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ORIGINAL PAPER

In vitro amoebicidal activity of four *Allium* species on *Acanthamoeba castellanii* and their cytotoxic potentials on corneal cells

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Abstract Amoebic keratitis is difficult to treat without total efficacy in some patients because of cysts, which is less susceptible than trophozoites to the usual treatments. We investigated the in vitro effectiveness of the methanolic extract of four *Allium* species from Turkey against *Acanthamoeba castellanii* and its cytotoxicity on corneal cells in vitro. Extracts were evaluated for their amoebicidal activities using an inverted light microscope. The effect of the *Allium* species with concentrations ranging from 1.0 to 32.0 mg/ml on the proliferation of *A. castellanii* trophozoites and cysts were examined in vitro. For the determination of the cytotoxicity of the *Allium* species on corneal cells, agar diffusion tests were performed. According to the results obtained from the tests, *A. scrodoprosum* subsp. *rotundum* showed remarkable amoebicidal effect on *A. castellanii*, while the others remained inactive. In the case of cyctotoxic activities, the methanolic extracts of *A. scrodoprosum* showed no cytotoxicity for the cells in the concentration of 32 mg/ml, while the others exerted significant cytotoxic activities between the concentrations of 1 and 16 mg/ml. As a result, the methanolic extract of *A. scrodoprosum* could be concluded as a new natural agent against *Acanthamoeba*. On the other hand, it still needs to be further evaluated by in vivo test systems to confirm the efficiency of its biological effect.

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Introduction

Free-living amoebae belonging to the genus *Acanthamoeba* are opportunistic pathogens isolated from a number of different ecological environments. The life cycle of *Acanthamoeba* consists of two stages: an actively feeding, dividing trophozoite and a dormant cyst. The trophozoite varies in size from 25 to 40 μm and feeds on bacteria, algae, and yeast in the environment but also can exist axenically on nutrients in liquid taken up through pinocytosis (Ma et al. 1990; Saragoza 1994). In the encysted state, they are protected from unfavorable environmental conditions and are resistant to extremes of temperature, desiccation, and microbial agents (Biddick et al. 1984; Kilvington 1989; Turner et al. 2000).

Acanthamoeba is the causative agent of granulomatous amebic encephalitis (GAE), a fatal disease of the central nervous system (CNS) and amebic keratitis (AK), a painful sight-threatening disease of the eyes (Martinez and Janitschke 1985; Naginton et al. 1974). They were also identified as the agent of cutaneous nodules and abscesses, arthritis, and rhinosinusitis. Immunocompromised individuals, including AIDS patients, are particularly susceptible to infections of *Acanthamoeba* (Dunand et al. 1997; Gullett et al. 1979; Torno et al. 2000). *Acanthamoeba* keratitis is a rare disease that occurs most commonly among healthy individuals and

seems to be associated with minor trauma to the eye and the use of contact lenses. The species detected in patients with keratitis are commonly *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, *Acanthamoeba rhysodes*, *Acanthamoeba culbertsoni*, *Acanthamoeba hatchetti*, and *Acanthamoeba griffini* (Ahearn and Gabriel 1997; Marciano-Cabral and Cabral 2003). Whether cysts or trophozoites, or both, are responsible for keratitis is unclear (Ahearn and Gabriel 1997; Kilvington and White 1994).

The genus *Allium* is one of the major sources of dietary flavonoids, which are a group of polyphenolics, in many countries (Hertog et al. 1995; Knekt et al. 1996) The genus *Allium* is very large consisting of more than 700 species of bulbous perennials and biennials that occur in temperate regions of the northern hemisphere (Könemann 1999). There are 164 *Allium* species available in Turkish flora and 65 of them are endemic (Davis 1984, 1998; Guner et al. 2000). Since antiquity, *Allium* plants were believed to be beneficial to human health. Garlic, a member of this genus, has held a special position as a prophylactic and therapeutic agent in cultures of Egypt, India, Greece, and Rome, a position it still holds in present-day societies. Anectodal evidence supports the important roles of the members of this genus in the prevention and treatment of pathogenic infections, tumors and cardiovascular diseases (Cao et al. 1996; Gazzani et al. 1998; Yin and Cheng 1998). In addition, they are thought to also have anticoagulant, anticancer, and antiparasitic effects, which are probably due to the specific organosulphur compounds such as ajoene, alliine, and allicine (Dorant et al. 1993; Mansell and Reckless 1991; Lun et al. 1994; Block 1985; Sendl et al. 1992).

The aim of the present study was to evaluate the in vitro effectiveness of the methanol extracts of four *Allium* species (*Allium sivasicum*, *Allium dictyoprosum*, *Allium scrodoprosum* subsp. *rotundum*, and *Allium atroviolaceum*) on the growth and adherence of *A. castellanii* trophozoites and cysts and their cytotoxic potentials on corneal cells.

Materials and methods

Collection of plant material

Localities and collection periods of the *Allium* species studied are given below:

1. *A. sivasicum*: Celalli-Hafik road, Karayun, Sivas, Turkey; July 10, 2003
2. *A. dictyoprosum*: Gurun turn off, Kangal, Sivas, Turkey; July 19, 2003
3. *A. scrodoprosum subsp. rotundum*: Bolucan, Zara, Sivas, Turkey; July 14, 2003
4. *A. atroviolaceum*: Celalli-Hafik road, Karayun, Sivas, Turkey; July 10, 2003

The voucher specimens were deposited at the Herbarium of the Department of Biology, Cumhuriyet University, Sivas, Turkey (CUFH-Voucher Nos. 1-AA3337; 2-AA3351; 3-AA3341; 4-AA3335, respectively).

Preparation of the methanol extracts

The air-dried and finely ground samples were extracted by using a method described elsewhere (Sokmen et al. 1999). Briefly, the sample, weighing about 100 g, was extracted in a Soxhlet apparatus with methanol (MeOH) at 60°C for 6 h. The extract was then filtered and concentrated in vacuo at 45°C, yielding a waxy material (14.36%, 10.49%, 11.22%, and 11.39% *w*/*w*, respectively). Finally, the extracts were then lyophilized and kept in the dark at +4°C until tested.

Trophozoites

Vegetative forms of *A. castellanii* 1BU strains (Walochnik et al. 2000) were obtained from axenic cultures in 25-cm^2 corning flasks containing 10 ml of protease peptone, yeast extract, and glucose (PPYG) medium (Schuster 2002) and kept at 37°C. Trophozoites in the stage of exponential growth (72 to 96 h) were concentrated by centrifugation at 500×*g* for 10 min. The amoebae, which were washed twice in sterile Neff's saline solution (1.2 g NaCl, 0.4 g $MgSO_4 \cdot H_2O$, 0.4 g $CaCl_2 \cdot 2H_2O$, 1.42 g Na_2HPO_4, 1.36 g $KHPO_4$ in 100 ml distilled water), counted in a hemacytometer, adjusted to a final concentration in Neff's saline solution at a density of 8×10^5 amoebae/ml (95.0% trophozoites), and used immediately for testing.

Cysts

Cyst inocula were prepared by incubating the cultures in flasks for 6 weeks and harvested as described above. Inocula containing at least 85.0% cysts were used in the comparative experiments. The cysts were harvested, washed in sterile Neff's saline solution, and adjusted to a final concentration of 8×10^5 cysts/ml.

Experimental design

Experiments were performed in sterile 96-well plates (Corning). Of the calibrated trophozoite and cyst suspension, 100 µl was added to the each well and then the plates were left for 30 min to avoid disturbance of the adherence of amoebae onto the wells' surface. Then, the saline solution was removed and 100 µl of the test solutions were added into the 4 wells of a row, the plates were sealed and incubated at 37°C in an atmosphere containing 5.0% CO_2 for 1, 3, 6, 12, 24, 48, and 72 h. Same procedure was

applied for the controls containing only the parasites in Neff's saline solution. All of the tests were repeated four times.

Effects of the *Allium* species on the trophozoite stage

After the incubation period at 37°C, a sterile cell scraper was used to gently remove the trophozoites adhered to the base of the tissue culture flask. Then, it was agitated by pipetting up and down for 1 min for counting the test and control wells in the presence of 0.1 ml of 0.3% basic methylene blue. Unstained (viable) and stained (nonviable) trophozoites were enumerated in the hemocytometer, 10 min later after stain addition. More than 100 *A. castellanii* trophozoites were examined in each of the 4 samples.

Effects of the *Allium* species on the cyst stage

After each incubation period, for counting the test and control wells, 0.1 ml of the test solution was transferred into 0.1 ml of 0.3% basic methylene blue media. Unstained (viable) and stained (nonviable) cysts were enumerated in the hemocytometer, 2 min after stain addition. More than 100 *A. castellanii* cysts were examined in each of the 4 samples. For cultures containing no viable cysts, an additional test was performed to confirm the results obtained. To evaluate their viability, cysts were inoculated onto a non-nutrient agar plates covered with a lawn of *Escherichia coli* after the each incubation period. The culture plates were incubated at 30°C for 14 days. Amoebic growth was observed daily by phase contrast microscopy (Nikon, Eclipse TS 100) (Schuster 2002).

Primary epithelial cell culture

A limbal biopsy was performed on the healthy eyes of a New Zealand white rabbit. The eyelid was sterilized with povidon iodine and 1–2 mm of limbal tissue containing epithelial cells and part of the corneal stroma was separated from the limbal margin and excised from the superficial corneal stroma by lamellar keratectomy. Tissue samples were air-dried for 10 to 15 min and then covered with primary culture medium consisting of equal parts of Hams F-12 and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-buffered Dulbecco's modified eagle medium (GIBCO Laboratories, Grand Island, NY) enriched with 5% Nu-Serum (Collaborative Research, Bedford Park, MA), 50 ug of Gentacin (GIBCO) per ml, 5.0 ug of insulin (Collaborative Research) per ml, and 10 ng of epidermal growth factor (Collaborative Research) per ml. The dishes were incubated at 37°C in a humidified incubator containing 5.0% CO_2. Epithelial outgrowth from the explant was usually apparent within 72 h. The medium was sub-cultured three times a week regularly.

Agar diffusion method

The agar diffusion tests were performed according to international standards (International Standard ISO 7405 1997). Briefly, the cultures were harvested using 0.25% trypsin solution (Gibco, Germany). Stock cultures were seeded in 35 mm diameter of cell culture petri dishes (Nunc, Wiesbadan, Germany) at a density of 1×10^6 cells per petri dish and sub-cultured once a week. After the formation of confluent cell layer, the medium was removed and replaced with complete medium containing 1.5% agarose (FMC BioProducts, Rockland, ME, USA). After solidifying the agarose, the cells were stained with a vital dye (neutral red; Sigma). During the experimental procedures, the cells were protected from light to prevent cell damage elicited by photo-activation of the stain. Experimental solutions were applied by using sterile round Whatman papers with a diameter of 6 mm. For each *Allium* solution, four replicate dishes and four additional dishes containing positive and negative control materials were prepared. As negative control, DMEM (Sigma Chemical, St. Louis, MO) was used, while absolute phenol was used as positive control. After an exposition period of 24 h at 37°C, the cell responses were evaluated by inverted microscope observation. In this study, cell lysis was scored as follows: 0=no cell lysis detectable; 1=less than 20% cell lysis; 2=20% to 40% cell lysis; 3=>40% to <60% cell lysis; 4=60% to 80% cell lysis; 5=more than 80% cell lysis. For each sample, one score was given and the median score value for all parallels from each samples was calculated for the lysis zone. Cytotoxicity was then classified as follows: 0–0.5=non-cytotoxic; 0.6–1.9=mildly cytotoxic; 2.0–3.9=moderately cytotoxic; 4.0–5.0=markedly cytotoxic. The median (instead of the mean) was calculated to describe the central tendency of the scores because the results were expressed as an index in a ranking scale.

Statistical analysis

Data were presented as the mean±SD and analyzed by repeated measures of ANOVA followed by the Tukey test for post hoc pairwise comparisons. The difference was considered significant when the P value was <0.05.

Results

Extracts obtained from the *Allium* species were evaluated for their amoebicidal activity against *A. castellanii* using an

Table 1 Effect of *A. scrodoprosum* on the proliferation of *A. castellanii* trophozoites

A. scrodoprosum dose (mg/ml)	Measurement times (h)						
	1	3	6	12	24	48	72
32	9.6±3.7	0	0	0	0	0	0
16	30±7.2*	17±7.9	0	0	0	0	0
8	40±7.2**	31±6.5	17±5.5	0	0	0	0
4	50±7.9**	37±3.6***	28±4.6****	18±3.9	9±1.5	0	0
2	59±7.2**	47±8.6***	35±6.0****	27±4.6*****	17±1.5	7.1±1.5	0
1	68±7.4**	54±4.7***	47±6.7****	37±6.0*****	27±5.8	17±5.8	16±5.0
Control	80±7.9	80±7.9	80±11.7	76±11.4	78±4.6	76±5.8	74±5.8

Data were expressed as the mean±SD.
*$P<0.05$ vs 3–72 h.
**$P<0.05$ vs 6–72 h.
***$P<0.05$ vs 12–72 h.
****$P<0.05$ vs 24–72 h.
*****$P<0.05$ vs 48 and 72 h.

inverted light microscope. According to the results obtained from the tests, *A. scrodoprosum* subsp. *rotundum* showed remarkable amoebicidal effect on *A. castellanii*, while the others remained inactive (Tables 1 and 2).

The effect of the methanolic extract of *A. scrodoprosum* on the proliferation of *A. castellanii* trophozoites is shown in Table 1.

Data presented in Tables 1 and 2 showed similarities from the point of viable cell patterns. For all of the concentration values, viable trophozoites were detected within the first hour. On the other hand, after the first hour, no viable trophozoites were observed in the presence of the methanolic extract of *A. scrodoprosum* in a concentration of 32 mg/ml. Viable trophozoite numbers showed increase with the decreasing concentrations of the extract. In the case of 1 mg/ml, 16±5.0 viable trophozoites were detected at the end of 72 h.

The effect of the methanolic extract of *A. scrodoprosum* on the proliferation of *A. castellanii* cysts is shown in Table 2.

As can be seen from the Table 2, viable cysts were detected only within the first hour (23±6.0). The cysts were not able to live in the rest of the experimental procedure. In the concentrations ranging from 2 to 32 mg/ml, no viable cysts were observed at 72 h. On the other hand, in the case of the 1 mg/ml concentration, 29±2.2 viable cysts were detected at the end of the incubation period. Viable cysts within the control media did not show any significant changes during the test (values were ranged from 74 to 80).

In the case of the cytotoxic acitivities, methanolic extracts of *A. scrodoprosum* showed no cytotoxicity for the cells in the concentration of 32 mg/ml, while the others exerted significant cytotoxic activities between the concen-

Table 2 Effect of *A. scrodoprosum* on the proliferation of *A. castellanii* cysts

A. scrodoprosum dose (mg/ml)	Measurement times (h)						
	1	3	6	12	24	48	72
32	23±6.0*	7.5±2.0	0	0	0	0	0
16	29±3.8**	14±3.9	6.6±1.5	0	0	0	0
8	45±3.8**	35±1.9***	24±2.0***	8.2±5.5	0	0	0
4	56±11.4**	44±2.0***	35±1.5****	20±1.9	8.9±1.5	0	0
2	65±1.5**	55±1.5***	45±1.5****	35±1.5	24±2.0	23±3.4	0
1	74±4.1**	65±1.5***	55±1.5****	45±1.5*****	35±3.7	34±2.0	29±2.2
Control	80±7.9	80±7.9	80±11.7	76±11.4	79±6.5	76±3.3	74±3.1

Data were expressed as the mean±SD.
*$P<0.05$ vs 3–72 h.
**$P<0.05$ vs 6–72 h.
***$P<0.05$ vs 12–72 h.
****$P<0.05$ vs 24–72 h.
*****$P<0.05$ vs 72 h.

trations of 1 and 16 mg/ml. There was no decolorization zone around the samples. Although the cells are directly in contact with the methanol extracts of the *Allium* species in the culture media, they did not showed any signs of injury and kept their morphological characteristics and wholeness like those in the controls. Extracts of *A. sivasicum*, *A. dictyoprosum*, and *A. atroviolaceum* in the 32 mg/ml concentration, showed moderate cyctotoxicity for the cells. Overall, the lysis index score was 5 (markedly cytotoxic) for the positive control group, while it was 0 (non-cytotoxic) for the negative control group.

Discussion

As can be seen from the results presented in Tables 1 and 2, the number of viable trophozoites of *A. castellanii* decreased with exposure to increasing concentrations of *A. scrodoprosum* and generally with increased times of exposure. As expected, the sensitivity of *Acanthamoeba* trophozoites to *A. scrodoprosum* was higher than that of the cysts. No cytotoxic activity was observed on the cultures of corneal epithelium with the 32 mg/ml dose of *A. scrodoprosum*.

Acanthamoeba species are an important cause of microbial keratitis that may cause severe ocular inflammation and visual loss (Khan 2003). Contact lens wear and corneal trauma are the leading risk factors for *Acanthamoeba* keratitis (Marciano-Cabral and Cabral 2003). As cysts and active trophozoites attach to the surface of contact lenses, *Acanthamoeba* can easily be transmitted from the storage case onto the eye (Kilvington and Larkin 1990). In a series of in vitro studies using corneal organ cultures, Clarke and Niederkorn (2006) demonstrated that *Acanthamoeba* trophozoites can penetrate the corneal endothelium and the underlying Descemet's membrane, suggesting that the trophozoites can, therefore, enter the anterior chamber. The increasing incidence of *Acanthamoeba* keratitis among contact lens wearers and other diseases caused by *Acanthamoeba* has necessitated the evaluation and development of potential contact lens disinfectants and therapeutic agents active against the resistant cyst stage of the organism (Buck et al. 1998; Kilvington et al. 2002; Borazjani and Kilvington 2005).

Although this infective keratitis is a relatively rare corneal disease, the emergence of drug-resistant strains and the recurrence of dormant infectious forms underscore the need for more effective treatments (Khan 2003). A number of drugs (cationic antiseptics, azole antifungals, and antibiotics such as macrolides) were tested in vitro and in vivo with varying degrees of efficacy, leading to some recovery (Lloyd et al. 2001; Radford et al. 1998; Duguid et al. 1997), but also to some failures (Mattana et al. 2004). These treatments are poorly effective against the cystic stages of the parasite. In vivo, sufficient efficacy is achieved in only half of the patients and resistance to propamidine was observed to develop in a temporal series of *Acanthamoeba* isolates from one patient (Ficker et al. 1990). Single therapy with chlorhexidine alone was also applied successfully. Chlorhexidine (0.02%) in particular has become the favored therapy among many clinicians because it appears to be less toxic than polyhexamethylene biguanide (Kosrirukvongs et al. 1999). The encysted stage of *Acanthamoeba* species appears to cause most of the problems, so an important consideration is to use drugs that are not only amoebicidal, but also cysticidal. From this point of view, our study indicates the significant cysticidal effect of *A. scrodoprosum* on the cyst stage of *A. castellanii.*

As far as our literature survey could ascertain, this is the first report presenting the results of the study concerning the effect of the *Allium* species against *A. castellanii*. Our findings indicated that the methanolic extract of *A. scrodoprosum* has amoebicidal effect and cysticidal properties on the trophozoites and cysts of *Acanthamoeba*. On the other hand, it did not show cytotoxicity on the cultures of the corneal epithelium with a dose of 32 mg/ml. It is assumed from the results presented that the methanolic extract of *A. scrodoprosum* can be applied for the prevention and therapy for *Acanthamoeba* infections under controlled conditions. But, further in vitro and in vivo studies are needed to define the exact efficacy and ophthalmic side effects of extract.

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