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In vitro biological activity screening of *Lycopodium complanatum* L. ssp. *chamaecyparissus* (A. Br.) Döll

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This article reports the results of selected biological activities, including anticholinesterase, antioxidant, antibacterial, antifungal and antiviral properties, of the petroleum ether, chloroform and methanol extracts as well as the alkaloid fraction of *Lycopodium complanatum* L. ssp. *chamaecyparissus* (A. Br.) Döll (LCC, Lycopodiaceae) growing in Turkey. Anticholinesterase effect of the extracts was tested against both acetylcholinesterase (AChE) and butyryl-cholinesterase (BChE) at concentrations of 0.2 and 1 mg mL^{-1} using microplate-reader assay based on Ellman method. Antioxidant activity of the LCC extracts was evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method at 0.2 mg mL^{-1} using microplate-reader assay. Both DNA virus *Herpes simplex* (HSV) and RNA virus *Parainfluenza* (PI-3) were employed for antiviral assessment of LCC exracts using Madin-Darby Bovine Kidney and Vero cell lines. Antibacterial and antifungal activities of the extracts were screened against the bacteria: *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Acinobacter baumannii*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis* as well as the fungi: *Candida albicans* and *C. parapsilosis*. Only the petroleum ether extract of LCC possessed remarkable activity against both AChE and BChE at 1 mg mL^{-1} (76.5 and 69.6%, respectively), whereas LCC extracts showed low free radical-scavenging activity. All of the extracts were found to be more effective against the ATCC strains than isolated ones, particularly *S. aureus*, while the extracts had moderate antifungal activity. On the other hand, we found that only the petroleum ether extract was active against HSV. In addition, we also analysed the content of the alkaloid fraction of the plant by capillary gas chromatography-mass spectrometry (GC-MS) and identified lycopodine as the major alkaloid (60.8%).

Keywords: *Lycopodium complanatum* spp. *chamaecyparissus*; lycopodiaceae; bioactivity; GC-MS; alkaloid

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1. Introduction

The genus *Lycopodium* L. (syn. *Huperzia* Bernh. and *Diphasia* Presl.) from the clubmoss family (Lycopodiaceae) consists of low terrestrial or epiphytic plants with approximately 100 species, occurring over most of the earth (Lawrence, 1989). In Turkey, this genus is represented by five species (*Lycopodium clavatum* L., *L. selago* L., *L. annotinum* L., *L. alpinum* L. and *L. complanatum* L. ssp. *chamaecyparissus* (A. Br.) Döll), distributed only in the northern Black Sea region (Davis & Cullen, 1984). On the other hand, previous investigations have shown that *Huperzia serrata* (Thunb.) Trev. (*syn. Lycopodium serratum* Thunb.), a plant used for improvement of memory and learning in Chinese traditional medicine for a long time, afforded the alkaloid Huperzine A, which has been proved to be effective in treatment of Alzheimer's Disease (AD) via inhibiting acetylcholinesterase (AChE) (Liu et al., 1986). AD is a progressive and neurodegenerative disorder illustrated by memory insufficiency and cognition loss and its pathogenesis is still unclear (Iwata, Higuchi, & Saido, 2005). At the moment, AChE inhibitors (e.g. donepezil, rivastigmine and galanthamine) and *N*-methyl-D-aspartate (NMDA) receptor antagonists (e.g. memantine) as well as antioxidants (e.g. *Ginkgo biloba* extract, EGb761) are the only drug classes in clinical practice for AD treatment (Karcmaz, 1998; O'Neill, 2005).

For this purpose, we herein examined the anticholinesterase potential of the petroleum ether (PE), chloroform (CHL) and methanol (MET) extracts as well as the alkaloid fraction of *L. complanatum* L. ssp. *chamaecyparissus* (A. Br.) Döll (LCC), growing in Turkey, against both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) using ELISA technique with the modified method of Ellman. Besides, antioxidant activity of these extracts was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging method using ELISA microplate-reader assay. Additionally, antibacterial, antifungal and antiviral properties of LCC extracts were also assessed and the alkaloid fraction of LCC was analysed by capillary gas chromatography-mass spectrometry (GC-MS).

2. Results and discussion

The extracts prepared with PE, CHL, MET and the alkaloid (ALK) extract from LCC were screened against both AChE and BChE at concentrations of 0.2 and 1 mg mL^{-1} by using microtiter plate assay based on the Ellman method. Galanthamine, used as the reference drug, exhibited 100% of inhibitory activity against AChE at both 0.2 and 1 mg mL^{-1} concentrations, while it showed 98.6 and 100% of inhibition against BChE at 0.2 and 1 mg mL^{-1} (Table 1). Among the extracts screened, LCC-PE had a quite significant activity against AChE with 76.5% of inhibition at 1 mg mL^{-1} concentration, whereas the rest of the extracts were found to be ineffective against AChE at 0.2 and 1 mg mL^{-1}. Interestingly, LCC-PE also seemed to be considerably active against BChE at the two tested concentrations (51.3 and 69.6%, respectively). Although LCC-CHL extract was shown to moderately inhibit BChE at 1 mg mL^{-1} with 48.7% of inhibition, rest of the extracts did not exhibit any noticeable inhibition on AChE and BChE.

The extracts were also screened for their antioxidant capacity by DPPH radical-scavenging activity (Table 1). BHA, a well-known antioxidant, was used as reference drug. The results demonstrated that all of the extracts did not present any notable radical-scavenging activity, ranging between 22.7 and 38.6%, while BHA displayed 92.7% of scavenging activity against DPPH.

Table 1. Anticholinesterase and DPPH radical-scavenging activities of the LCC extracts.

	Inhibition %				
	AChE		BChE		
Extracts	0.2 mg mL^{-1}	1 mg mL^{-1}	0.2 mg mL^{-1}	1 mg mL^{-1}	DPPH (0.2 mg mL^{-1})
LCC-PE	21.4 ± 3.02***	76.5 ± 0.71*	51.3 ± 3.73***	69.6 ± 1.11***	22.7 ± 1.83***
LCC-CHL	28.9 ± 3.21***	27.4 ± 1.86***	34.4 ± 5.14***	48.7 ± 1.12***	42.3 ± 1.56***
LCC-ALK	22.6 ± 0.51***	–	–	–	41.8 ± 0.50***
LCC-MET	7.6 ± 0.49***	–	–	–	38.6 ± 0.74***
Galanthamine	100.0 ± 0.73	100.0 ± 0.88	98.6 ± 0.23	100.0 ± 0.56	–
BHA	–	–	–	–	92.7 ± 0.21

Notes: –, No inhibition. *$p < 0.05$; ***$p < 0.001$.

The extracts were also tested for their antibacterial, antifungal and antiviral activities. Antibacterial activity of the extracts was compared to ampicilline, ofloxacine and levofloxacine as the reference drugs as listed in Table 2 by microdilution method. All of the extracts had the same minimum inhibitory concentration (MIC) values against the whole panel of the bacteria and were more successful on the ATCC strains than their isolated counterparts, while they were comparatively active against ATCC strain of *S. aureus* with the lowest MIC value ($4\,\mu g\,mL^{-1}$). On the other hand, LCC extracts had conspicuous antifungal activity against both *C. albicans* and *C. parapsilosis*, having MIC value of $16\,\mu g\,mL^{-1}$ (Table 2). Apart from the abovementioned activities, antiviral activity of the extracts was assessed against both DNA (*Herpes simplex*, HSV) and RNA viruses (*Parainfluenza-3*, PI-3) using MDBK and Vero cell cultures, respectively, and their maximum non-toxic concentrations (MNTC) were calculated (Table 3). Acyclovir for HSV and oseltamivir for PI-3 were employed as reference drugs. Our data revealed that the extracts were completely inactive against PI-3, whereas only LCC-PE exhibited significant inhibition against HSV.

Searching for enzyme inhibitors from natural sources is a modern approach in drug discovery and development. The major reason such an examination became opportune was the worldwide interest on finding new drugs for AD treatment from plants. The selection of LCC for the anticholinesterase and antioxidant screening in this survey was due to the reason that huperzine A, a very promising drug candidate for AD treatment, was previously isolated from *Huperzia serrata* (syn. *L. serratum*), a Chinese traditional medicine used against amnesia (Foster, 1989). Although huperzine A is an alkaloid-type of compound, PE extract of LCC, instead of the LCC-ALK extract, was found to be effective against both AChE and BChE at $1\,mg\,mL^{-1}$ concentration (76.5 and 69.6%, respectively), which may lead the speculation that nonpolar components found in the PE extract could be responsible of the anticholinesterase activity of LCC. The occurrence of anticholinesterase activity in ALK extract of LCC was considerably lower than we expected and, obviously, the alkaloidal constituents do not show evidence of anticholinesterase activity in this study. However, there have been several reports on cholinesterase inhibitory activity of non-polar compounds. In one of the earliest studies, *N*-[β-(dialkoxyphosphinylmercaptoethyl)] decahydroquinolines were shown to be inhibitors of AChE (Dalimov, Abduvakhabov, Aslanov, Godovikov, & Sadykov, 1978). Later, Tlegenov et al. (1991) reported that isovaleroyl, cinnamoyl, trichloroacetyl, trimethylacetyl derivatives of lupinine and epilupinine were active against AChE. Besides, the steroidal alkaloids (impericine and forticine) with cevanine skeleton from the bulbs of *Fritillaria imperialis*, were also stated to inhibit both AChE and BChE (Atta-ur-Rahman et al., 2002). Besides, one recent report on the AChE and BChE inhibitory activities of sterols concluded that new C-24 sterols, named as haloxysterols A-D, together with five known sterols, were tested by the Ellman method and all of these sterols were found to be the noncompetitive inhibitors of AChE and BChE in a concentration-dependent manner (Ahmed, Nawaz, Malik, & Choudhary, 2006).

Conversely, PE extract of LCC exerted the lowest free radical-scavenging activity (22.7%) against DPPH, while LCC-ALK and LCC-CHL had almost the same radical-scavenging capacity.

In addition to anticholinesterase and anticholinesterase activities, a range of antimicrobial screens were also included. Accordingly, in the antibacterial screening, there was a relatively low occurrence of antibacterial activity with LCC extracts, excepting *S. aureus*, in which they somewhat showed a comparatively higher inhibition.

Table 2. Antimicrobial activity of the extracts and the standards as (MICs) ($\mu g\,mL^{-1}$).

Micro-organism	*E. coli*		*P. aeruginosa*		*P. mirabilis*		*K. pneumoniae*		*A. baumannii*		*S. aureus*		*E. faecalis*			
Extracts	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	ATCC	Isol.	*C. albicans*	*C. parapsilosis*
LCC-PE	32	64	32	64	16	32	16	32	16	64	4	16	16	32	16	16
LCC-CHL	32	64	32	64	16	32	16	32	16	64	4	16	16	32	16	16
LCC-ALK	32	64	32	64	16	32	16	32	16	64	4	16	16	32	16	16
LCC-MET	32	64	32	64	16	32	16	32	16	64	4	16	16	32	16	16
AMP	2	64	–	–	2	4	2	4	2	4	<0.12	8	0.5	1	–	–
OFX	0.12	1	1	4	<0.12	1	<0.12	1	0.12	2	0.5	4	1	2	–	–
LVX	<0.12	0.25	1	2	<0.12	1	<0.12	1	0.12	2	0.5	4	0.5	2	–	–
KET	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1	1
FLU	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	4

Notes: –: No activity observed. AMP: Ampicilline, OFX: Ofloxasine, LVX: Levofloxacine, KET: Ketoconazole, FLU: Fluconazole.

Table 3. Antiviral activity of the extracts and the standards.

	MDBK cells ($\mu g\,mL^{-1}$)			Vero cells ($\mu g\,mL^{-1}$)		
		CPE inhibitory concentration			CPE inhibitory concentration	
		HSV			PI-3	
Extracts	MNTC ($\mu g\,mL^{-1}$)	Maximum	Minimum	MNTC ($\mu g\,mL^{-1}$)	Maximum	Minimum
LCC-PE	32	32	16	16	–	–
LCC-CHL	64	–	–	16	–	–
LCC-ALK	64	–	–	32	–	–
LCC-MET	8	–	–	32	–	–
Acyclovir	16	16	<0.25	–	–	–
Oseltamivir	–	–	–	32	32	<0.25

Notes: –: No activity observed. MNTC: Maximum non-toxic concentration, CPE: Cytopathogenic effect.

Another feature was the occurrence of noteworthy antifungal activity observed in LCC extracts. Interestingly, LCC–PE extract also revealed a good antiviral effect against HSV as compared to acyclovir with a better MNTC than that of acyclovir. Up to date, there has been no report on antimicrobial activity of LCC, however, methanolic extracts of some *Lycopodium* species (*L. cernuum*, *L. deuterodensum*, *L. laterale* and *L. scariosum*) were formerly tested for their antimicrobial activity (Bloor, 1995). These data demonstrated that the abovementioned species did not exert any antiviral effect against HSV, while they did not inhibit the growth of *S. aureus*, *P. aeruginosa*, *K. pneumoniae* and *Enterobacter agglomerans*, nor of *C. albicans*. Conversely, in a later study, ethanolic extract of *L. cernuum*, along with 10 serratene-type triterpenes and a flavonoid glucoside were tested against *C. albicans* secreted aspartic proteases (SAP) (Zhang et al., 2002). After testing the isolated compounds, only lycernuic acid C and apigenin-4′-*O*-(2′′,6′′-di-*O*-p-coumaroyl)-*β*-D-glucopyranoside were found to inhibit *C. albicans* SAP with IC50 of 20 and 8.5 $\mu g\,mL^{-1}$, respectively, while the other compounds were inactive.

The *Lycopodium* alkaloids represent a large family of plant constituents isolated from the clubmosses belonging to Lycopodiaceae (Morita et al., 2005). In continuation of the present work, we aimed to investigate the ALK extract of LCC by GC-MS in order to elucidate its main alkaloidal constituents, since no phytochemical work has been previously carried out on LCC. Our GC-MS analysis demonstrated that LCC-ALK consisted of lycopodine (m/z 247, 60.8%), as the major alkaloid, along with dihydrolycopodine (m/z 249, 8.0%) and lycodine (m/z 242, 3.2%). Lycodine, along with complanadine A and B, were previously isolated from *L. complanatum*, which is in consistance with our data (Morita et al., 2005; Kobayashi, Hirasawa, Yoshida, & Morita, 2000).

The complex nature of plant extracts and the high probability of competing or synergistic bioactivities within the same extract mean that these results represent the starting point for an activity-guided search for active plant metabolites. Especially, LCC-PE extract could be considered worthy of further investigation to uncover new agents with anticholinesterase or antiviral activities and this work is under progress in our laboratory. This is the first report describing anticholinesterase, antioxidant, antibacterial, antifungal and antiviral activities of LCC and also explaining the alkaloidal content of this subspecies.

3. Material and methods

3.1. *Plant material*

Plant material was collected from the Bagirankaya plateau near Ikizdere town of Rize province in the Northern Anatolia at approximately 1500 m of altitude. Voucher specimen was authenticated by Dr S. Terzioglu from the Department of Forest Botanics, Faculty of Forestry, Karadeniz Technical University, Trabzon (Turkey) and was deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara (Turkey) (GUE 2215).

3.2. *Preparation of the extracts*

Air-dried and powdered plant material was accurately weighed (173.4 g), macerated with petroleum ether (PE, 3×1 L), and sequentially with chloroform (CHL, 3×1 L)

and methanol (MET, 3×1 L). The organic phases were filtrated, combined, and evaporated until dryness to afford PE (10.5%, w/w), CHL (30.4%) and MET (20.2%) extracts of LCC.

In order to obtain the alkaloid extract of LCC according to Ortega, Agnese and Cabrera (2004)'s method, 40 g of dried the plant material was macerated with boiling water (500 mL $\times$ 2) for 1 h at each time. Later, the aqueous phases were filtrated and alkalinised to pH = 12 by 0.1 N NaOH and partitioned with dichloromethane in a separation funnel. The organic phase was concentrated under vacuum to dryness to yield the crude total alkaloid extract (LCC-ALK, 31.1%) and also employed in GC-MS analysis.

3.3. *Determination of AChE and BChE inhibitory activities*

Aetylcholinesterase and BChE inhibitory activities were measured by slightly modifying the spectrophotometric method developed by Ellman, Courtney, Andres and Featherstone (1961). Electric eel AChE (Type-VI-S, EC 3.1.1.7, Sigma) and horse serum BChE (EC 3.1.1.8, Sigma) were used, while acetylthiocholine iodide and butyrylthiocholine chloride (Sigma, St. Louis, MO, USA) were employed as substrates of the reaction. 5,5′-Dithio-*bis*(2-nitrobenzoic) acid (DTNB, Sigma, St. Louis, MO, USA) was used for the measurement of the cholinesterase inhibitory activity. All the other reagents and conditions were the same as described in a previous publication (Atta-ur-Rahman, Parveen, Khalid, Farooq, & Choudhary, 2001). Briefly, in this method, 140 μL of 0.1 mM sodium phosphate buffer (pH 8.0), 20 μL of DTNB, 20 μL of test solution and 20 μL of AChE/BChE solution were added by multichannel otomatic pipette (Gilson Pipetman, France) in a 96-well microplate and incubated for 15 min at 25°C. The reaction was then initiated with the addition of 10 μL of acetylthiocholine iodide/butyrylthiocholine chloride. The hydrolysis of acetylthiocholine iodide/butyrylthiocholine chloride was monitored by the formation of the yellow-coloured 5-*thio*-2-nitrobenzoate anion as a result of the reaction of DTNB with thiocholines, catalyzed by enzymes at a wavelength of 412 nm utilising a 96-well microplate reader (SpectraMax Plus-384, Molecular Devices, CA, USA). The measurements and calculations were evaluated by using Softmax PRO 4.3.2.LS software. Percentage of inhibition of AChE/BChE was determined by comparison of rates of reaction of samples relative to blank sample (ethanol in phosphate buffer pH = 8) using the formula $(E-S)/E \times 100$, where E is the activity of enzyme without test sample and S is the activity of enzyme with test sample. The experiments were done in triplicate. Galanthamine, purchased from Sigma (St. Louis, MO, USA), was used as the reference drug.

3.4. *DPPH free radical-scavenging assay*

The antiradical activity of the plant extracts and the reference were assessed on the basis of the radical scavenging effect of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) free radical (Lee et al., 1998). The concentration of DPPH was kept to 300 μM. The extracts and reference were dissolved in DMSO, while the DPPH solution was prepared in ethanol. 10 μL of each extract and reference were allowed to react with 200 μL of stable free radical DPPH at 37°C for 30 min in a 96-well microtiter plate. After incubation, decrease in absorption for each solution was measured at 515 nm

using ELISA microplate reader (Spectra MAX-340 Molecular Devices, USA). The corresponding blank readings were also taken and the remaining DPPH was calculated. Percent radical scavenging activity by samples was determined in comparison with a DMSO treated control group. Butylated hydroxyanisol (BHA, Sigma) was used as reference. Inhibition of DPPH in percent ($I\%$) was calculated in following way:

$I\% = (1 - A_{\text{sample}}/A_{\text{blank}}) \times 100$, where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} is the absorbance of the extracts/reference.

3.5. *Microbiological studies*

The LCC extracts were dissolved in ethanol:hexane (1 : 1) by using 1% tween 80 solution at a final concentration of $1024\,\mu g\,mg\,mL^{-1}$ and sterilised by filtration using $0.22\,\mu m$ Millipore (MA 01730, USA), and used as the stock solutions. Standard antibacterial powders of ampicillin (AMP, Fako), ofloxacin (OFX, Hoechst Marion Roussel), levofloxacin (LVX, Fako) standard antifungal powders of ketoconazole (KET, Bilim) and fluconazole (FLU, Pfizer) were obtained from their respective manufacturers and dissolved in phosphate buffer solution (ampicillin, pH: 8.0, $0.1\,mol\,L^{-1}$), dimethylsulphoxide (DMSO) (ketoconazole), in water (fluconazole, ofloxocin and levofloxacin). The stock solutions of the agents were prepared in medium according to the NCCLS recommendations (National Committee for Clinical Laboratory Standards, 1996).

3.6. *Microorganisms*

Standard strains of the following bacteria, namely *Escherichia coli* (ATCC 35218), *P. aeruginosa* (ATCC 10145), *P. mirabilis* (ATCC 7002), *K. pneumoniae* (RSKK 574), *Acinetobacter baumannii* (RSKK 02026), *Staphylococcus aureus* (ATCC 25923) and *Enterococcus faecalis* (ATCC 29212) for the determination of antibacterial activity, and standard strains of *C. albicans* (ATCC 10231) and *C. parapsilosis* (ATCC 22019) were used for the determination of antifungal activity.

3.7. *Inoculum preparation*

Mueller-Hinton Broth (Difco) and Mueller-Hinton Agar (Oxoid) were applied for growing and diluting of the bacteria. Sabouraud liquid medium (Oxoid) and Sabouraud dextrose agar (SDA) (Oxoid) were applied for growing and diluting of the fungi. The medium RPMI-1640 with L-glutamine was buffered pH : 7 with 3-[*N*-morpholino]-propansulfonic acid (MOPS). Prior to the test, strains of fungi and bacteria were cultured on media and passaged at least twice to ensure purity and viability at 35°C for 24–48 h. Culture suspensions were prepared according to the NCCLS M27-A (National Committee for Clinical Laboratory Standards, 1996). The bacterial suspensions used for inoculation were prepared at $10^5\,cfu\,mL^{-1}$ by diluting fresh cultures at McFarland 0.5 density ($10^8\,cfu\,mL^{-1}$). The fungi suspension was prepared by the spectrophotometric method of inoculum preparation at a final culture suspension of $2.5 \times 10^3\,cfu\,mL^{-1}$ (Özçelik, Küsmenoglu, Türköz, & Abbasoglu, 2004b).

3.8. *Antibacterial and antifungal tests*

The microdilution method was employed for antibacterial and antifungal activity tests. Media were placed into each well of the microplate. Extract solutions at 1024 μg mL^{-1} were added into first raw of microplates and 2-fold dilutions of the compounds (512–0.25 μg mL^{-1}) were made by dispensing the solutions to the remaining wells. 10 μL culture suspensions were inoculated into all the wells. The sealed microplates were incubated at 35°C for 24 and 48 h in humid chamber. The lowest concentration of the extracts that completely inhibit macroscopic growth and MICs were determined. Antibacterial activity of the extracts was tested against two Gram-positive, five Gram-negative bacterial strains and their isolates, using ampicillin, ofloxacin and levofloxacin as the standards. Two yeast-like fungi, *C. albicans* and *C. parapsilosis*, were included in the array of antifungal screens with the standards ketoconazole and flucanazole (National Committee for Clinical Laboratory Standards, 2002; Özçelik, Citak, Cesur, Abbasoglu, & Icli, 2004a).

3.9. *Cytotoxicity and antiviral tests*

African green monkey kidney (Vero cell line) and Madin-Darby bovine kidney (MDBK) used in this study were obtained from the Department of Virology, Faculty of Veterinary, Ankara University (Turkey). The culture of the cells were grown in Eagle's minimal essential medium (EMEM) enriched with 10% foetal calf serum (FCS) (Biochrom, Germany), 100 mg mL^{-1} of streptomycin and 100 IU mL^{-1} of penicillin in a humidified atmosphere of 5% CO_2 at 37°C. The cells were harvested using trypsin solution (Bipco Life Technologies, UK).

3.10. *Test viruses*

In order to determine the antiviral activity of the extracts, *Herpes simplex virus* (HSV) and *Parainfluenza*-3 *virus* (PI-3) were employed. The test viruses were obtained from the Department of Virology, Faculty of Veterinary, Ankara University (Turkey).

3.11. *Antiviral activity*

Media (EMEM) were placed into each well of the 96-well microplates (Greiner^{R}, Germany). Stock solutions of the extracts were added first raw into microplates, and two-fold dilutions of the extracts (512–0.25 μg mL^{-1}) were made by dispensing the solutions to the remaining wells. Two-fold dilutions of each material were obtained according to Log_2 on the microplates. Acyclovir (Biofarma) and oseltamivir (Roche) were used as the control agents. Strains of HSV and PIV titers were calculated by the Frey and Liess method as $TCID_{50}$ (Frey & Liess, 1971). They were inoculated into all the wells. The sealed microplates were incubated in 5% CO_2 at 37°C for 2 h to detect the possible antiviral activities of the samples. After incubation, 50 μL of the cell suspension of 300.000 cells/mL^{-1}, which were prepared in EMEM together with 5% foetal bovine serum, was put in each well and the plates were incubated in 5% CO_2 at 37°C for 48 h. After the end of this time, the cells were evaluated using cell culture microscope (×400), comparing with treated-untreated control cultures and with acyclovir and oseltamivir as the control agents.

Consequently, maximum cytopathogenic effect (CPE) concentrations as the indicator of antiviral activities of the extracts were determined (Özçelik et al., 2004a,b).

3.12. *Cytotoxicity*

The MNTC of each sample was determined by the method described previously by Özçelik and co-workers, based on cellular morphologic alteration (Özçelik, Aslan, Orhan, & Karaoglu, 2005). Several concentrations of each sample were placed in contact with confluent cell monolayer and incubated in 5% CO2 at 37°C for 48 h. MNTCs were determined by comparing treated and controlling untreated cultures.

3.13. *Conditions of gas chromatography-mass spectrometry (GC-MS) analysis*

Chromatographic analysis was carried out on Agilent 6890N Network GC system combined with Agilent 5973 Network Mass Selective Detector (GC-MS). The capillary column used was an Agilent 19091N-136 (HP Innowax Capillary; 60.0 m × 0.25 mm × 0.25 μm). Helium was used as carrier gas at a flow rate of 2.4 mL min^{-1} for MS and 2.3 mL min^{-1} for FID columns with 1 μL injection volume. Samples were analysed with the column started initially at 150°C, then increased to 250°C with 5°C min^{-1} heating ramp and kept at 250°C for 15 min. The injection was performed in split mode (20 : 1). Detector and injector temperatures were 250 and 250°C, respectively. Run time was 35 min. MS interface temperature was 280°C. MS scan range was (m/z): 15–450 atomic mass units (AMU) under electron impact (EI) ionisation (70 eV).

3.14. *Identification of the alkaloidal components*

Identification of the peaks was done using Wiley and Nist Library, comparison of retention time (*Rt*) and comparison of standard compounds with mass spectrums. Relative contents of % alkaloids were determined with area under peaks using Agilent software. The results were expressed as an average of three determinations in all cases.

3.15. *Statistical analysis of data*

Data obtained from *in vitro* experiments were expressed as mean standard error (±SEM). Statistical differences between the treatments and the control were evaluated by ANOVA test. $p < 0.05$ was considered to be significant ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

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