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In vitro study of carbonyl thiourea derivatives' cytotoxicity on human corneal epithelial cells

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Abstract

Introduction: The global rise of *Acanthamoeba* keratitis infections has highlighted the shortcomings in the current treatments and prevention approaches for the disease. Therefore, this study is a continuation of our prior work and is intended to investigate the possible adverse effects of using carbonyl thiourea derivatives as alternative ocular drugs for amoebic keratitis on non-targeted human corneal epithelial cells (HCEC) in vitro. **Methods:** The efficacy and safety profiles of two carbonyl thiourea derivatives, 2-(3-benzoylthioureido)-3-mercaptopropanoic acid (M1) and 2-(3-benzoylthioureido-4-(methylthio)butanoic acid (M2), were tested on HCEC lines using the cytotoxicity assay and microscopic analysis. **Results and Discussion:** The carbonyl thiourea compounds were found to be non-toxic on HCEC with IC_{50} of 37.73 µg.mL^{-1} and 30.68 µg.mL^{-1} on M1 and M2, respectively. These compounds have selectivity indices (SI) of 14.74 (M1) and 11.38 (M2), indicating moderate selectivity for the targeted *Acanthamoeba* cells. HCEC-treated cells continued to proliferate in a time-dependent manner without significantly altering the cell population by retaining 90% viability. However, the microscopic examination revealed that HCECs were poorly differentiated and disintegrated, with irregular forms after the compound treatment, as opposed to their original hexagonal morphology of the healthy viable cells. The AO/PI staining indicated that the thiourea compounds impaired HCEC membrane integrity. **Conclusion:** This study's findings are critical in providing relevant information on carbonyl thiourea compounds if they were to be proposed as anti-amoebic agents in treating or preventing *Acanthamoeba* keratitis infection.

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Introduction

Human corneal epithelium is the first layer of *Acanthamoeba's* adherence and pathogenesis in host cells to cause amoebic keratitis (de Lacerda *et al.,* 2021). Normally, the protozoan parasite *Acanthamoeba* invades eyes through corneal abrasion. This is followed by the secretion of pathogenic proteases to degrade the basement membrane of the cornea layer which then causes cytolysis and culminates in the dissolution of collagenous cornea stroma (Lorenzo-Morales *et al.,* 2015). According to a previous study, the breach of corneal integrity which is caused by trauma or contact lens wear would lead to a greater chance of getting this sight-threatening amoebic infection (Fleiszig *et al.,* 2019).

Current treatments for *Acanthamoeba* keratitis infection are chlorhexidine gluconate and polyhexamethylene biguanide which have been applied since the 1990s (Alkharashi *et al.,* 2015). However, they were reported to cause various side effects on the human corneal epithelium (Siddiqui *et al.,* 2016). Furthermore, the current pharmaceutical industry barely focuses on ocular drug invention. Most of the existing ocular drugs were adapted from other therapeutic applications that were originally intended for other therapeutic areas but converted to ophthalmic applications following accidental observations that indicated their potential usefulness (Sangkanu *et al.,* 2021).

This situation occurs because ocular drug development requires a thorough understanding of ophthalmology, associated eye structures, pathophysiology, genetic makeup of ocular diseases, and a solid knowledge of the ability of the eye to respond to certain physical and pharmacologic interventions (Anwar *et al.,* 2020). Thus, ocular drug development remains a big challenge. Consequently, an attempt to search for new potential agents that could be developed as new alternatives for the treatment of *Acanthamoeba* keratitis is highly demanded. Therefore, the investigation presented in this study is relevant to meet the purpose. It is very crucial to understand the nature of the proposed carbonyl thiourea derivatives from our previous work against the non-target cell, human corneal epithelial cell (HCEC). In this study, the effects of these compounds were evaluated by conducting practical cytotoxic tests parallel with the experimental design conducted in our previous separate study on *Acanthamoeba* species (Ibrahim *et al.,* 2014). Two selected carbonyl thiourea derivatives from the previous work; 2-(3-benzoylthioureido)-3-mercaptopropanoic acid and 2-(3-benzoyl thioureido-4-(methylthio)butanoic acid which were labeled as M1 and M2 were treated on HCEC lines (Ibrahim *et al.,* 2022). The assessment is crucial to supplement safety profiles for the projected thiourea compounds before being further developed as new agents or ocular drugs for *Acanthamoeba* keratitis disease.

Materials and methods

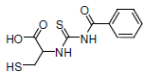
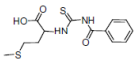
Preparation of working stock solution for carbonyl thiourea derivatives

The molecular structures of 2-(3-benzoylthioureido)-3-mercaptopropanoic acid, and 2-(3-benzoylthioureido)-4-(methylthio) butanoic acid were obtained from our previous study in Ibrahim *et al.* (2022). The thiourea derivative compounds that were labeled as M1 and M2 were freshly prepared prior to every experiment (Ibrahim *et al.,* 2014). The compound's structures are illustrated in Table 1.

Culturing and maintenance of human corneal epithelial (HCEC)

HCEC progenitor cells (CellnTec, Switzerland) were used and maintained throughout the experiments as instructed by the manufacturer's protocol. The cells were grown at 37oC in a humidified atmosphere (95%) incubator with 5% CO2. The cells approached confluence on the sixth day. Changing the medium was done every third day. Specialized CnT-20 culture medium (CellnTeac, Switzerland) was used for trypsinization to dislodge the cells from the

Table 1: Compounds of M1 and M2 carbonyl thiourea derivatives.

Compound	Compound name	MW	Molecular structure
M1	2-(3-benzoylthioureido)-3-mercaptopropanoic acid	284.35	
M2	2-(3-benzoylthioureido)-4-(methylthio) butanoic acid	312.41	

surface of the culture flask and rinsed with sterile phosphate-buffered saline (PBS) before adding 2 mL of Trypsin/EDTA (Sigma, USA) into the flask. The cells were incubated at 37°C for 6 minutes until the cells became rounded and detached from the surface of the flask. After the cells were completely disaggregated, 3 mL of Trypsin Neutralizer (Sigma, USA) was added to stop the reaction of Trypsin/EDTA solution**.**

Determination of cell population inhibition (IC_{50})

Cells density of 5 x 10^4 cells.mL^{-1} was used for seeding before the experiment which was conducted in the 96-microtitre plate. CnT-30 specialized media (CellnTeac, Switzerland) was used throughout the experiment. They were incubated for 24 hours in a 37°C of humidified atmosphere (95%) incubator with 5% CO_2. Before 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay, 20 µL from the prepared 1 mg.mL^{-1} compound stock (M1 and M2) was added into each well of 96-wells microtitre plate, making final concentrations for compound in well of 200 µ L as the final volume with the following; 100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, and 0.39 µg.mL^{-1}. A baseline control containing HCEC without any treatment was included. In this assay, 3% hydrogen peroxide (H_2O_2) (Sigma, USA) was used as a positive control in nine different concentrations; 881.8, 440.9, 220.45, 110.23, 55.11, 27.56, 13.78, 6.89 and 3.44 µM. The preparations were done in three replicates. The well plate was then incubated again for 72 hours.

After the incubation, the medium was discarded and HCEC was gently rinsed with PBS. After that, 20 µL of MTT solution (5 mg.mL^{-1} in PBS) was added into each well and the plate was further incubated for 3 hours at 37°C in a CO_2 incubator. Later, the supernatant was removed from each well. DMSO (100 µL) of was added to solubilize formazan crystals. After the formazan blue was completely dissolved, the absorbance was measured at 595 nm with a reference wavelength of 690 nm by a microplate reader (Thermo, Finland). The amount of formazan produced is proportional to the number of viable cells present in the well plate (Kamiloglu *et al.*, 2020). Cytotoxicity which was based on the absorbance reading was expressed as IC_{50} value which was concentration causing 50% inhibition of the cell's population with reference to untreated cells. T-test (SPSS version 11.5) was used to compare means between HCEC of thiourea-treated cells to non-treated ($p < 0.05$). The IC_{50} values were calculated using GraphPad Prism software ver. 5.03 (San Diego, USA). Consequently, the selectivity index (SI) was calculated using the formula as follows to determine the potency and selectivity of the proposed thiourea compounds (Nguyen *et al.*, 2016):

$$SI = \frac{\text{IC50 for mammalian cell line}}{\text{IC50 for protozoan parasite}}$$

Observation of cell monolayer effect by light microscopy

For morphological observation, HCEC with 5 x 10^4 cells.mL^{-1} in density were supplemented with M1 and M2 thiourea derivatives and H_2O_2 at IC_{50} concentrations in 6-well plates. The untreated HCEC in CnT-30 medium were used as negative controls. The cells were incubated for 72 hours in humidified atmosphere (95%) incubator with 5% CO_2. Later, they were observed under a light inverted microscope (Olympus IX51, USA). The images were captured by AnalySIS Research (version 5.0) software.

Observation of membrane integrity and mode of cell death by AO/PI staining

The membrane integrity of thiourea-treated HCECs was evaluated with fluorescence microscopy technique by applying simultaneous staining of acridine orange and propidium iodide (AO/PI). Duration of 24 hours-seeded HCEC at 5×10^4 cells.mL^{-1} of density were treated with M1 and M2 thiourea derivative and H_2O_2 at their IC_{50} concentrations. Meanwhile, the healthy untreated cells in CnT-30 medium were prepared as negative controls. This test was carried out in 6-well plates as described by the previous study (Hussain et al., 2019). Later, the well-plates were incubated for 72 hours at 37^{o}C in a humidified atmosphere (95%) incubator with 5% CO_2.

After the incubation, the cells from the media were removed and transferred into 1.5 mL Eppendorf tubes. The remaining cells that adhered to the bottom of the wells were removed by trypsinization. The trypsinized corneal epithelium was later collected and transferred into 1.5 mL Eppendorf tubes. After that, the cells in the tubes were centrifuged at 1000 rpm for 3 minutes and the supernatant was discarded. Acridine orange (Sigma-Aldrich, USA) and propidium iodide (Sigma-Aldrich, USA) for AO/PI solution were individually prepared from 1 mg AO and PI powder dye in 999 µL PBS to prepare 1 mg.mL^{-1} solution. For the assessment, 50 µL of AO/PI dye solution was mixed with HCEC pellets and transferred onto microscope slides. The cells were immediately visualized under a fluorescent microscope attached with a DAPI-blue filter (Olympus IX51, USA) and captured using AnalySIS Research (version 5.0) software. For quantitative analysis, 100 cells were randomly calculated and recorded as viable, apoptotic, or necrotic cells. Tukey's test (SPSS version 11.5) was used to classify the group of the distribution of each type of cell for every treatment ($\alpha = 0.05$).

Results and discussion

The corneal epithelium is the first layer affected in the early pathogenesis of the protozoan parasite *Acanthamoeba* to cause *Acanthamoeba* keratitis infection. The corneal epithelium is a non-keratinized stratified squamous epithelium composed of five to six layers of tightly adherent cells that undergo a continuous process of self-renewal and regeneration (Sridhar, 2019). According to Fanselow et al. (2021), the pathogenesis of *Acanthamoeba* leads to epithelial thinning and necrosis of human corneal epithelial cells (HCEC). This suggests that this parasite produces direct cytopathic effects during the initiation of infection. In this case, the application of a topical drug is the most relevant route to treat and manage this ocular disease. Accordingly, in this present research, the safety of the proposed carbonyl thiourea derivatives on HCEC was investigated and evaluated to introduce a new agent for the amoebic keratitis treatment. Well-maintained HCEC lines in medium culture with optimum conditions in a laboratory environment are stable to be conducted in the laboratory for various experiments which include investigation of chemicals and components effects, inflammation, wound healing, and other studies into ocular function. Therefore, the cell lines were in this present study where the density of 5×10^4 cells per mL for HCEC was used as it is a stage where the cells would reach confluency without excessive growth after three days of incubation time (Alsam et al., 2008; Chen et al., 2021).

Inhibition concentration of 50% cell population (IC_{50}) and Selectivity Index (SI)

Dye-technique and fluorescence-based cell viability and proliferation assays are generally less hazardous, less expensive techniques, and more convenient than animal testing methods (Ghasemi *et al.,* 2021). These *in vitro* cytotoxicity assays do not require large samples and are easy to perform. Considering these factors, these kinds of assays were adapted in this current study. MTT calorimetric assay was applied for IC_{50} determination since it offered a quantitative and convenient method for evaluating the cell population's response to the investigated compounds (Rai *et al.,* 2018; Gabriel *et al.,* 2021).

The IC_{50} obtained for M1 and M2 thiourea derivatives on HCEC are depicted in Figure 1. The

inhibition of the corneal cell population indicates the potential of these thiourea derivatives to cause adverse effects on the non-target host's corneal epithelium by suppressing the growth of the cell culture.

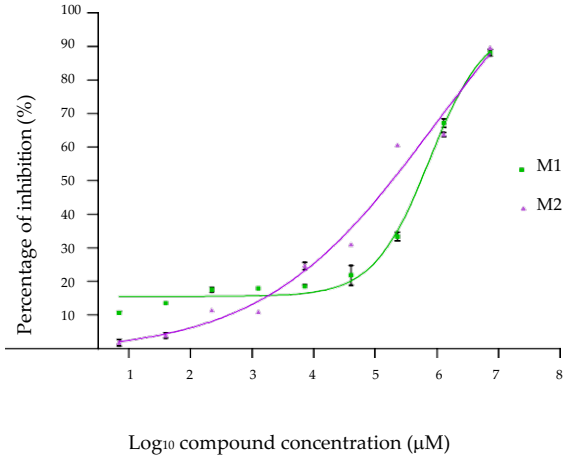


Fig. 1: Dose-response curve for M1- and M2-treated HCEC by MTT assay from absorbance reading ± S.E.M of three replicates.

The IC_{50} values of the compounds are 37.73 for M1, and 30.68 μg.mL^{-1} for M2 thiourea derivatives which are equivalent to 132.69 μM and 98.20 μM respectively (Table 2). Statistical analysis by independent sample T-test ($p < 0.05$) showed that these thiourea compounds are statistically significant as compared to the untreated culture. Meanwhile, the inhibition effect between M1 and M2 compounds was found to be not significant. Comparatively, M1 thiourea showed higher IC_{50} than M2 on HCEC.

Table 2: IC_{50} values of investigated thiourea derivatives on HCEC.

Compound	IC_{50} (μg.mL^{-1})	IC_{50} (μM)
M1	37.73 ±0.42	132.69
M2	30.68 ±0.87	98.20

Positive control, H_2O_2 gave 148 μM of IC_{50}. It was used as a positive control since it is one of the most common elements in contact lens disinfectant solutions (Gabriel *et al.*, 2021), where it is an effective microbial disinfectant that destroys pathogens by oxidation (Yoo, 2018). However, according to the US FDA, H_2O_2 is toxic to human cornea and must be neutralized before lens wear to avoid pronounced stinging, lacrimation, hyperemia, and possible corneal damage (US FDA, 2022). Abbasi *et al.* (2021) revealed that concentrations of H_2O_2 to be cytotoxic on cells can be found ranging from less than 10 μM to more than 1000 μM. Intracellular steady-state concentrations of H_2O_2 above 1 μM are considered to cause oxidative stress inducing growth arrest and cell death (Konno et al., 2021).

In order to assess the thiourea derivatives' potentiality as new agents, the Selectivity Index (SI) was used for easy evaluation of compounds of interest based on their activity toward target and non-target cells (Radha Abbas Hasoon et al., 2021). Table 3 portrays the SI of M1 and M2 thiourea derivatives, with their IC_{50} values for the tested thiourea compounds on HCEC and protozoan *Acanthamoeba* which had been tested previously (Ibrahim *et al.*, 2014). SI classification for the compounds was made by following the proposed categorization by Rolon *et al.* (2019) with SI ≤ 10 is considered as low in selectiveness, ≤ 24 as moderately selective, and SI ≥ 25 is classed as very selective.

According to de Souza et al. (2019), higher SI shows higher selectivity for the anti-parasite activity of the investigated compound. SI values obtained in the present study were in the range of 9.55-21.20 for M1, and 8.76-14.21 for M2 thiourea. These compounds are concluded to be moderately selective toward the targeted *Acanthamoeba*. This study result also shows that M1 and M2 carbonyl thiourea are not highly cytotoxic on non-target HCEC, but cytotoxic on the targeted protozoan parasite, *Acanthamoeba*.

Table 3: Selectivity Index (SI) for M1 and M2 thiourea derivatives

Compound	IC_{50} HCEC (μm)	IC_{50} amoeba (μM)*	SI
M1	132.69	9.00	14.74
M2	98.20	8.63	11.38

Monolayer effects of cells

Monolayer of HCEC after the treatment with M1 and M2 thiourea derivatives showed population inhibition compared to the untreated cells that

produced a confluent culture after 72 hours of incubation. The corneal cells were observed to transform from a well-differentiated combination of hexagonal shape for untreated cells to become disintegrated with M1 and M2 thiourea derivatives' treatment. Several rounded and detached cells were also observed in the treated culture, indicating they became unhealthy. However, the numbers were notsignificant.

Meanwhile, H_2O_2-treated HCEC's monolayer was also seen to become irregular in shape through cells' elongation. More rounded and detached cells were detected compared to M1 and M2-treated HCEC. From the observation, it could be concluded that the investigated thiourea derivative compounds suppress the HCEC differentiation and proliferation without significantly affecting the cells' cellular morphology. The microscopic observation of thiourea-treated HCEC was almost identical as seen in a study by Trocme et al. (1997) where the study on the effect of human eosinophil major basic protein toward HCEC noted a minimal extent of cytoplasmic granularity with loss of cell adhesion in the treated cells. The morphological microscopy result is shown in Figure 2.

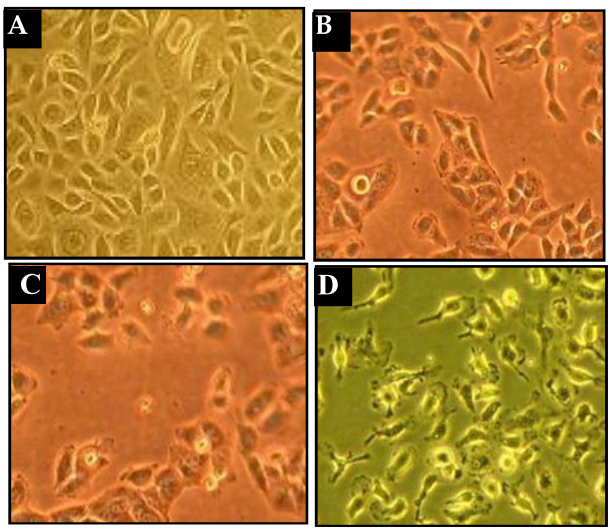


Fig. 2: Phase contrast micrographs of human corneal epithelial cells (HCEC) under inverted light microscopy. (A) Untreated HCEC; (B) M1-treated HCEC; (C) M2-treated HCEC; (D) H_2O_2-treated HCEC. Magnification: 200×

Membrane integrity and mode of cell death

Cavet *et al.* (2012) measured the protease activity associated with the loss of membrane integrity in HCEC and proposed that a decrease in the cell's viability may be due to an increase in apoptosis. Accordingly, in this current research, a quantitative study from the fluorescence dual staining technique was utilized to evaluate the effect of the proposed thiourea derivatives on HCEC since fluorescent viability staining provides information beyond morphology. This technique visualizes functional alterations in cells' membrane integrity which is related to the mode of cell death.

The untreated HCEC produced a green fluorescence color under a fluorescence microscope, which indicates that the cell membrane was still intact. Meanwhile, thiourea-treated HCEC demonstrated that they had compromised membranes which allowed for the penetration of PI exclusion dye, exhibiting both green and orange fluorescence color of cells. H_2O_2-treated HCEC also emitted both green and orange colors under the fluorescence microscope. The microscopy result is shown in Figure 3.

Galluzzi *et al.* (2018) highlighted that dying cells demonstrate distinctive morphological features depending on the mechanism of cell death. In the present study, thiourea-treated HCEC exhibited apoptotic characteristics, particularly the loss of membrane integrity, which allowed the penetration and diffusion of both acridine orange (AO) and propidium iodide (PI) dyes. PI is known to penetrate only cells with severely damaged membranes (Crowley *et al.*, 2016), and a similar staining pattern was observed in HCEC treated with H_2O_2.

According to Hu *et al.* (2021), intact membranes indicate viable or early apoptotic cells, whereas loss of membrane integrity is a hallmark of late apoptosis and necrosis. In this study, HCEC emitting green fluorescence were classified as viable, while cells displaying both green and orange fluorescence were considered apoptotic due to compromised membranes. Cells showing uniform

orange fluorescence were identified as necrotic, indicating complete membrane rupture and full PI penetration. These findings suggest that thiourea compounds primarily induce apoptosis, a regulated form of cell death that minimizes damage to surrounding cells.

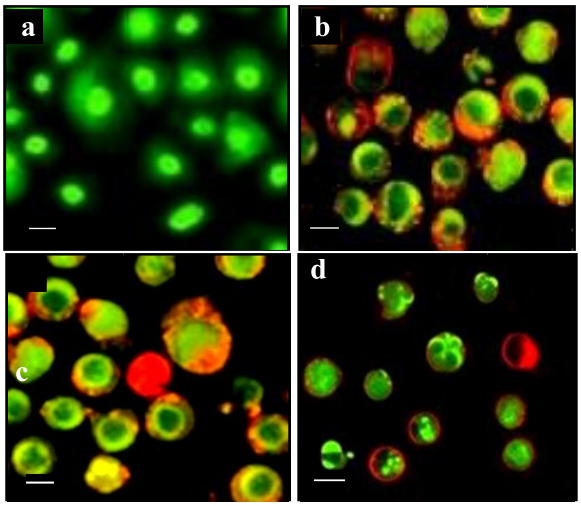


Fig. 3: Fluorescence microscopy images of AO/PI-stained human corneal epithelial cells (HCEC) (a) Untreated-HCEC; (b) M1-treated HCEC; (c) M2-treated HCEC; (d) Positive control, H_2O_2-treated HCEC. Magnification: 300×

The quantitative result from the quantification of 100 cells is shown in Figure 4. The given treatments with M1, M2, and H_2O_2, caused HCEC to become non-viable and exhibited indicators that the compounds promote apoptosis in HCEC. Statistical analysis showed that apoptotic cells were significant in the effect caused by the thiourea derivatives and H_2O_2. In the untreated culture, HCEC showed almost total viability of the cells' population. Tukey-test (α = 0.05) showed different groups of distribution between viable, apoptotic, and necrotic cells for HCEC treated with M1-thiourea and M2-thiourea.

From the cytotoxicity and morphological study for both carbonyl thiourea derivatives on HCEC, these compounds are found to be poorly cytotoxic on the non-target HCEC which are comparable to H_2O_2 that have been widely used in contact lens solutions in the current market. The data gained through this study is very important to provide useful information on the proposed carbonyl thiourea compounds to avoid non-desirable effects on the non-target cells from its application as an anti-amoebic agent to treat *Acanthamoeba* keratitis.

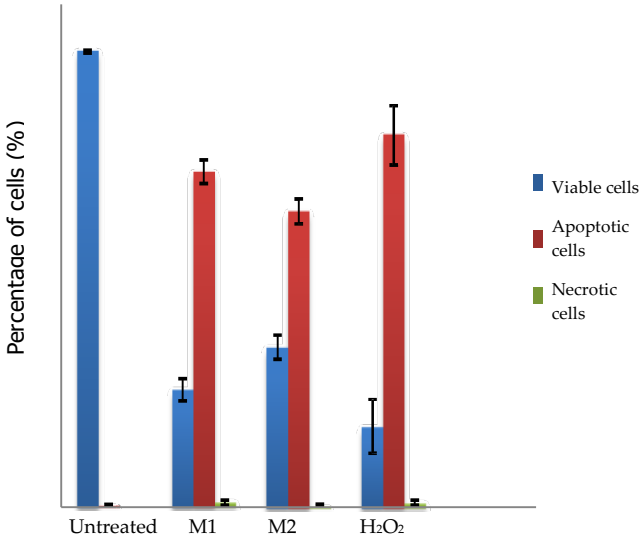


Fig. 4: Percentage of viable, apoptotic, and necrotic HCEC (+ S.E.M three replicates).

Conclusion

Two selected thiourea derivatives 2-(3-benzoylthioureido)-3-mercaptopropanoic acid, and 2-(3-benzoylthioureido)-4-(methylthio)butanoic acid, labeled as M1 and M2 respectively, were further tested on non-target human corneal epithelial cells (HCEC). These thiourea derivatives showed low cytotoxicity on HCEC with IC_{50} at 37.73 µg.mL^{-1} (148 µM) for M1 and 30.68 µg.mL^{-1} (98.20 µM) for M2 thiourea. The Selectivity Index described these derivatives were moderately selective compounds toward the targeted *Acanthamoeba* cells with SI at 14.74 for M1, and 11.38 for M2 thiourea. Microscopic observation showed HCECs were not well-differentiated and disintegrated, having irregular shapes as compared to their normal hexagonal form. AO/PI staining concluded that the compounds disrupted the cell's membrane integrity and caused apoptosis in the HCEC.

Authors contributions

Conceptualization, M.A.I. and M.S.M.Y.; methodology, M.A.I.; validation, M.A.I.; formal analysis, M.A.I.; data curation, N.H.Z.; writing—original draft preparation, M.A.I. and N.H.Z.; writing—review and editing, N.H.Z. and N.H.M.H.; visualization, F.A.A.M. All authors have read and agreed to the published version of the manuscript.

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Ethical approval statement

Not applicable.

Informed consent statement

Not applicable.

Conflict of interest

No conflicts of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

Not applicable.

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