.83	1.67
Octadecane	13.59	3.19	Octacosane	24.88	1.77
Nonadecane	14.57	2.01	Nonacosane	25.22	2.39
Eicosane	15.58	11.53	Cholesterol	29.49	11.15
Heneicosane	16.31	2.79	Stigmasterol	32.37	4.72
Docosane	18.12	1.86	β-Sitosterol	34.75	1.71
Tricosane	19.12	10.49	β-Amyrin	35.82	7.79
Tetracosane	19.83	11.20	α-Amyrin	36.83	5.20
Pentacosane	21.45	8.21			

Rt: retention time.

TABLE 5. The Composition of the Fatty Acids

Acid type	Rt, min	%	Acid type	Rt, min	%
12:0	4.72	5.03	18:2 ω6	7.47	5.22
12:1 ω9	5.05	1.07	18:3 ω3	7.51	5.85
14:0	5.52	3.10	20:0	7.84	2.54
15:0	5.86	8.00	20:3 ω6	8.43	2.48
15;1	5.90	6.38	20:4 ω6	8.46	2.32
16:0	6.27	11.98	22:0	8.82	2.10
16:1 ω7	6.35	2.18	22:1 ω9	9.34	9.13
18:0	7.01	3.48	24:0	9.89	13.6
18:1 ω9	7.11	4.46	24:4	10.09	0.68
18:2 ω6	7.33	2.09	25:0	10.48	1.98

Rt: retention time.

TABLE 6. The Cytotoxic Activity of the $CHCl_3$ Fraction

Compound	IC_{50}, μg/mL		
	HeLa cells	U 251	HEPG2
$CHCl_3$ fraction	5	>10	>10
Cisplatin	2.34	2.37	9.83

IC_{50}: dose that reduces the survival of cells to 50%.

The unsaponifiable matter USM was analyzed by GLC, and the results are shown in Table 4, revealing the presence of hydrocarbons ranging from C_{16}-C_{29} and sterols; eicosane 11.53%, tetracosane 11.20%, cholesterol 11.15%, tricosane 10.49%, pentacosane 8.21%, and β-amyrin 7.79% were the major constituents.

The saponifiable matter SM was analyzed by GC-MS, and the results are shown in Table 5, revealing the presence of 20 fatty acid ranging from $C_{12:0}$-$C_{25:0}$ consisting of saturated fatty acids, e.g., lauric acid, myrisitic acid, pentadecanoic acid, palmitic acid, stearic acid, arachidic acid, behenic acid, and lignoceric acid, together with unsaturated fatty acids, e.g., oleic acid and linoleic acid. The qualitative identification of the FAME was carried out by comparing their retention times and mass fragmentations with the reported published data [8–10].

The triterpene was hydrolyzed, isolated using HPLC, and identified as 3-*epi*-betulin by comparison of its ^{1}H NMR, MS, and specific rotation with the published data [11, 12]. The bond between the triterpene and the fatty acids was identified by comparing the spectral data and the chemical shifts before and after hydrolysis. There was a significant downfield shift for C-28 protons, while the C-3 proton was not changed at all, indicating that the fatty acids were bound to the C-28 OH group. The fatty acid mixtures were isolated and converted into FAMEs and identified as linoleic, palmitic, and stearic acids from their MS spectra [8–10].

The cytotoxic activity (Table 6), revealed the significant cytotoxicity of the $CHCl_3$ fraction with IC_{50} 5 μg/mL against HeLa cells and IC_{50} > 10 μg/mL for both U251 and HEPG2 compared with cisplatin (positive control) which showed IC_{50} 2.34, 2.37, and 9.83 μg/mL against the cell lines respectively.

EXPERMENTAL

Plant Material. The leaves of *Cupressus sempervirens* L. (Cupressaceae) were collected in January 2002 from El-Orman garden, Giza governorate, Egypt. The plant samples were kindly identified by Miss Tressa labib, the head of specialists

at the same garden. A voucher specimen of the plant was kept at our Lab.; the collected samples were air-dried, powdered, and kept for chemical analysis.

Extraction and Isolation. The air-dried powdered leaves (550 g) of *Cupressus sempervirens* L. were subjected to successive extraction with $CHCl_3$ using a Soxhlet apparatus. The $CHCl_3$ extract (38.00 g) was dissolved in a suitable amount of hot distilled H_2O–MeOH (95:5 v/v, 200 mL), then partitioned between petroleum ether and $CHCl_3$. The petroleum ether (20.50 g) and $CHCl_3$ (17.10 g) fractions were individually concentrated under reduced pressure.

Hydrodistillation. The air-dried leaves (300 g) of *Cupressus sempervirens* L. were cut into small pieces and then subjected to hydrodistillation for 3 h using a Clevenger-type apparatus, The oils, taken in 2 mL of capillary GC grade *n*-pentane and dried over anhydrous sodium sulphate, were subsequently analyzed by GC and GC-MS and stored at –20°C. The percent yield of oils was 0.26%.

GC-MS Analysis of the Essential Oil. Hewlett Packard gas chromatograph model (5890) series II plus, equipped with a carbowax 20 M capillary column (50 m × 0.32 mm i.d.), flame ionization detector (FID), helium as carrier gas at a flow rate of 1 mL/min, initial column temperature 60°C increasing to 200°C at a rate of 3°C/min and held at 200°C for 40 min, injector and detector temperatures 200 and 250°C, respectively. Hewlett Packard mass spectrometry model 5970. Temperature ionization detector (TIC) was used, carbowax 20 M capillary column (50 m × 0.32 mm i.d.), temperature increasing from 60 to 200°C by 3°C/min, and MS ionization voltage 70 eV. The identification of the components was based on comparison of their mass spectra with those of the Wiley and NBS Libraries [13] and those described by Adams [14].

GC of the Unsaponifiable Matters (USM). Hewlett Packard HP 6890 apparatus equipped with HP-1 methylsiloxane capillary column (30 m × 0.25 μm i.d.) and flame ionization detector (FID); nitrogen as carrier gas, hydrogen, and air set at flow rates 30, 30, and 300 mL/min, respectively. Oven temperature was programmed from 70–280°C at a rate 8°C/min. Temperatures of detector and injector were 300 and 250°C, respectively.

GC-MS of the Fatty Acid Methyl Esters (FAME). Voyager quadropole (Thermo-Finnigan Inc, CA, USA) full scan electron impact mode, under the following conditions: 2 μL of the sample was injected by split-less injection into a 30 m HP-5 MS capillary column (0.25 mm i.d.) and 0.25 μL film thickness (Agilent Technologies, CA, USA). The injector temperature was 200°C, ion source 230°C, column temperature was held at 100°C for 1 min and increased to 250°C at a rate of 30°C/min.

Instruments. ^1H NMR (Bruker DRX-400 spectrometer at 400 MHz). Optical rotation (Perkin–Elmer 241 polarimeter, at 25°C). HPLC (Shimadzu LC-10 system equipped with an SPC-H10Avp photodiode array detector). UV data were collected between 200 and 300 nm. Digital melting apparatus (Model 1A 8103, Electrothermal Engineering Ltd., U.K.).

Antimicrobial Activity of the Essential Oil.

Test Organisms. The Gram-negative bacterium *Escherichia coli* was used, as well as the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus,* and two fungi, *Aspergillus niger* and *Candida albicans,* which were obtained from Northern Regional Research Laboratory (NRRL), USA.

Screening Test. For bacterial strains, the organisms were grown in 1% (w/v) Oxoid Nutrient Broth (peptone 5 g, meat extract 3 g, and NaCl 2 g) supplemented separately with heat- sterilized glucose solution (0.5%, w/v final concentration). An inoculum (1.0 mL) of c.107 cells/mL was added to 9.0 mL of the glucose-nutrient broth. During incubation, tubes containing the microorganisms were shaken at 200 rpm in an incubator shaker for 24 h at the optimum growth temperature for each organism, after which the tubes were examined for visible growth [15].

Preparation of the Plates. The sterilized nutrient agar medium (50 mL) (Bacto-beef extract 0.3 g, Bacto-peptone 0.5 g, and Bacto-agar 15.0 g) was dissolved and maintained at 50–70°C; 1 mL of each test organism was added separately and 20 mL was distributed into Petri dishes with an inner diameter of 9 cm to provide thin agar plates of thickness 3.4–3.5 mm after being solidified. Then, hollows of 10 mm in diameter were made using a cork pourer [16]. An amount from the test solution (0.1 mL) was poured inside the hollows. Three hollows were made for each sample to be assayed. The Petri dishes were incubated at 5–8°C for 2–3 h to permit good diffusion and then transferred to an incubator of 28°C for 16 h. The diameter of the clear inhibition zone was measured for each sample.

Estimation of Minimum Inhibitory Concentrations (MIC). Due to the nature of the essential oil under investigation, serial dilutions of the oil sample in paraffin oil were prepared to give from 1:10 to 1:100 v/v dilutions. After complete mixing, 0.1 mL of each diluted solution was separately tested for its activity against the test organisms. MIC was recorded as the minimum level preventing growth after 24 h [17].

Antiviral Activity of the Essential Oil.

Cytotoxicity Assay. Cytotoxicity was assayed in African green monkey kidney cells [18] for both DMSO and the essential oil of *Cupressus sempervirens* L.; serial dilutions were prepared (1:32, 1:64 and 1:128) and inoculated on vero cells

grown in 96-well tissue culture plate. The maximum tolerated concentration (MTC) of the compounds was determined microscopically and also by cell counting using Trypan blue dye vital stain (0.4% Trypan blue dye in 0.81% NaCl and 0.06% K_2HPO_4, sterile and filtered).

Virucidal Assay of the Essential Oil. The direct effect of the volatile oil on HSV-1 was determined by incubating this virus for 1 h at 37°C with the essential oil of *Cupressus sempervirens* L. The essential oil was removed by ultracentrifugation at 45.000 rpm. The virucidal activity was determined by plaque reduction assay. Control was performed by incubating the virus with saline–phosphate buffer (PBS–A, pH 7.5), 16.0 mL of solution A (0.2 M NaH_2PO_4) added to 84.0 mL of solution B (0.2 M Na_2HPO_4), and treated identically.

Fractionation of the $CHCl_3$ Fraction. The $CHCl_3$ fraction was subjected to silica gel 60 column chromatography (550 g, mesh size 0.063–0.200 mm, Merck) (150 cm × 3 cm i.d.) eluted with *n*-C_6H_{12}, gradually increasing the polarity with EtOAc, which resulted in the isolation of two triterpene esters of fatty acids which were subjected to transesterification, then purified and isolated using HPLC, while the fatty acids were prepared as fatty acid methyl esters (FAMEs) and analyzed with GC-MS.

Antitumor Activity of the Chloroform Fraction. The potential cytotoxic activity of the $CHCl_3$ fraction was tested against human tumor cells HeLa, U251, and HEPG2 at concentrations between 1.00–10.00 μg/mL using the SRB assay at the National Cancer Institute and the previously reported method [19].

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