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Antiprotozoal activity and cytotoxicity of *Lycopodium clavatum* and *Lycopodium complanatum subsp. chamaecyparissus* extracts

[*Lycopodium clavatum* ve *Lycopodium complanatum subsp. chamaecyparissus* ekstrelerinin antiprotozoal aktivitesi ve sitotoksisitesi]

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ABSTRACT

Objective: We assessed *in vitro* antiprotozoal activity of the petroleum ether (PE), chloroform ($CHCl_3$), methanol (MeOH) and alkaloid (ALK) extracts of the ferns *Lycopodium clavatum* L. (LC) and *L. complanatum* L. subsp. *chamaecyparissus* (A. Br.) Döll. (LCC).

Methods: Antiprotozoal activity of the extracts was assessed against *Trypanosoma brucei rhodesiense*, *T. cruzi*, *Leishmania donovani*, and *Plasmodium falciparum* and their cytotoxicity was tested on rat skeletal myoblast (L6) cells.

Results: All extracts inhibited the growth of *T. brucei rhodesiense* with IC_{50} values of 9.3 to 47.0 µg/ml. The LC-$CHCl_3$ extract had the best trypanocidal activity against *T. cruzi* (IC_{50} 15.3 µg/ml), whereas the LCC-PE extract displayed the highest antileishmanial activity (IC_{50} 4.5 µg/ml). The most potent activity against *P. falciparum* was exhibited by LCC-ALK (IC_{50} 2.7 µg/ml) and LCC-PE (IC_{50} 2.8 µg/ml) extracts. No cytotoxicity for any of the extracts was detected at the highest concentration tested (IC_{50} > 90 µg/ml).

Conclusion: Both fern species possibly contain antiprotozoal compounds with no cytotoxicity.

Key Words: *Lycopodium*, Lycopodiaceae, antiprotozoal activity, cytotoxicity

Conflict of Interest: We declare no conflict of interest.

ÖZET

Amaç: İki eğrelti olan *Lycopodium clavatum* L. (LC) and *L. complanatum* L. subsp. *chamaecyparissus* (A. Br.) Döll.'un (LCC) petrol eteri (PE), kloroform ($CHCl_3$), metanol (MeOH) ve alkaloit (ALK) ekstrelerinin *in vitro* antiprotozoal aktivitesi tayin edilmiştir.

Yöntem: Ekstrelerin antiprotozoal aktivitesi, dört parazitik protozoaya (*Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*) karşı, sitotoksisiteleri ise sıçan iskelet miyoblast (L6) hücreleri üzerinde test edilmiştir.

Bulgular: Tüm ekstreler, IC_{50} değerleri 9.3 µg/ml ile 47.0 µg/ml arasında değişmek üzere, *T. brucei rhodesiense*'nin gelişimini inhibe etmiştir. LC-$CHCl_3$ ekstresi *T. cruzi*'ye karşı en iyi tripanosidal aktiviteye sahipken (IC_{50} 15.3 µg/ml), LCC-PE ekstresi en yüksek antileşmaniyal aktiviteyi göstermiştir (IC_{50} 4.5 µg/ml). *P. falciparum*'a karşı en potent aktivite LCC-ALK (IC_{50} 2.7 µg/ml) ve LCC-PE (IC_{50} 2.8 µg/ml) ekstreleri tarafından gösterilmiştir. Test edilen en yüksek konsantrasyonda, ekstrelerin herhangi biri için sitotoksisite tespit edilmemiştir (IC_{50} > 90 µg/ml).

Sonuçlar: Muhtemelen her iki eğrelti türü de sitotoksik olmayan antiprotozoal bileşikler taşımaktadır.

Anahtar Kelimeler: *Lycopodium*, Lycopodiaceae, antiprotozoal aktivite, sitotoksisite

Introduction

The genus *Lycopodium* (syn. *Huperzia* Bernh. and *Diphasia* Presl.) (Lycopodiaceae), usually known as "club moss, ground pine, devil's claw or devil ash" in, is a pteridophyte found abundantly in subtropical and tropical forests and is currently under risk of extinction [1]. The genus is represented by five species in Turkish flora, namely *L. alpinum* L., *L. annotinum* L., *L. clavatum* L., *L. complanatum* subsp. *chamaecyparissus* (A. Br.) Döll, and *L. selago* L. [2]. Of these species, *L. clavatum* (LC) is the most common in Anatolia and has been used in herbal tea form as well as its for wound-healing effect in powder form against nappies occurring in babies and, therefore, also called "belly powder" [3]. Spores of the plant as dusting powder have been stated to be protective of tender skin [4].

Protozoal diseases and infections are a major health problem in many parts of the world as the parasites develop resistance against the available drugs. Malaria caused by the protozoa *Plasmodium falciparum*, *P. ovale*, *P. vivax*, and *P. malariae* is the most important of these diseases [5], followed by African trypanosomiasis (sleeping sickness caused by *Trypanosoma brucei)* [6], Chagas' disease caused by *T. cruzi* [7], and leishmaniasis caused by *Leishmania* species [8], all of which can be fatal if untreated. Conventional therapy against leishmaniasis consists of pentavalent antimonials and recently liposomal amphotericin B [9]. Nevertheless, antimonials lead to drug resistance and liposomal amphotericin B is quite expensive. Treatment of African trypanosomiasis, another fatal parasitic disease, has also been problematic because of severe adverse effects, drug resistance and high cost, although discovery of melarsoprol in 1949 provided improvement to some extent in the treatment [10,11]. Hence, new, safe, effective, and affordable drugs are urgently needed for therapy of theSE diseases.

We previously reported antibacterial, antifungal and antiviral activities of the petroleum ether (PE), chloroform ($CHCl_3$), methanol (MeOH),and alkaloid (ALK) extracts of *Lycopodium clavatum* (LC) and *L. complanatum* L. subsp. *chamaecyparissus* (LCC) growing in Turkey [12,13]. Encouraged by the records on traditional use of several *Lycopodium* species as anti-infective [3,4,14], we have assessed *in vitro* growth-inhibitory activity of these extracts against clinically relevant stages of *Trypanosoma brucei rhodesiense* (bloodstream forms), *Trypanosoma cruzi* (intracellular amastigotes in L6 rat skeletal myoblasts), *Leishmania donovani* (axenic amastigotes) and *Plasmodium falciparum* (blood stage forms of K1 strain resistant to chloroquine and pyrimethamine), by determining their IC_{50} values, using chloroquine (*P. falciparum,*), benznidazole (*T. cruzi*), melarsoprol (*T. brucei rhodesiense*) and miltefosine (*L. donovani*) as reference drugs. Cytotoxicity of the extracts, in comparison with podophyllotoxin as reference, was evaluated using a rat skeletal myoblast (L6) cell line.

Materials and Methods

Plant materials

Lycopodium clavatum L. (LC) and *L. complanatum* L. subsp. *chamaecyparissus* (A. Br.) Döll. were collected from the Bagirankaya plateau nearby Ikizdere in Rize province of Northern Anatolia in 2011. Voucher specimens were authenticated by Dr. S. Terzioglu from the Department of Forest Botany, Faculty of Forestry, Karadeniz Technical University, Trabzon (Turkey) and were deposited at the Herbarium of the Department of Pharmacognosy, Faculty of Pharmacy, Gazi University, Ankara (Turkey) (GUE 2215).

Preparation of EXTRACTS OF LC and LCC

The air-dried and powdered plant materials of LC and LCC were accurately weighed (253 g and 127 g, respectively). The PE extracts were prepared by maceration (2 × 1 L) for each sample at room temperature, filtrating through filter paper and concentrating to dryness under reduced pressure, which were extracted sequentially by $CHCl_3$ (2 × 2 L) and MeOH (2 × 2 L). In order to prepare their alkaloid (ALK) extracts, the $CHCl_3$ extracts from both species using 100 g of each plant material extracted by $CHCl_3$ (1 L × 3) were prepared and treated with 3% HCl and left for 2 days at +4º. The precipitates formed after the addition of HCl were removed by filtration and then, the organic phases were exposed to acid-base shifting (adjusted to pH 12 using 25% NH_4OH), which finally led to the ALK extracts. Yield percentages (w/w) of the extracts are given as follows: LC-PE (4.92%), LC-$CHCl_3$ (22.83%), LC-ALK (24.07%), LC-MeOH (53.04%), LCC-PE (11.6%), LCC-$CHCl_3$ (32.11%), LCC-ALK (33.38%), LCC-MeOH (19.99%).

Activity against P. falciparum

In vitro activity against erythrocytic stages of *P. falciparum* was determined by a modified [^{3}H]-hypoxanthine incorporation assay [15], using the chloroquine- and pyrimethamine-resistant K1 strain and the reference drug artemisinin. Briefly, parasite cultures incubated in RPMI 1640 medium with 5% Albumax (without hypoxanthine) were exposed to serial drug dilutions in microtiter plates. After 48 h of incubation at 37°C in a reduced oxygen atmosphere, 0.5 µCi ^{3}H-hypoxanthine was added to each well and incubated for a further 24 h before being harvested onto glass-fiber filters and washed with distilled water. The radioactivity was counted using a Betaplate™ liquid scintillation counter (Wallac, Zurich, Switzerland). Results were recorded as counts *per* minute (CPM) *per* well at each drug concentration and expressed as percentage of the untreated controls. IC_{50} values were calculated from the sigmoidal inhibition curves using Microsoft Excel.

Activity against Trypanosoma brucei rhodesiense

T. b. rhodesiense (STIB 900 strain) and melarsoprol were used for the assay. This stock was isolated in 1982 from a human patient in Tanzania and after several mouse passages cloned and adapted to axenic culture conditions [16,17]. Minimum Essential Medium (50 µl) supplemented with 25 mM HEPES, 1g/l additional glucose, 1% MEM non-essential amino acids (100×), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and heat-inactivated horse serum (15%) was added to each well of a 96-well microtiter plate. Serial drug dilutions of seven 3-fold dilution steps from 90 to 0.123 µg/ml were prepared. Then 10^4 bloodstream forms of *T. b. rhodesiense* STIB 900 in 50 µl was added to each well and the plate incubated at 37°C under a 5% CO_2 atmosphere for 72 h. 10 µl Alamar Blue (resazurin, 12.5 mg in 100 ml double-distilled water) was then added to each well and incubation continued for a further 2-4 h [18]. The plates were THEN read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

Activity against Trypanosoma cruzi

Rat skeletal myoblasts (L6 cells) were seeded in 96-well microtitre plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% FBS and 2 mM l-glutamine. After 24 h, the medium was removed and replaced by 100 µl *per* well containing 5000 trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (Lac Z) gene [19]. After 48 h, the medium was removed from the wells and replaced by 100 µl fresh medium with or without a serial drug dilution of seven 3-fold dilution steps from 90 to 0.123 µg/ml. After 96 h of incubation, the plates were inspected under an inverted microscope to ensure growth of the controls and sterility. Then, the substrate CPRG/Nonidet (50 µl) was added to all wells. A color reaction developed within 2-6 h and could be read photometrically at 540 nm. The results were transferred into the graphic program Softmax Pro (Molecular Devices), which calculated IC_{50} values. Benznidazole was the reference drug used.

Activity against Leishmania donovani

Amastigotes of *L. donovani* (strain MHOM/ET/67/L82) were grown in axenic culture at 37°C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum under an atmosphere of 5% CO_2 in air. 100ul of culture medium with 10^5 amastigotes from axenic culture with or without a serial drug dilution were seeded in 96-well microtiter plates. Serial drug dilutions from 90 to 0.123 µg/ml were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to ensure growth of the controls and sterile conditions. 10 µl of Alamar Blue (12.5 mg resazurin dissolved in 100 ml distilled water) [19] was then added to each well and the plates incubated for another 2 h. The plates were THEN read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. The results were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (*i.e.*, inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves, the IC_{50} values were calculated. Miltefosine was used as reference drug.

Cytotoxicity against L6 cells

Assays were performed in 96-well microtiter plates having each well containing 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% fetal bovine serum, and 4 × 10 4 L-6 cells (a primary cell line derived from rat skeletal myoblasts). Serial drug dilutions of seven 3-fold dilution steps, from 90 to 0.123 µg/ml, were prepared. After 72 h of incubation, the plates were inspected under an inverted microscope to ensure growth of the controls and sterile conditions. 10 µl of Alamar Blue solution was then added to each well and the plates incubated for another 2 h. The plates were then read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the microplate reader software Softmax Pro. Podophyllotoxin was the reference drug used.

Statistical Analysis

The IC_{50} values of the extracts and the references in these experiments are mean values from at least two replicates of duplicates.

Results

The antitrypanosomal activities of LC and LCC extracts and reference compounds are listed in Table 1. All extracts showed moderate activity against African trypanosomes (*T. b. rhodesiense*), and only the MeOH extracts of both LC and LCC were inactive (IC_{50} >90 µg/ml) when tested against American trypanosome, *T. cruzi*. Generally, the growth inhibition was higher against *T. b. rhodesiense* than against *T. cruzi*, and the extracts displayed moderate activity having IC_{50} values in the range 9.3-47.0 µg/ml (*T. b. rhodesiense*) and 15.3-59.6 µg/ml (*T. cruzi*), while the references; melarsoprol and benznidazole showed IC_{50} values of 0.003 and 0.35 µg/ml, respectively. The most active extracts against these flagellates were LCC-PE (IC_{50} 9.3 µg/ml) and LC-$CHCl_3$ (IC_{50} 15.3 µg/ml), respectively.

All extracts of LC, except the MeOH extract, displayed leishmanicidal effect. LCC-PE, LCC-$CHCl_3$, and LCC-ALK, exerted remarkable activity against *L. donovani* (IC_{50} 4.5, 6.7, and 7.2 µg/ml, respectively) (Table 1). LC-

Table 1. *In vitro* antiprotozoal and cytotoxic activity of the extracts of *L. clavatum* (LC) and *L. complanatum* subsp. *chamaesyparissus* (LCC) as IC_{50} in µg/ml.

Extracts	Trypanosoma b. rhodesiense	*Trypanosoma cruzi*	*Leishmania donovani*	*Plasmodium falciparum*	Cytotoxicity (L6 cells)
LC-PE	13.3	53.6	**8.6**	**4.6**	>90
LC-$CHCl_3$	12.4	**15.3**	**8.5**	**6.2**	78.7
LC-ALK	23.1	59.6	20.9	12.5	>90
LC-MeOH	44.3	>90	>90	>20	>90
LCC-PE	9.3	**19.5**	**4.5**	**2.8**	>90
LCC-$CHCl_3$	15.8	45.0	**6.7**	**4.0**	>90
LCC-ALK	14.9	22.9	**7.2**	**2.7**	>90
LCC-MeOH	47.0	>90	17.2	>20	>90
Reference	0.003[a]	0.35[b]	0.20[c]	0.056	0.004[e]

[a]melarsoprol, [b]benznidazole, [c]miltefosine, [d]chloroquine, [e]podophyllotoxin.

PE and LC-$CHCl_3$ extracts had notable antileishmanial activity (IC_{50} ~ 8 µg/ml), while miltefosine had IC_{50} value of 0.20 µg/ml.

The highest antiplasmodial activity was that of LCC-ALK (IC_{50} 2.7 µg/ml), LCC-PE (IC_{50} 2.8 µg/ml), and LCC-$CHCl_3$ (IC_{50} 4.0 µg/ml) extracts. LC-PE and LC-$CHCl_3$ extracts had also noteworthy inhibition against *P. falciparum* (IC_{50} 4.6 and 6.2 µg/ml, respectively) as compared to the reference (chloroquine), which displayed IC_{50} value of 0.056 µg/ml. No cytotoxicity was exerted by LC and LCC extracts (IC_{50} >90 µg/ml) on rat skeletal muscle myoblasts (L6 cells), with the exception of LC-$CHCl_3$, which appeared to possess very low toxicity at 78.7 µg/ml.

Discussion

Searching for new antiprotozoal agents from natural sources is a reasonable approach in drug discovery and development. Quinine and artemisinin, the historical and modern antimalarial agents, are good examples of this strategy [20]. *Lycopodium* (*Huperzia*) is a genus of clubmosses, *i.e.* flowerless and primitive fern plants, rich in so-called "*Lycopodium* alkaloids" that have quinolizine, pyridine, and alpha-pyridone chemical skeletons [21]. These plants have a long history of use in Chinese folk medicine for the treatment of many ailments [22], but the genus has gained a worldwide reputation after the isolation of huperzine A from *Huperzia serrata* [23,24]. Huperzine A is a potent, reversible and selective acetylcholinesterase inhibitor [25] and is promising for treatment of symptoms of Alzheimer's disease [23]. *Lycopodium clavatum* is available as homeopathic remedy for treatment of mental, liver, eye, skin, urological and inflammatory diseases, respiratory allergies as well as eczema, otitis media, tumor, and cough [26-29]. *L. complanatum* has been also used against some skin diseases in Russia and central Asia [30].

We previously screened for antibacterial, antifungal, and antiviral activities of LC and LCC extracts [12,13]. Despite the lack of notable antibacterial and antifungal activities, the PE and $CHCl_3$ extracts exhibited significant antiviral activity against *Parainfluenza* virus (*e.g.* LC-PE) and *Herpes simplex* virus (*e.g.* LC-$CHCl_3$, LCC-PE). In the current study on the antiprotozoal activity of these extracts against four highly pathogenic parasitic protozoa, we observed a similar trend, the non-polar and middle polarity extracts being generally active, whereas the MeOH extract had low or no activity (IC_{50} value against *T. brucei rhodesiense* 44.3 µg/ml). The LC-PE and LCC-$CHCL_3$ extracts exerted similar activity profiles, especially against *L. donovani* and *P. falciparum*, which may result from having a similar phytochemistry. This also suggests that nonpolar and middle polarity components found in the PE and $CHCl_3$ extracts may be responsible for the antiprotozoal activity of LC and LCC. While *Lycopodium* species have been extensively searched for their alkaloid contents [22-24], we have encountered few studies on its nonpolar components; namely sterols, hydrocarbons, and fatty acids [31,32]. We also reported the isolation of some non-polar compounds; namely palmitic acid, cerotic acid, tetradecyl acetate, and 24-alpha-methylcholest-5-enol, and a serratane-type triterpene, alpha-onocerin [33,34]. It has been reported that amphotericin B complexed with cholesterol is at least four times more active than amphotericin B alone in *L. donovani*-infected hamsters [35]. Moreover, some fatty acid derivatives have been reported with antileishmanial and antimalarial activity [36-39]. Hence, remarkable antiprotozoal activity of the PE extracts of LC and LCC could be due to their fatty acids and sterol constituents. Along with their prosperous alkaloid contents, LC and LCC also contain triterpenes [40-42] and flavonoid derivatives [43]. Therefore, noteworthy inhibitory effect of the $CHCl_3$ extracts of both LC and LCC could be related to their middle-polarity components, such as terpenes, flavonoids, and alkaloids. In a comparable relation with

this statement, apigenin-4′-O-(2″,6″-di-O-p-coumaroyl)-β-D-glucopyranoside and lycernuic acid (a serratane-type triterpene) isolated from *L. cernuum* were reported to strongly inhibit aspartic protease secreted by *Candida albicans* [44].

In the current study, an interesting trend was seen with the alkaloid extracts of LC and LCC plants. The LCC-ALK extract was two to five times more active than LC-ALK against all protozoan species, which is suggestive of differences in the alkaloid profile of the species. However, our recent studies, which analyzed the alkaloid extracts of LC and LCC by gas chromatography coupled with mass spectrometry (GC-MS) [9,10], showed great chemical similarities between these two extracts. LCC-ALK extract contained lycopodine (60.8%) as the major alkaloid along with dihydrolycopodine (8.0%) and lycodine (3.2%), while LC-ALK consisted of lycopodine (89.7%), dihydrolycopodine (7.6%), and lycodine (2.7%). Therefore, we can speculate that those alkaloids could be responsible for the high antileishmanial and antiplasmodial activities of LCC-ALK. The lower bioactivity profile observed with LC-ALK however might be due to some other additional minor components present in LCC-ALK that contribute to the high antiprotozoal activity of this extract. Another important information gained from the present study is the absence (or very low) cytotoxicity of all extracts against a mammalian cell line (L6), indicating a good selectivity. It also shows that the antiprotozoal activities of the extracts are not due to a non-selective toxicity, opening new doors for the discovery of safe antiprotozoal agents. Toxicity is an important issue in the treatment of parasitic diseases, as many leishmanicidal and trypanocidal drugs currently in use have very low therapeutic windows, limiting their use particularly in children.

Hence, the present study has revealed some interesting clues about the antiprotozoal potential of Turkish *Lycopodium* species. Recently, four huperzine derivatives have been reported to possess strong *in vitro* antitrypanosomal activity against *T. brucei* [41]. Our previous liquid chromatography-mass spectrometry (LC-MS) analyses have ruled out the presence of huperzine A in the alkaloid extracts of either LC or LCC [42], which may explain to some extent the low potential of LC and LCC extracts against *T. brucei rhodesiense* and *T. cruzi*.

Conclusion

Our findings indicate that LC and LCC extracts may have the potential to provide novel antiprotozoal agents and deserve further phytochemical investigations. Based on our extensive literature survey, our study appears to be the first reporting the antiprotozoal activity of *Lycopodium* species, including *Lycopodium clavatum* and *L. complanatum* L. subsp. *chamaecyparissus*.

Conflict of Interest: We declare no conflict of interest.

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