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The amoebicidal effect of New Bioactive o-Phenylenediamine Surface Functionalized Poly (Maleic Anhydride-alt-Vinyl Acetate) and Citric Acid Derived Carbon Quantum Nanodot-Based Conjugates on *Acanthamoeba castellanii* cysts and trophozoites and its cytotoxic potentials

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Abstract

Objective: This study aimed to evaluate the amoebicidal activity of o-phenylenediamine (o-PDA)-conjugated carrier systems, including maleic anhydride-vinyl acetate alternating copolymer (MAVA) and citric acid-derived carbon quantum dots (CNDs), against *Acanthamoeba castellanii* cysts and trophozoites. Additionally, the cytotoxicity of these compounds was assessed on the HT-22 Mouse Hippocampal Neuronal cell line.

Method: Two non-toxic carrier systems were synthesized: MAVA via charge transfer complex (CTC) radical polymerization, and CNDs via rapid thermal pyrolysis of citric acid. o-PDA surface conjugation was achieved through an amide mechanism after the ring-opening reaction. The synthesized compounds were tested for their amoebicidal effects on *A. castellanii* cysts and trophozoites, while cytotoxicity analyses were conducted using the HT-22 neuronal cell line.

Results: According to our findings, the cytotoxicity of all compounds at the tested concentrations was within an acceptable range. When examining the amoebicidal activities, it was observed that MAVA at concentrations of 0.01 g/mL and 0.005 g/mL did not have any viable trophozoites, while CNDs at a concentration of 0.01 g/mL did not have any viable cysts. Overall, all compounds showed significant amoebicidal activity.

Conclusion: Our findings demonstrate a strong dose-dependent amoebicidal effect of these substances on *A. castellanii* cysts and trophozoites. It is believed that these substances, supported by in vivo experiments, could potentially serve as new therapeutic agents.

Keyword: Carbon quantum nanodots (CNDs), maleic anhydride-vinyl acetate alternating copolymer (MAVA), surface modification, o-phenylenediamine (o-PDA), A. castellanii, Ameboicidal activity, Cytotoxicity.

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INTRODUCTION

Acanthamoeba spp. are free-living amoebae isolated from various ecological sources such as swimming pools (1), dams and lakes (2), soil (3) and even air filters (4). They can also be found in natural and artificial water reservoirs, as well as in contact lens solutions, dental units, dialysis stations, and medical equipment. Additionally, they have been isolated from sandboxes, faucets, shared sewage, and tap water. Furthermore, these amoeba species have been isolated from fruits, vegetables, plants, fungi, and certain animal species (5). The increased interaction between humans and the environment has increased the likelihood of contamination with free-living amoebae, and diseases caused by free-living amoebae have gained importance over time (6, 7). *Acanthamoeba castellanii* (A. castellanii) can cause skin and brain infections, but it is typically associated with Acanthamoeba keratitis (AK), a painful and severe infection of the cornea that can lead to blindness or visual impairment. Although AK is rare, its incidence has been steadily increasing in recent decades, making it an emerging disease (8). Acanthamoeba spp. is also the causative agent of granulomatous amebic encephalitis (GAE), a multifocal encephalitis. *A. castellanii* can also affect the lungs, liver, kidneys, adrenal glands, heart, skin, and bones. These are rare infections that mainly occur in immunocompromised patients, including that post-organ transplantation (5). Due to the lack of appropriate diagnostic methods and effective treatment, Acanthamoeba infections are characterized by high mortality rates (5).

The poor prognosis of Acanthamoeba infections, the difficulty in diagnosis, the lack of successful treatment, the resistance to chemotherapeutic agents, and the increasing cases of keratitis have further emphasized the importance of developing new therapeutics against this parasite.

Among biomedical applications, polymer-based carriers with active pharmaceutical ingredients are of great importance for controlled drug delivery systems. Covalent copolymer-drug conjugates, which are produced by a covalent bond between the drug and a polymer backbone, were first studied by Helmut Ringsdorf in 1975 (9). On the other hand, carbon quantum dots (CNDs) offer a unique combination of multiple attractive properties on a single molecule, and these nanomaterials, CNDs, have attracted great interest in various industrial fields in recent years due to their unique physical and chemical potentials (10). CNDs are interesting fluorescent hemispherical carbon-based nanoparticles less than 10 nm in size that can be prepared under mild conditions from inexpensive carbon sources with high water-soluble and biochemical properties. Furthermore, they also accepted harmless eco-friendly materials for bio-applications due to their desirable behaviors such as water stability, chemical inertness, biocompatibility and non-toxicity (11). Furthermore, CNDs are generally preferred as nano-based materials compared to the toxic heavy metal quantum dots in view of many superior properties (12). They are usually made from organic precursor molecules with a bottom-up approach (13). Furthermore, their surface chemistry can change depending on the fabrication method and precursor molecules (14) and the tunable surface-functional CNDs can be fabricated without using chemical polymer linkers (15).

CDs are generally made from many different chemicals and organic sources as precursor molecules that occur either individually or together as the main constituent of the structure. Citric acid (CA) is the most commonly used precursor molecule for biomedical applications, especially because of their desired low toxicity, good biocompatibility and low-cost properties (16). As CNDs consist of a carbonaceous carbon core with attached organic functional groups containing heteroatoms (17), they gain greater functionality as special compounds with high reactivity to produce unique next-generation materials with desirable properties (18). CNDs are generally classified between organic (polymers) and inorganic materials (black carbon), macromolecules and nanoparticles, between "bottom-up" (polycyclic aromatic compounds) and "top-down" synthesis products (graphene) (19,20).

Surface passivation and (bio) functionalization reactions of CNDs are applied as indispensable tools for the producer of high-performance CNDs-based materials (21). The particular polar (hydrophilic) character of their external surfaces gains importance due to their high solubility character in water or polar solvents. During the passivation process, reactive functional groups such as hydroxyl (-OH), amine (-NH2), and carboxyl (-COOH) groups are added to the CNDs surface.

In this study, o-phenylenediamine (o-PDA) was chosen as the conjugating or surface passivating agent for the synthesis of the o-PDA surface functionalized MAVA and CNDs derivative as MAVA/o-PDA and CNDs/o-PDA, respectively. In medicinal chemistry, aromatic diamines are known as important precursor molecules for the produce of many heterocyclic compounds for drug discovery. Organic compounds with heterocyclic scaffolds, available both naturally and synthetically, especially with five-membered rings are of critical importance in biological chemistry (22).

This study was designed to generate new polyanhydride-based formulations by both rational and spherical post-functionalization of the MAVA copolymer and CNDs surface with a small molecule and to elucidate the cell cytotoxicity mechanism and to test amoebicidal effects on Acanthamoeba cysts and trophozoites. Five-membered aromatic heterocyclic compound, 2,5-dione-substituted form of the furan molecule, is almost identical to the acid anhydride backbone. Therefore, the passivation process was carried out on an anhydrite-like ring with a regular distribution on the surface of both copolymer and nanodots. Ring-opening reaction for the acid anhydride functional group performed according to our previous studies (23). As a result, the conjugated form of MAVA and CNDs prepared from the biocompatible, less toxic, and low-cost maleic anhydride and citric acid precursor molecules, respectively. They can also be developed for various targeted drug release applications due to the compatibility of their molecular structures.

METHODS

Materials

Maleic anhydride (MA), benzoyl peroxide (BPO), and methyl ethyl ketone (MEK) were purchased from Merck (Schuchardt, Germany). Vinyl acetate (VA), ethyl acetate, and petroleum ether were supplied from Sigma-Aldrich (St. Louis, USA). Anhydrous citric acid was provided by Merck (Schuchardt, Germany). The fluorescent 1,2-phenylenediamine molecule (o-PDA), N,N-dimethylformamide (DMF) and triethyamine (TEA) were provided by Merck (Darmstadt, Germany). Ethanol (95 %) and diethyl ether were provided by Carlo-Erba (Rodano, Italy). The reagents and chemicals used for the synthesis, passivation, and purification process were of analytical quality.

Synthesis of MAVA Copolymer

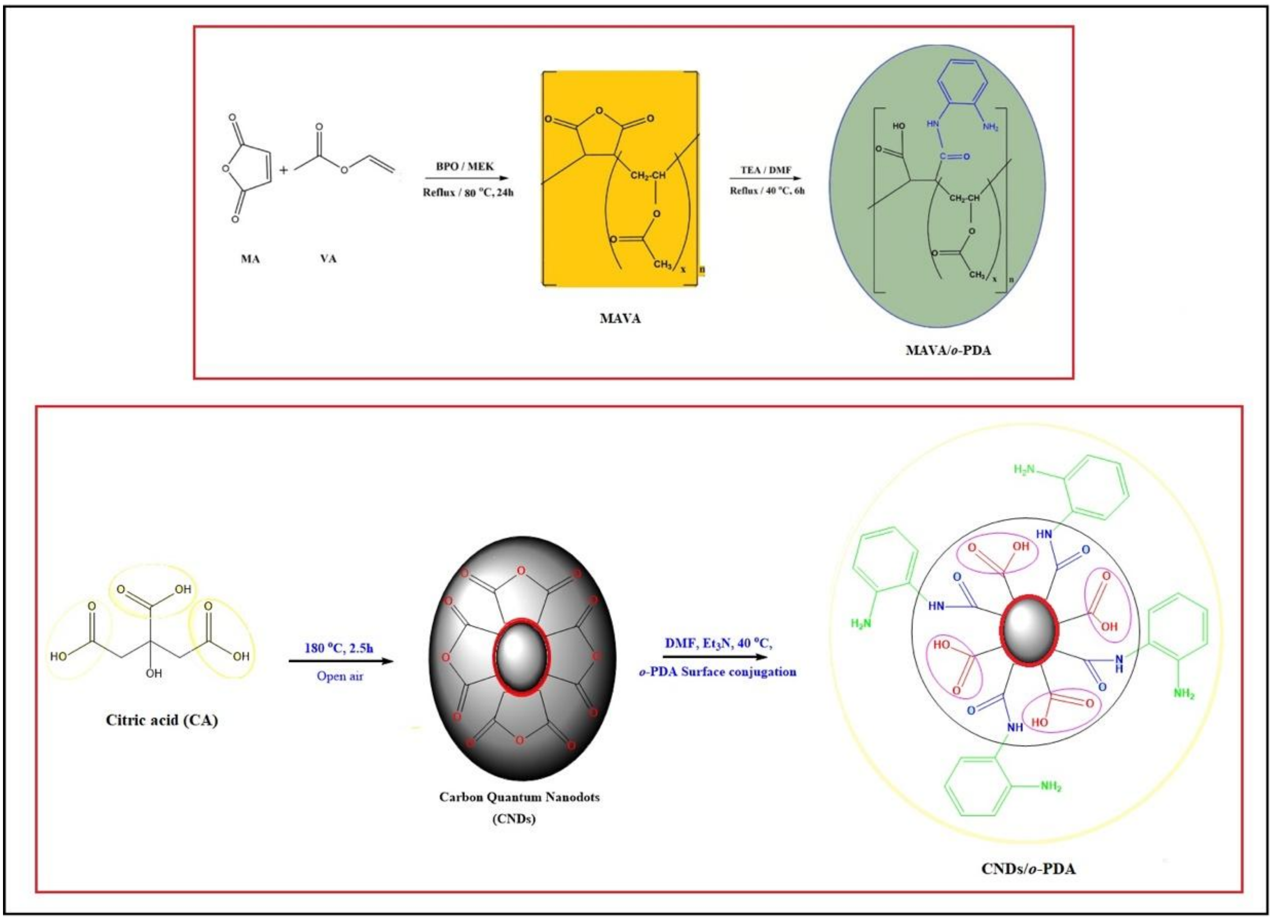
Maleic anhydride-vinyl acetate (MAVA) copolymer was synthetically prepared according to our previous study using the in situ charge transfer complex (CTC) radical polymerization process (24). Briefly, MAVA was synthesized by free-radical polymerization from maleic anhydride (MA) and vinyl acetate (VA) monomers with 1:1 (MA:VA) monomer feeding mole ratio in MEK by BPO free radical initiator for 24h at 80 oC (Scheme 1).

Preparation of the CNDs

Citric acid (CA) derived from CNDs was prepared by thermal pyrolysis through gradual thermal decomposition of CA. Briefly, anhydrous CA (10 g) was heated continuously to 180 °C in a Radleys reaction system. The color change that occurs in several steps during the preparation of CNDs was carefully controlled. Acid anhydride-CNDs are synthesized by heating anhydrous CA at 180 °C for 2 hours until the characteristic brown powder form of nanodot residue is obtained (14), (Scheme 1).

Surface Functionalization of MAVA Copolymer and CNDs-based Carriers with o-PDA Molecule

Surface-functionalized citric acid-based CNDs in conjugated form were prepared according to our previous studies by modifying CNDs with o-PDA fluorescent dye precursor molecules in a molar ratio of 1:1 in anhydrous dimethylformamide (DMF) using triethylamine (Et3N) for 16 hours at 40 °C (24-27), (Scheme 1).



Scheme 1. Schematic representation of MAVA, MAVA/*o*-PDA, CNDs and CNDs/*o*-pDA preparation.

Structural Characterization

MAVA and CNDs carriers, MAVA/o-PDA and CNDs/o-PDA conjugates were synthesized in our research laboratory (23). Their chemical structures and conjugation mechanism were elucidated by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR), nuclear magnetic resonance (1H-NMR), UV-visible spectroscopy, and fluorescence spectroscopy. The crystal structure of the samples was characterized using X-ray diffractometers (XRD-MF). The physical characterizations were carried out using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC). Q-TOF LC/MS (liquid chromatography quadrupole time-of-flight mass spectrometry) and size exclusion chromatography (SEC) were also used as chromatographic methods.

Ameboicidal effect

The Acanthamoeba castellani (ATCC 30010) strain was used in the study. The study tested the amoebicidal effects of MAVA, o-PDA, MAVA/o-PDA, CNDs and CNDs/o-pDA on *A. castellanii.* Because effective chemotherapeutic against *A. castellani* hasn't yet been found, Positive control wasn't used.

Parasite culture

Axenic trophozoite culture

In this study, the axenic culture method described by Jensen et al. (28) was employed. For the preparation of the trophozoite axenic medium, 20 g of Mycological Peptone (Oxoid), 20 g of Yeast Extract (Difco), 18 g of glucose, 120 mg of NaCl, 3 mg of $MgCl_2 \cdot 6H_2O$, 142 mg of Na_2HPO_4, 136 mg of KH_2PO_4, 3 mg of $CaCl_2$, and 3 mg of $FeSO_4$ were weighed and dissolved in one liter of distilled water. The prepared mixture was autoclaved at 121 °C for 15 minutes, cooled to room temperature, and the pH was adjusted to 5.6 before use.

Cyst production

In the study, it was used the axenic culture described by Moon et al (29). For the medium of the cyst; 5,5519 g NaCl, 0,3728 g KCl, 0,963 g MgSO4, 0,084 g NaHCO3, 0,044 g CaCl2, 3,152 g Tris-HCl were dissolved in one-liter distilled water after weighed and it was autoclaved at 121 °C for 15 minutes. The mixture was allowed to cool to room temperature. The prepared mixture pH was adjusted to 9.

Experimental design

Trophozoites

The experiments were conducted using 24-well sterile plates, each well with a volume of 2 mL. A total of 20 mL of axenic trophozoite culture was transferred into 25 cm² culture flasks, and *Acanthamoeba castellanii* trophozoites were inoculated into the cultures. The flasks were then incubated at 25 °C for 96 hours. Following incubation, the parasite concentration was adjusted to 1×10^5 cells/mL and subsequently used in the study.

The concentrations of 0.01 g/mL, 0.005 g/mL, 0.0025 g/mL, 0.00125 g/mL, 0.000625 g/mL, 0.000312 g/mL of substances in 24-well plates were adjusted using calibrated trophozoite culture containing *A. castellani* trophozoites. The substances in the study were used by dissolving in 100 µL DMSO. The same amount of DMSO was added to control culture wells without substances derived from benzothiazole. The viability of the parasites was analyzed using the XTT method after 24 hours.

Cyst

Experiments targeting the cyst form of the parasite were carried out in 24-well sterile plates, each with a volume of 2 mL. Initially, 20 mL of axenic trophozoite culture was transferred into 25 cm^2 culture flasks. The *Acanthamoeba castellanii* strain was inoculated into the axenic trophozoite culture and incubated at 25 °C for 96 hours. Following incubation, the culture was centrifuged at 500 g for 10 minutes.

Subsequently, 20 mL of pre-prepared axenic cyst medium was added to a 25 cm^2 culture flask. The pellet obtained after centrifugation was resuspended in the cyst medium and transferred into the cyst culture. The culture was then incubated at 25 °C for six days. Microscopic examination confirmed that the trophozoites had completely transformed into cysts. The final parasite concentration was adjusted to 1×10^5 cells/mL using the axenic cyst culture, and this preparation was employed in subsequent experiments. The concentrations of 0.01 g/mL, 0.005 g/mL, 0.0025 g/mL, 0.00125 g/mL, 0.000625 g/mL, 0.000312 g/mL of substances in 24-well plates were adjusted using calibrated cyst medium contained *A. castellanii* cysts. Substances were dissolved in 100 µL DMSO. The same amount of DMSO was added to control cultures without substances. The viability of the parasites was analyzed using the XTT method after 24 hours.

XTT (sodium 3,39-[1-(phenylaminocarbonyl)-3,4-tetrazolium]- bis(4-methoxy-6-nitro) amethyne sulonic acid hydrate) test

After 24 hours of exposure, a new plate with 96 wells was taken, and 100 µL of the culture from each well was transferred to the new plate. In a separate location, a mixture of 5 mL of XTT reagent and 0.1 mL of electron-binding reagent was prepared and 50 µL of the mixture was added to each well. After 4 hours of incubation at 25 °C, the plate was read at 450 nm using an ELISA reader, and the absorbance values were recorded. The viability rates were calculated based on the formula (viability (%) = [(OD450 (sample)/OD450 (negative control)) × 100]).

Cytotoxicity

The XTT assay was conducted to evaluate the cytotoxic effects of the tested substances on the HT-22 mouse hippocampal neuronal cell line. Cells were cultured in phenol red-free DMEM medium at a density of 1×10^5 cells/mL. The medium was supplemented with 10% FBS and 1% Penicillin-Streptomycin to prepare the suspension. A volume of 100 µL of this suspension (corresponding to 1×10^4 cells/well) was seeded into each well of a 96-well plate. After confirming equal distribution of cells under the microscope, the plates were incubated in a CO_2 incubator at 37 °C for 24 hours.

At the end of incubation, cell morphology and attachment were examined and verified as normal. Subsequently, five replicates of five different test substances, each prepared at six different concentrations (0.01 g/mL, 0.005 g/mL, 0.0025 g/mL, 0.00125 g/mL, 0.000625 g/mL, 0.000312 g/mL), were added to the wells at a volume of 2 µL per well. The plates were then incubated again for 24 hours at 37 °C in a 5% CO_2 environment.

For the positive control, 200 µL of phenol red-free DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin was added, while 2 µL of DMSO was used for the negative control. The XTT reagents used were Reagent I (XTT labeling reagent, Roche) and Reagent II (electron coupling reagent, Roche), mixed at a ratio of 50:1 to prepare the working solution. Following removal of the culture medium, 30 µL of the XTT solution was added to each well, along with 170 µL of phenol red-free DMEM. Plates were incubated for 4–6 hours at 37 °C. After incubation, absorbance was measured using an ELISA microplate reader at 450 nm. Cell viability was calculated using the formula:

Cell viability (%) = [(OD_{450} (sample) / OD_{450} (negative control)) × 100]

Based on the cytotoxicity evaluation, values below 10% were considered non-cytotoxic, values between 10–25% were classified as low cytotoxicity, values between 25–40% as moderate cytotoxicity, and values above 40% as high cytotoxicity (30).

Statistical analysis

The data obtained in our study were evaluated in the SPSS (Ver: 22.0) program and the One-way ANOVA followed by Tukey’s post hoc test was used, the level of error was taken as 0.05.

RESULTS

The a amoebicidal activity of MAVA, o-PDA, MAVA/o-PDA, CNDs and CNDs/o-pDA on *A. castellanii* trophozoite and cyst forms is shown in Table 1, Figure 1.

According to the results of the study, when examined for their effects on *A. castellanii* trophozoite forms, it was observed that in the group treated with MAVA at concentrations of 0.01 g/mL and 0.005 g/mL, no viable trophozoites were found. At concentrations of 0.0025 g/mL and 0.00125 g/mL, it was determined that the number of parasites significantly decreased. The viability rates of trophozoites at these concentrations were 48% and 85%, respectively. When the first four concentrations included in the study were statistically compared to the control group, it was observed that the difference was significant ($p<0.05$). When comparing the groups, it was found that there was no significant difference between the concentrations of 0.01 g/mL and 0.005 g/mL ($p>0.05$), but there was a significant difference compared with the other groups ($p<0.05$). In the last two concentrations included in the study (0.000625 g/mL, 0.000312 g/mL), the viability rates were 95% and 96%, respectively, and it was observed that the difference between these groups and the control group was not significant ($p>0.05$). When examining the effects of o-PDA on *A. castellanii* trophozoites, it was found that the viability rate was 28% at a concentration of 0.01 g/mL, 31% at a concentration of 0.005 g/mL, and 86% at a concentration of 0.0025 g/mL. When the first three concentrations included in the study were statistically compared to the control group, it was observed that the difference was significant ($p<0.05$). When comparing the groups, it was found that there was a significant difference between these concentrations ($p<0.05$). When examining the effects of MAVA/o-PDA on *A. castellanii* trophozoites, it was found that the viability rate was 18% at a concentration of 0.01 g/mL, 28% at a concentration of 0.005 g/mL, and 52% at a concentration of 0.0025 g/mL. When the first three concentrations included in the study were statistically compared to the control group, it was observed that the difference was significant ($p<0.05$). When examining the effects of CNDs on *A. castellanii* trophozoites, it was observed that the substance had a significant amoebicidal effect in the first four concentrations. At concentrations of 0.01 g/mL, 0.005 g/mL, 0.0025 g/mL, and 0.00125 g/mL, the viability rates of *A. castellanii* trophozoites were 8%, 20%, 58%, and 65%, respectively. The difference between these concentration groups and the control group was statistically significant ($p<0.05$). When examining the effects of CNDs/o-PDA on *A. castellanii* trophozoites, it was observed that the substance had a significant amoebicidal effect in the first three concentrations. At concentrations of 0.01 g/mL, 0.005 g/mL, and 0.0025 g/mL, the viability rates of *A. castellanii* trophozoites were 12%, 22%, and 60%, respectively. The difference between these concentration groups and the control group was statistically significant ($p<0.05$). (Table 1, Figure 1).

When examining the effects of MAVA, o-PDA, MAVA/o-PDA, CNDs, and CNDs/o-PDA on *A. castellanii* cysts, it was observed that in the group treated with MAVA at a concentration of 0.01 g/mL, no viable cysts were found. At a concentration of 0.005 g/mL, it was found that only 2% of the cysts were alive. This rate was 65% at a concentration of 0.0025 g/mL. When the intergroup data were statistically analyzed, it was found that there was no significant difference between the first two concentrations ($p>0.05$), but there was a significant difference between the concentration of 0.0025 g/mL and the others ($p<0.05$). When compared to the control group, it was found that there was a significant difference between all three concentrations ($p<0.05$). When examining the effects of o-PDA on *A. castellanii* cysts, it was found that at concentrations of 0.01 g/mL, 0.005 g/mL, and 0.0025 g/mL, the viability rates of *A. castellanii* cysts were 30%, 32%, and 80%, respectively, and the difference between these concentrations and the control group was statistically significant ($p<0.05$). In the same concentrations, *A. castellanii* cysts exposed to MAVA/o-PDA for 24 hours had viability rates of 55%, 85%, and 90%, respectively. The difference between these groups and the control group was significant ($p<0.05$). When examining the effects of CNDs on *A. castellanii* cysts, it was observed that there was a strong amoebicidal effect in the first three concentrations. It was found that at a concentration of 0.01 g/mL, there were no viable cysts of *A. castellanii.* At concentrations of 0.005 g/mL and 0.0025 g/mL, the viability rates were 6% and 69%, respectively. When comparing the data at this concentration with the control group, it was found that the difference was significant ($p<0.05$). When examining the effects of CNDs/o-PDA on *A. castellanii* cysts, it was found that at concentrations of 0.01 g/mL, 0.005 g/mL, and 0.0025 g/mL, the viability rates of *A. castellanii* cysts were 4%, 10%, and 75%, respectively, and the difference between these concentrations and the control group was statistically significant ($p<0.05$). (Table 1, Figure 1)..

Table 1. The amoebicidal effect of MAVA, o-PDA, MAVA/o-PDA, CNDs and CNDs/o-PDA after 24 hours.

	Concentration (g/mL)	**Viability Rate (%) MAVA**	***o*-PDA**	**MAVA/*o*-PDA**	**CNDs**	**CNDs/*o*-PDA**	**DMSO (10%)**	**Control**
A. castellanii Trophozoite	0.01	$0\pm0.00^{a*}$	$28\pm0.55^{a*}$	$18\pm0.56^{a*}$	$8\pm0.60^{a*}$	$12\pm0.58^{a*}$	97±0.82	100±0.82
	0.005	$0\pm0.00^{a*}$	$31\pm0.58^{b*}$	$28\pm0.58^{b*}$	$20\pm0.71^{b*}$	$22\pm0.60^{b*}$		
	0.0025	$48\pm0.56^{b*}$	$86\pm0.58^{c*}$	$52\pm0.58^{c*}$	$58\pm0.71^{c*}$	$60\pm0.60^{c*}$		
	0.00125	$85\pm0.58^{c*}$	98 ± 0.71^{d}	$94\pm0.72^{d*}$	$65\pm0.75^{d*}$	$76\pm0.71^{d*}$		
	0.000625	95 ± 0.62^{d}	99 ± 0.71^{d}	98 ± 0.72^{e}	90 ± 0.78^{e}	$92\pm0.71^{e*}$		
	0.000312	96 ± 0.62^{d}	99 ± 0.71^{d}	98 ± 0.72^{e}	96 ± 0.78^{f}	99 ± 0.78^{f}		
A. castellanii Cys	0.01	$0\pm0.00^{a*}$	$30\pm0.55^{a*}$	$55\pm0.58^{a*}$	$0\pm0.00^{a*}$	$4\pm0.60^{a*}$	100±0.88	100±0.88
	0.005	$2\pm0.48^{a*}$	$32\pm0.58^{a*}$	$85\pm0.68^{b*}$	$6\pm0.58^{b*}$	$10\pm0.71^{b*}$		
	0.0025	$65\pm0.67^{b*}$	$80\pm0.75^{b*}$	$90\pm0.75^{c*}$	$69\pm0.71^{c*}$	$75\pm0.75^{c*}$		
	0.00125	$90\pm0.71^{c*}$	98 ± 0.75^{c}	$94\pm0.75^{c*}$	$90\pm0.78^{d*}$	$92\pm0.75^{d*}$		
	0.000625	$92\pm0.71^{c*}$	100 ± 0.78^{c}	100 ± 0.78^{d}	95 ± 0.78^{e}	99 ± 0.71^{e}		
	0.000312	99 ± 0.78^{d}	100 ± 0.78^{c}	100 ± 0.78^{d}	99 ± 0.78^{e}	99 ± 0.71^{e}		

Data were expressed as mean ± SD. Differences between columns marked with different letters were statistically significant ($p<0.05$)., *; significant compared to the control group ($p<0.05$).

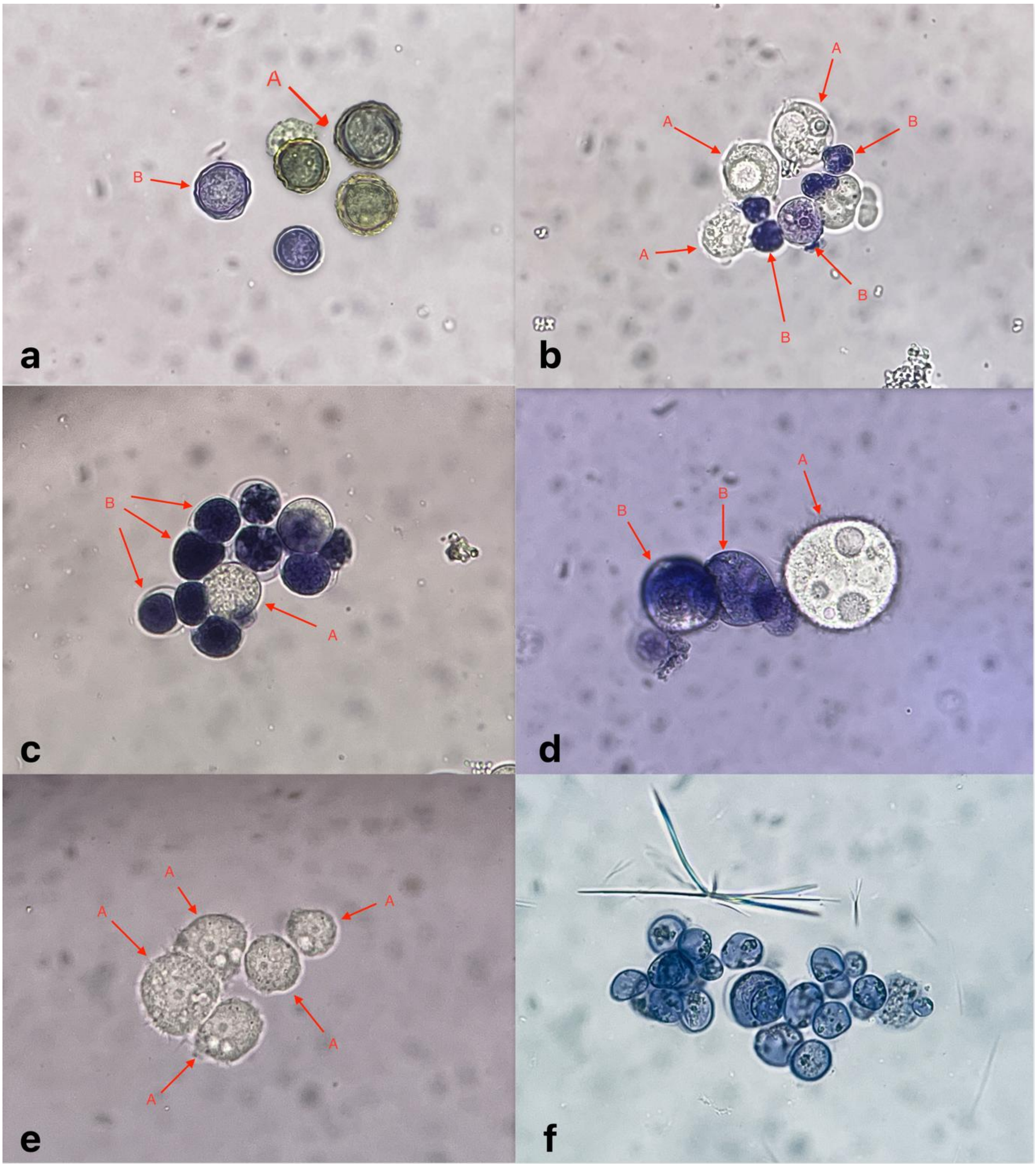


Figure 1: Microscopic view of *A. castellanii* cysts and trophozoites exposed to active substances after 24 hours (40x), a; cysts, MAVA, 0.005 g/mL, b; trophozoites *o*-PDA, 0.01 g/mL, c; trophozoites CNDs, 0.01 g/mL, d; trophozoites CNDs/*o*-PDA, 0.01 g/mL, e; trophozoites, control, f; trophozoites, MAVA, 0.01 g/mL. (Arrows: A; live parasites, B; dead parasites)

The results of the cytotoxicity analysis conducted with the HT-22 Mouse Hippocampal Neuronal Cell Line are shown in Table 2. According to the results, MAVA and o-PDA exhibited low-level cytotoxicity at concentrations of 0.01 g/mL, 0.005 g/mL, 0.0025 g/mL, 0.00125 g/mL, and 0.000625 g/mL. On the other hand, CNDs and CNDs/o-PDA showed no cytotoxicity at the concentrations included in the study (Table 2).

Tablo 2. Cytotoxicity analysis results with HT-22 Mouse Hippocampal Neuronal Cell Line (% viability)

Concentration (g/mL)	MAVA	*o*-PDA	MAVA/*o*-PDA	CNDs	CNDs/*o*-PDA
0.01	75	78	82	90	92
0.005	76	79	90	92	98
0.0025	82	85	92	95	98
0.00125	86	86	94	98	99
0.000625	88	89	99	98	100
0.000312	91	94	100	100	100
Pozitive Control (DMEM+FBS+PS)	100	100	100	100	100
Negative Control (DMSO)	0	0	0	0	0

DISCUSSION

Free-living amoebae (FLA) are protists that are commonly found in water and soil (31). Some FLA can cause both opportunistic and non-opportunistic infections. It has been reported that the Acanthamoeba genus can cause lethal amoebic meningoencephalitis (32). It has been reported that several species of Acanthamoeba can cause a painful corneal disease called Acanthamoeba keratitis (33). This disease typically occurs in contact lens users, often due to poor contact lens hygiene. However, it can also occur after trauma or contact with contaminated water (34).

There are very few reports indicating successful treatment of Acanthamoeba infection. In human infections, combination therapies have been proven to be more successful than single-drug treatments, as many drugs exhibit amebostatic activity but not amoebicidal activity. It has not yet been demonstrated that a single drug is effective against both the trophozoite and cyst stages of Acanthamoeba. Ketokonazole, pentamidine, hydroxychloroquine, paromomycin, 5-fluorocytosine, polymyxin, sulfadiazine, trimethoprim-sulfamethoxazole, azithromycin, and medicinal plant extracts have been reported to be active against Acanthamoeba in vitro, but there is no direct evidence of their effectiveness (33, 35, 36). Despite treatment attempts, the cyst formation of these amoebae in stressful environments is a major reason for resistance to many therapies (37). Additionally, when the stressful environment is eliminated, the amoebae often transition back to the trophozoite form, leading to recurrent infections. The main obstacle in the treatment of Acanthamoeba infections is the robust structure of the cysts, which are highly resistant to physical and chemical agents. Targeting the cyst stage is necessary for a successful prognosis.

In our study, 5 previously untested chemical agents were tested in vitro on *A. castellanii* trophozoite and cyst forms. Additionally, the cytotoxic activity of these agents on healthy cells was observed. It was found that all 5 substances included in the study were effective against both the parasite's trophozoite and cyst forms, and the cytotoxic effect on healthy cells was found to be within acceptable levels.

In a study conducted with MAVA, the amoebicidal activity of MAVA on *A. castellanii* cyst and trophozoite forms was mentioned (38). Furthermore, there are several data in the literature regarding cytotoxicity studies with MAVA, indicating that the cytotoxic activity is within acceptable levels (24).

The main idea of this study is that MAVA is a straight-chain rational copolymer and CNDs are spherical quantum nanodots that are selected after surface functionalization with the chemical agent o-PDA to synthesize MAVA/o-PDA and CNDs/o-PDA conjugates. In addition, both carriers have a regular anhydride functionality (-CO-O-CO-) on the surface of the main structure, and also the o-PDA molecule is a precursor molecule used in the synthesis of many drugs currently used in clinical trials. When carefully examining the results obtained by comparing CNDs/o-PDA and MAVA/o-PDA conjugates developed as candidate molecules, it is important that the structure is flat or spherical, in particular in terms of influencing the shape of the cyst and the CNDs /o-PDA has a dramatic effect, and seems to dominate. This is believed to be due to a better interaction between the spherical nanoscale CNDs/o-PDA conjugate (CNDs are about 4 nm in size) and the cyst or trophozoite form of the parasite. On the other hand, in the case of the straight-chain MAVA/o-PDA conjugate, the parasite is thought to partially affect the cyst or trophozoite shape due to the degradation of the surface functionality. Considering all the results, it can be said that particularly environmentally friendly CNDs, which are accepted as green materials and are obtained homogeneously and stably from fruit acid in a short time under moderate conditions without the use of additional chemical agents, are good carriers as drug delivery systems. In addition, since the cytotoxic effect of CNDs and CNDs/o-PDA conjugate is acceptable in healthy cells, it can be proposed that CNDs/o-PDA conjugate can be developed as a candidate molecule for the next-generation carrier-based drug systems that are particularly effective against the cyst form of the parasite..

CONCLUSION

As it is well known, effective treatment for *A. castellanii* infections has not yet been found. These amoeba-related cases continue to increase over time. In this study, we tested the amoebicidal effects of five previously untested substances on *A. castellanii* cysts and trophozoites. Our findings demonstrate a strong dose-dependent amoebicidal effect of these substances on *A. castellanii* cysts and trophozoites. It is believed that these substances, supported by in vivo experiments, could potentially serve as new therapeutic agents.

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Peer-review: Externally peer-reviewed

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