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ORIGINAL STUDY

Potential of Critical Mineral and Cytotoxicity Activity of Gadolinium Complex as Anti-amoebic Agent by Viability Studies and Flow Cytometry Techniques

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ABSTRACT

The screening of lanthanide as a critical mineral and used as an anti-amoebic agent is important to identify the safest method and mode of cell death to eliminate this microorganism from the host. In this study, gadolinium ion (Gd^{3+}) that complexed with a short acyclic triethylene glycol (EO3) and in the presence of picric acid (HPic) as counter anion for anti-amoebic activity was explored. The trivalent Gd^{3+} complexed with EO3 and HPic via *one-pot* synthesis reaction using a mole ratio of gadolinium nitrate salt, EO3 ligand and HPic in a mole ratio 1:3:4 to produce $[Gd(Pic)_2(H_2O)(EO3)]^+(Pic)^-.CH_3OH$. The viability of cells was assessed by using an MTT assay after 24 hours exposed to the Gd complex. The cytotoxicity of the Gd complex has an IC_{50} value of 23.44 μg/mL. The treated cells display orange organelles in the green cytoplasm after 24 h of incubation time and indicated that the cells experienced autophagic cell death. The externalisation of phosphatidylserine was not observed by flow cytometry techniques using Annexin V-FITC. Overall, the Gd complex is potent as anti-amoebic agent and is possible to use for the treatment of *Acanthamoeba* infection in future.

Keywords: Acanthamoeba, Antiamoebic agent, Critical Mineral, Cytotoxicity, Gadolinium

1. Introduction

The important pharmacological requirement for a drug and a contract agent is the limited toxicity and stability of compounds [1]. For instance, the relaxation of the complex can be adjusted by increasing the number of inner-sphere coordination using water molecules. Lanthanides, well-known as rare earth elements (REEs), are critical minerals and have many applications [2]. These metals and their complexes showed excellent biological activity especially as an anti-amoebic activity, and

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have attracted great attention to find new candidate drugs for *Acanthamoeba* keratitis infection. These metals are unrenewable products and very important materials for advanced technologies in petroleum productions, energy, synthetic products, medical applications, lamps, lasers, magnets, projectors, and X-ray intensification screens.

Design of the macromolecule complexes based on REE ions and hydrophilic ligands is important to produce anti-amoebic agent, which could be functionalized in clinical applications. It is noted that some nanoparticles, including synthetic compounds, drugs, and natural products have been reported to be promising antiamoebic agents against free-living amoebae. For instances, quinazolinones, amphotericin B, and quercetin were coated with nanoparticles, and were used as effective antiamoebic agents against free-living amoebae in vitro, as have been reported by Mungroo et al. [3], Rajendran et al. [4], Anwar et al. [5], respectively. Tavassoli et al. [6] reported some options of using other types of chemical compounds, such as aminoglycosides, aromatic diamidines, biguanide (polyhexamethylene biguanide, chlorhexidine), imidazoles (fluconazole, ketonazole) as anti-amoebic agents.

It is important to highlight that Acanthamoeba *sp.* is one of free-living protozoa that causes *Acanthamoeba* keratitis (AK), which is a serious eye infection that can affect both contact lens and non-contact lens wearers. This infection is caused by poor personal hygiene, wearing contact lenses for a long period of time, or being exposed to contaminated water (Khan, 2006). Most of the AK cases are related to inappropriate ways of cleaning the contact lenses and contamination with bacteria and amoeba, as reported by Trevisan (2010). The eye drops for treating AK infections using the biguanides, namely chlorhexidine digluconate (CHX, 0.02%) or polyhezamethyle biguanide (0.02%) have been reported by Cucina et al. [7]. This formulation comprised by 0.02% CHX-0.1% ethylenediaminetetraacetic acid disodium salt (Na_2EDTA) loaded ocular gel [7]. However, the use of these antiseptics have some side effects on humans.

In contrast to antiseptics, the advantages of lanthanide complexes as anti-amoebic agents have been demonstrated, especially relatively low IC_{50} values, less toxicity, and unique structure that can be adjusted and affect their capabilities as antimicrobial activities. Kusrini and co-workers have reported both light and heavy lanthanide complexes as anti-amoebic agents, such as the terbium-trinitrato-triaquo-18-crown-6 $[Tb(NO_3)_3(OH_2)_3.(18C6)]$ [8], the acyclic [bis(picrato)(pentaethylene glycol)samarium(III)] picrate, $[Sm(Pic)_2(EO5)]^+(Pic)^-$, and the cyclic [bis(picrate)(18-crown-6)samarium(III)] picrate, $[Sm(Pic)_2(18C6)]^+(Pic)^-$ [9], where Tb is terbium, Sm is samarium, 18C6 is 18-crown-6; EO5 is pentaethylene glycol, and Pic is picrate anion. Based on those previously reported studies, the surrounding of Ln^{3+} ions in the inner-sphere and outer-sphere coordination significantly affect their toxicities and anti-amoebic activities. According to Kusrini et al. [8], the level of cytotoxicity of lanthanide metal ions against *Acanthamoeba* sp was different from those in their salts, their complexes, and free ligands.

Kusrini et al. [14] have reported a molecular structure of gadolinium (Gd) complex that coordinated with an acyclic triethylene glycol (EO3) ligand, picrate anion (Pic) in the form of $[Gd(Pic)_2(H_2O)(EO3)]^+(Pic)^-.CH^3OH$ complex. To our knowledge, there is no report regarding the anti-amoebic activity of this Gd complex. This complex is of interest in the areas of critical minerals and advanced materials for biomedical and biological applications. One of basic interesting ideas is the potential of Gd complexed with EO3 and picric acid as counter anion for anti-amoebic agent.

CHX is an amphiphilic and cationic compound that has a positively charge in both hydrophilic and lipophilic sides. Similarly, $[Gd(Pic)_2(H_2O)(EO3)]^+(Pic)^-.CH_3OH$ complex is salt type and has a positive and negative charge, making it capable to degrade and/or killing of amoeba cells. Thus, an identification and screening technique to evaluate this complex as anti-amoebic activity in vitro is urgent to investigate, especially the safety of this complex and mode of amoeba cell death. Therefore, in this study, the cytotoxicity assessment of $[Gd(Pic)_2(H_2O)(EO3)]^+(Pic)^-.CH_3OH$ complex was conducted. The percentage of inhibition of 50% inhibitory concentration (IC_{50}) was determined by fitting the dose-response curves to a standard curve. It is noted that the highest IC_{50} value has the lowest toxicity level, thus, this complex has the potentially to use for further assessment as an anti-amoebic agent. The ability of the chemical compounds or any substances to damage a healthy living cell is called as cytotoxicity activity. Moreover, the Gd complex was investigated for the externalisation of phosphatidylserine (PS) by flow cytometry techniques using Annexin V-FITC.

2. Experimental

2.1. Materials

The triethylene glycol (EO3) ligand ($C_6H_{14}O_4$; 99%) was obtained from Acros. The $Gd(NO_3)_3.6H_2O$ salt with a purity of 99.9% was originated from

Johnson Matthey Electronics (New Jersey, U.S.A.). Picric acid with a chemical formula of $[(NO_2)_3C_6H_2OH]$ and purity of 98% was purchased from B.D.H. (Poole, England).

2.2. Preparation of Gd complex

The Gd complex was prepared in a one-pot reaction with the mole ratio of $Gd(NO_3)_3.6H_2O$ salt, EO3 ligand, and picric acid in a 1:3:4, according to the method reported by Kusrini et al. [14]. All components were dissolved in the 30 mL acetonitrile:methanol:water in a volume ratio of 3:3:1, and were stirred for 10 min at ambient temperature. The container was kept at room temperature and covered by aluminium foil or plastic to allow the slow evaporation process in the formation of the Gd crystalline complex. The crystalline was obtained after several recrystallization processes by sequencing solvents, namely methanol, acetonitrile, acetonitrile and methanol:acetonitrile (1:1 v/v). The Gd crystalline complex was obtained from methanol:acetonitrile (1:1 v/v) solvent after 10 weeks. This Gd complex was characterized and evaluated the biological property in vitro.

2.3. Preparation of Gd complex stock solution

The Gd complex (1 mg) was dissolved in 30 μL of dimethyl sulfoxide (DMSO) and 970 μL of Peptone Yeast Extract Glucose Broth (PYG) media with the final concentration of the stock solutions is 1000 μL/mL. The PYG medium is consisting of 2% proteose peptone, 0.2% yeast extract, and 0.1 M glucose. The Gd stock solution was vortexed and kept in the 4 °C refrigerator for further use.

2.4. Determination of IC_{50} Value by using MTT assay

The most common assays that were used for measuring the cell viability and cytotoxicity of chemical compounds towards *Acanthamoeba* sp. was MTT assays [15] using (3-(4,5-dimethlthizol-2-yl)-2, 5-diphenyltetrazolium bromide) tetrazolium as reagent. This reagent is able to detect cell viability via the formation of insoluble formazan blue crystals after the cells were incubated with the reagent for 4 h. The conversion of the yellow substrate colour to the formazan blue crystals is due to active metabolic activity of the viable cells [16]. For MTT solution preparation, 5 mg/mL of MTT was dissolved in 1 mL of phosphate buffered saline (PBS) solution and was filtered in order to remove the small amount of insoluble residue present in batches of MTT. Seeded *Acanthamoeba* in 96-well plate at 1×10^5 cell/plate was incubated at 30 °C for 8 h. After 8 h, the medium was replaced by solution of Gd complex ranging from 0.45 to 30 μg/mL. Then, 20 μL from MTT stock solution was added to a 96-well-plate, and the plate was incubated in a 30 °C incubator for 4 h. After the incubation, crystal visibility was presented, 150 μL of DMSO was added in each of the wells to dissolve the Gd complex. The well plate was then left for a few minutes at room temperature.

2.5. Morphological observation under an inverted light microscope

Cytotoxicity of the Gd complex on morphology of *Acanthamoeba* sp. was assessed by using an inverted light microscope. The cells were directly observed under an inverted light microscope after 24 h incubation with the Gd complex at its IC_{50}. Treatments of *Acanthamoeba* sp. were carried out in 6-well plates in a 3 mL culture medium containing Gd complex stock solution. It was incubated at 30 °C for 24 h, and an inverted light microscope was used to observe the morphological changes of cells.

2.6. Mode of cell death determination under fluorescence microscope

The treatments of *Acanthamoeba* sp. were carried out in 6-well plates in 3 mL culture medium containing the Gd complex at their IC_{50} values and incubated at 30 °C for 24 h. The untreated and Gd-treated *Acanthamoeba* cells were centrifuged at using speed of 3000 rpm for 15 mins. Supernatant and pellets were formed, and then they were re-centrifuged process using speed of 1000 rpm for 5 minutes. The supernatant and the pellets were separated and washed once using PBS solution. The pellets were resuspended in 100 μL acridine orange/propidium iodide (AO/PI) dye solution. The cells were incubated for 10 min in the dark condition. The cell suspension was placed onto a slide, covered with a cover slip and was observed and visualized under fluorescence microscopy to observe the mode of amoeba cell death.

2.7. Detection of the mode of cell death using Annexin V-FITC method

According to Vermes and Haanen [17], combinations of Annexin V-FITC with PI can differentiate the viable, apoptotic, and necrotic cells. The *Acanthamoeba* cell was put in 6-well plate containing 3 mL of PYG media with Gd complex at their IC_{50} value and incubated at 30 °C for 24 h. After that they were centrifuged 1 min at a speed of 3000 rpm. The supernatant was discarded, and the pellets

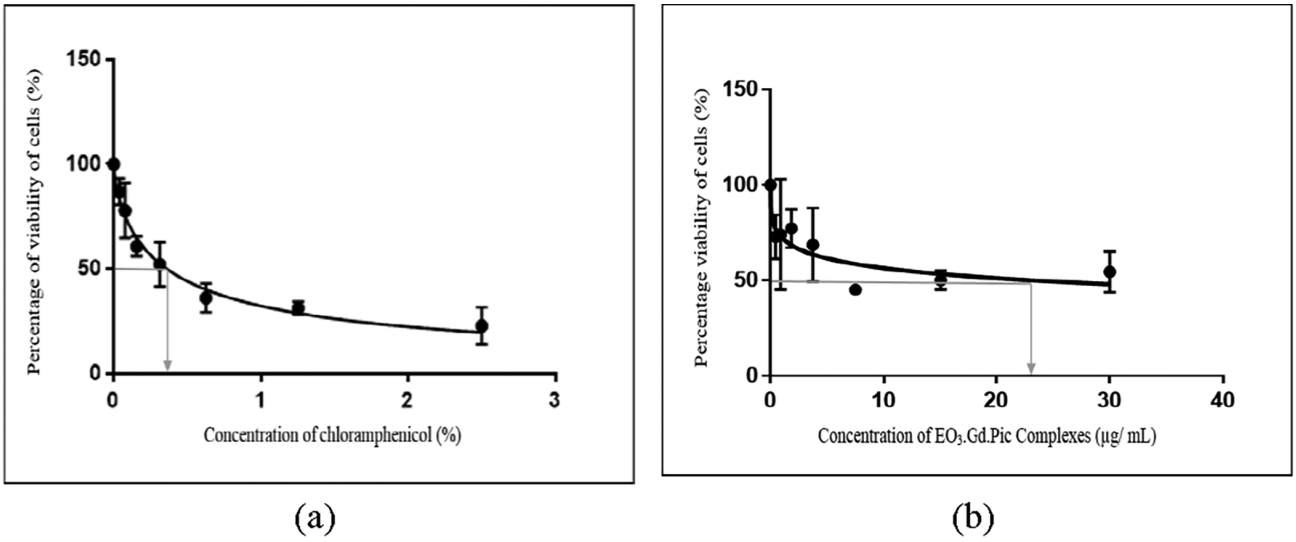

Fig. 1. Comparison of percentage viability of cells assessed by using MTT assay after 24 hours exposed to chloramphenicol with the IC_{50} value of 0.39 (%) (a), and (b) percentage viability of cells assessed by using MTT assay after 24 hours exposed to the Gd complex with the IC_{50} value of 23.44 µg/mL.

were washed in ice-cold PBS solution at pH 7.4. The cells were resuspended and recentrifuged at 3000 rpm for 1 min. The excess PBS was removed by pipette, and the cells were resuspended in a binding buffer. Then, 10 µL of Annexin V-FITC was added to 190 µL of cell suspension and mixed gently. The cells were incubated at room temperature and washed with a binding buffer solution. The first steps were repeated and re-suspended in 190 µL of binding buffer followed by 10 µL of 20 µg/mL PI stock solution, and the cells were analysed by using flow-cytometry.

2.8. Characterizations

To detect the cytotoxicity of chemical compounds and the viability of the treated cells, IC_{50} values of the Gd complex was determined using the MTT assays. IC_{50} value graph was plotted using Graph Pad version 7.0. A Dynatech MR580 MicroElisa reader was used to read the plates at a wavelength of 570 nm. After 24 h of exposure of *Acanthamoeba* cells with Gd complex in different concentrations were analysed using ELISA readings. Toxicity of the lanthanide complexes was observed under the light microscope. A fluorescence microscope detected changes on in the trophozoite shapes of *Acanthamoeba* sp. after being stained with fluorescence dyes to determine the mode of cell death. Confirmation of the mode of cell death, either apoptotic or necrotic, was determined via flow cytometry using the Annexin V-FITC staining.

3. Results and discussion

3.1. Studies of cell viability and cytotoxicity of Gd complex

A comparison of the percentage viability of cells assessed by using MTT assay after 24 hours of exposure to chloramphenicol and Gd complex is shown in Fig. 1(a). The toxicity of chloramphenicol showed IC_{50} value of 0.39 (%). The Gd complex is cytotoxic towards *Acanthamoeba* sp. with different concentrations from 0 to 30 µg/mL. The fifty percent inhibition concentration (IC_{50}) on *Acanthamoeba* sp. using the Gd complex is 23.44 µg/ mL. This value was needed to detect the concentrations of Gd complex that inhibited cells by 50%. The percentage of viability of cells decreases after has been exposed to increasing concentrations of the Gd complex. The Gd complex is only having one coordinated water molecule in the inner coordination sphere, this environment affected its performance as anti-amoebic agent. According to Huda et al. [18], cytotoxicity assessment on the treated cells is important in developing therapeutic drugs. The Gd metal has a high paramagnetic property, so it has used in biomedical applications such as magnetic resonance imaging (MRI) to obtain the clarity images of tissue and detection of abnormalities and diseases in the body [19].

The *Acanthamoeba* cells were treated for 24 hours because it was the exact incubation time for the treatments to react and perform their toxicity towards cells. If the *Acanthamoeba* cells were treated for less than 24 hours, most of the cells would remain viable,

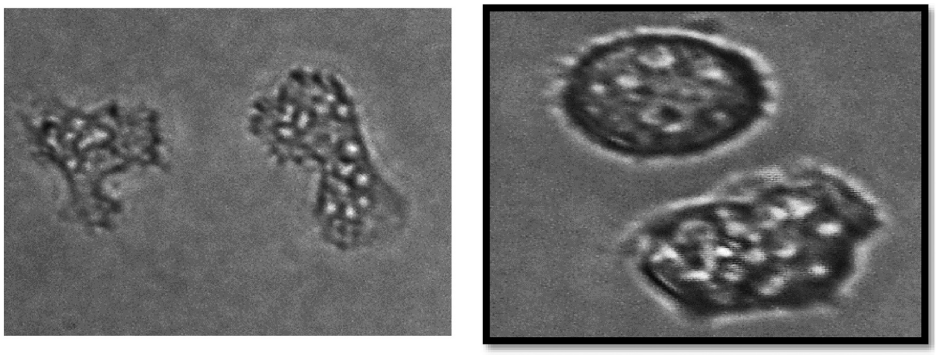

Fig. 2. Image of morphological structure of cells under light microscopy observation for (a) untreated cells has trophozoite active form, irregular shape with existence of acanthapodia and vacuoles, and (b) treated cells showing a dormant cyst form, round shape and decrease in size, structure of acanthapodia shorten or lost, cyst formed under unfavourable environmental conditions caused the encystment of the cells. Magnification was 400x.

and the cells that were killed did not reach fifty per cent of the population, showed that the chemical complex was not toxic at short incubation of time. Based on the graph, the percentage of cells decrease across the increasing concentration of the complex (see Figs. 1(a) and (b)).

The IC_{50} value of the Gd complex is higher than that found for chalcone derivatives [20]. The chalcone compound derivatives did not show significant cytotoxicity effects at different concentrations (100, 50, 25, 12.5, 6.25, 3.125, and 1.5625 μg/mL) compared to the studied Gd complex. The viability of *Acanthamoeba* cells using the chalcone compound derivatives remains higher than 50% after being treated with a specific incubation time, leading to no IC_{50} value to be determined [20].

3.2. Morphological observation under inverted light microscopy

The *Acanthamoeba* cell morphology were compared with the negative control, i.e. the untreated cells. As shown in Fig. 2(a), the untreated cells have an irregular shape with the formation of acanthapodia, finger-like projections, and also the presence of vacuoles. Meanwhile, the morphology of cells changed after being treated with the Gd complex, and showed the shortening of acanthapodia structure of the cells from the surface of *Acanthamoeba*. The sizes of cells treated with Gd complex were reduced from a normal and irregular shape to a round shape due to the shortening and loss of acanthapodia structure. A large vacuole was also observed in treated *Acanthamoeba* cells (see Fig. 2(b)).

Comparing the untreated *Acanthamoeba* cells under favourable conditions showed an active trophozoite formed. Meanwhile, under unfavourable conditions, the treated *Acanthamoeba* cells showed in the dormant cyst formed by decreasing its size and becoming round with the loss and shortening of acanthapodia structures. The treated cells were found floating in the culture medium due to the dysfunctions of acanthapodia that was short and loss to adhere on the surface of the culture flask. The surrounding conditions in the flask were in unfavourable conditions due to the presence of Gd complex leading to the encystment of *Acanthamoeba* cells. This statement was supported by Ibrahim et al. [21], where chemical compounds caused the encystment of the cells. Encystment is a process where the cells start to become inactive due to unfavourable environments by reducing their size from the trophozoite stage to form cyst stage with the presence of wall layers that can resist disturbance. The trophozoite stage is the active stage that is high in pathogenicity. The pathogenicity of *Acanthamoeba* sp. was linked with the existence of acanthapodia because the ability to adhere to the surface and invade tissues that contributes to infections host. It was supported by Marciano-Cabral and Cabral [22], who identified the trophozoites within the brain tissue of granulomatous amebic encephalitis (GAE) patient. The structure of acanthapodia plays a main role in the pathogenesis of AK that causes infections by binding to the corneal epithelium of the host, which then leads to secondary events like toxin secretions from *Acanthamoeba* to host and phagocyte of host cells caused cell death [23].

3.3. Mode of cell death determination using AO/PI staining

The toxicity of the Gd complex towards *Acanthamoeba* affects the cells' permeability. The

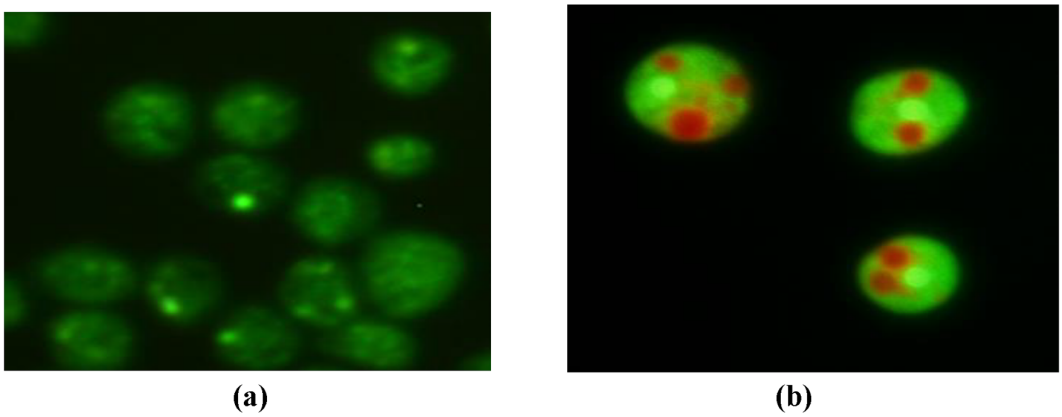

Fig. 3. Comparison images of stained Acanthamoeba sp. with AO/PI viewed under fluorescence microscopy for (a) untreated cells display green fluorescence nuclei and cytoplasm showed the healthy and viable cells, where AO is a membrane-permeable dye which emit green at high pH concentration and bind to healthy DNA and green fluorescence, and (b) the treated cells display cells with green nuclei and orange bits in cytoplasm = autophagic cell death, where AO stained lysosomes and emit orange fluoresce in low concentration of pH and at high positive charge in lysosomes inhibit other molecules to cross the vesicle membrane but allowed cationic AO to enter and orange fluorescence after exposure for 24 h of incubation time. Magnification was 400x.

permeability and viability of cells were assessed using the AO/PI staining. AO and PI dyes are the fluorescence dyes that are used to measure both viable and non-viable cells with also the specificity to visualize apoptosis and necrosis states. AO is a nucleic-acid selective dye used that helps in the detection of the mode of cell death by staining both viable and non-viable cells. According to Foglieni [24], the treated *Acanthamoeba* cells were compared with the untreated *Acanthamoeba* cells to observe the differences color of dyes that appeared on cells that indicated the different types of modes of *Acanthamoeba* cell death.

In this study, *Acanthamoeba* cells were stained with the combinations of AO/PI dyes, and then the cells were observed under fluorescence microscopy in order to differentiate the viable and non-viable *Acanthamoeba* cells. At high pHs, AO staining cells emit green, while at low pHs, AO staining cells will emit red colour. A membrane-permeable dye specifically binds to the native or denatured nucleic acid of a cell by crossing an intact membrane, thus, the changing of colour can be observed. According to Pierzynska et al. [25], AO staining can be used to differentiate the double stranded DNA by fluoresce green and single-stranded RNA by fluoresce red. However, for necrotic cell death, Mascotti et al. [26], state that PI is an impermeable dye to intact membrane, and it penetrates the non-viable cells by producing orange fluorescence. Non-viable cells mean the cells were completely damaged due to the exposure to disturbance. Al-Rubeai and Fussenegger [27] also state that PI interact with the nucleic acids of the cells in the lysed membrane and results in red fluorescence of necrotic cells. If penetration of PI dye occurred, the cell membrane of the cells was not intact.

The untreated *Acanthamoeba* cells demonstrated green fluoresce nuclei and the cytoplasm showed viable and healthy cells (see Fig. 3(a)). The emitted green fluoresce cells proved that the membrane of untreated *Acanthamoeba* was in intact conditions means that no rupture events of the membrane occur. However, the treated cells displayed with the orange organelles in green cytoplasm after 24 h of incubation time (see Fig. 3(b)), indicating that the cells experienced autophagic cell death.

The mode of *Acanthamoeba* cell death, namely (i) apoptosis or (ii) necrosis, can be detected by the colouration of the cytoplasm of cells viewed under a fluorescence microscope [28]. Autophagic cell death is the type of cell death that involve the engulfment of damaged materials into autophagosomes. This type of cell death does not involve or disrupt the membrane of cells because autophagosome was formed from the isolation of the membrane. The orange organelles in the cell cytoplasm was due to the AO stained lysosome due to the permeable-cationic dyes entering acidic organelles through intact membrane. AO is a dye that accumulates in acidic organelles such as lysosomes at low pH conditions and emits orange fluoresced [29]. This phenomenon was due to the high positive charge in lysosomes inhibiting the ability of other molecules to cross the vesicle membrane but allowing the cationic A.O. dye to enter. However, the dye molecules became protonated and were trapped inside the lysosome, thus, orange-stained vesicles were detected by a fluorescence microscope [25]. It is

Table 1. The comparison the IC_{50} values for lighter [30] and heavier lanthanide complexes using similar triethylene glycol (EO3) as ligand.

Name of lanthanide complexes	IC_{50} (μg/mL)
$[Pr(NO_3)(Pic)(H_2O)_2(EO3)]^+(Pic)^-$	1.72 [30]
$Nd(NO_3)(Pic)(H_2O)_2(EO3)]^+(Pic)^-$	1.69 [30]
$[Gd(Pic)_2(H_2O)(EO3)]^+(Pic)^-.CH_3OH$	23.44

strongly believed that the emitted orange fluoresced in lysosomes indicates autophagic cell death. It is also similarly observed for *Acanthamoeba* cells that were treated using $[Tb(NO_3)_3(OH_2)_3]$ complex [8] and showed that yellow-orange granules for an autophagic activity.

The Gd complex does not completely damage the *Acanthamoeba* cells after they have been treated for 24 h. This may be due to the decrease of toxicity levels of the Gd^{3+} ion and this data is agreed with the data of IC_{50} of 23.44 μg/mL. As reported by Kusrini et al. [14], the Gd^{3+} ion was coordinated with the EO3 ligand in the tetradentate manner through two oxygen (O_{eter}) and two $O_{hydroxyl}$ atoms. The presence of the water molecule in the Gd complex also helps to form the heavier lanthanide complex. The EO3 ligand presents an important class of compounds for new drug development because the hydroxyl group moieties were selected for hydrogen bonding interaction and provide high stability at physiological pH in acidic and basic conditions, fascinating for drug delivery system. These findings are consistent with earlier studies using REE complexes with acyclic and cyclic ligands [9], which showed anti-amoebic activities. Table 1 illustrates the IC_{50} values for lighter [30], and heavier lanthanide complexes using a similar EO3 ligand for comparison purposes. Significant differences in the IC_{50} values between lighter and heavier lanthanide complexes due to the surroundings in the inner-coordination sphere of Ln^{3+} ion.

Comparisons the IC_{50} with other compounds are summarized in Table 2. The $[Ce(Pic)_2(H_2O)(EO4)](Pic)H_2O$ complex, Ce salt and tetramethylene glycol (EO4) ligand showed IC_{50} of 3, 3.75 and 0 μg/mL [31]. On the other hand, the cytotoxicity assay of *Acanthamoeba* sp. suggested for Fe_3O_4-PEG, Fe_3O_4-PEG-Dy_2O_3, $Dy(NO_3)_3.6H_2O$ and $Dy(OH)_3$ nanoparticles have an anti-amoebic activity with the IC_{50} values of 4.5, 5.0, 9.5 and 22.5 μg/mL, respectively, where PEG is polyethylene glycol and Dy is dysprosium [32]. The Gd complex has the highest IC_{50} value compared to the other lanthanide complexes, lanthanide salts, and their ligands. This indicates that the Gd complex has mild cytotoxicity activity and the potential for anti-amoebic agents in the future.

3.4. Validation of apoptosis and necrosis by Annexin V-FITC

To confirm the presence of the mode of cell death of the treated *Acanthamoeba* cells either in an apoptosis or necrosis types, the Annexin V conjugated with fluorescein isothiocyanate (FITC) and nuclei stained with PI were investigated. Pathologies were associated with vascular damage [33]. Annexin V-FITC has been used as a marker for the detection of early apoptotic events of cells while PI was used to detect the necrosis events of cells. This assay can assess cell membrane integrity due to the cell death process. Annexin V is a cellular protein in a group of annexins and can be bonded with the phospholipid of the cell surface membrane phosphatidylserine (PS) of *Acanthamoeba* sp. It is a protein with a core structure and has the ability to bind with phosphatidylserine (PS) of the membrane. PS plays a key role in keeping the cells intact and control the movement of substances that cross the cell membrane.

The graph consists of four quadrants which first (I), second (II) and third (III) quadrants indicated the type of cell death necrosis, late apoptosis, and early apoptosis, respectively, while the fourth (IV) quadrant indicate viable cells (Fig. 4(a) and Fig. 4(b)). The untreated cell is shown in Fig. 4(a). The chloramphenicol-treated cells showed 0.89% of apoptosis (Fig. 3(b)). Treated cells for 24 h incubation time were detected by Annexin V-FITC staining and analysed using flow cytometry to undergo autophagy cell death. The results showed the apoptosis events of the treated cells were at low percentages with 0.10, 0.14, and 0.08% (Fig. 4(c)). The lower percentage of apoptosis in the Gd complex treated cells demonstrated that most of the *Acanthamoeba* cells undergo autophagy instead of apoptosis or necrosis. One of the early events of apoptosis is the alterations of the phospholipids in the plasma membrane of cells. There is no membrane externalisation because the Gd complex entered the cells and interacted directly with the lysosomes leading to autophagic cell death (Fig. 4(c)). The treated cells experienced autophagic cell death rather than apoptosis. The Annexin V-FITC technique was unable to detect autophagic cell death because no exposure of PS occurred. This method is only for the detection of apoptosis and necrosis of cells. Apoptosis was indicated by externalization of PS and annexin V-FITC bind to PS, whereas, autophagy is intercellular cell death, no externalization of PS occurred, and Annexin V-FITC unable to detect due to no exposure of PS. Autophagic cell death was one of the survival cell deaths that degrade the unwanted substances in the cells by engulfment and fused with lysosomes for digestion. Formation of the autophagosome, which is

Table 2. Comparison the cytotoxicity of lanthanide complexes as antiamoebic agent.

No	Name of compound	IC_{50} (μg/mL)	References
1	$Tb(NO_3)_3(OH_2)_3$.(18C6) complex	7	[8]
2	$Tb(NO_3)_3.6H_2O$ salt	2.6	[8]
3	18-Crown-6 molecule	1.2	[8]
4	$[Sm(Pic)_2(EO5)](Pic)$	0.7	[9]
5	$[Sm(Pic)_2(18C6)](Pic)$	6.5	[9]
6	$Ce(NO_3)_3.6H_2O$ salt	3.75	[31]
7	$[Ce(Pic)_2(H_2O)(EO4)](Pic)H_2O$ complex	3	[31]
8	EO4 molecule	0	[31]
9	Fe_3O_4-PEG	4.5	[32]
10	Fe_3O_4-PEG-Dy_2O_3	5.0	[32]
11	$Dy(NO_3)_3.6H_2O$	9.5	[32]
12	$Dy(OH)_3$ Nanoparticles	22.5	[32]
13	$[Gd(Pic)_2(H_2O)(EO3)]^+(Pic)^-.CH_3OH$ complex	23.44	In this study

Note: 18C6 = 18-crown-6, Ce = cerium, Pic = picrate anion, Tb = terbium, Sm = samarium, Gd = gadolinium, Dy = dysprosium, PEG = polyethylene glycol, EO 4 = Tetraethylene glycol EO4

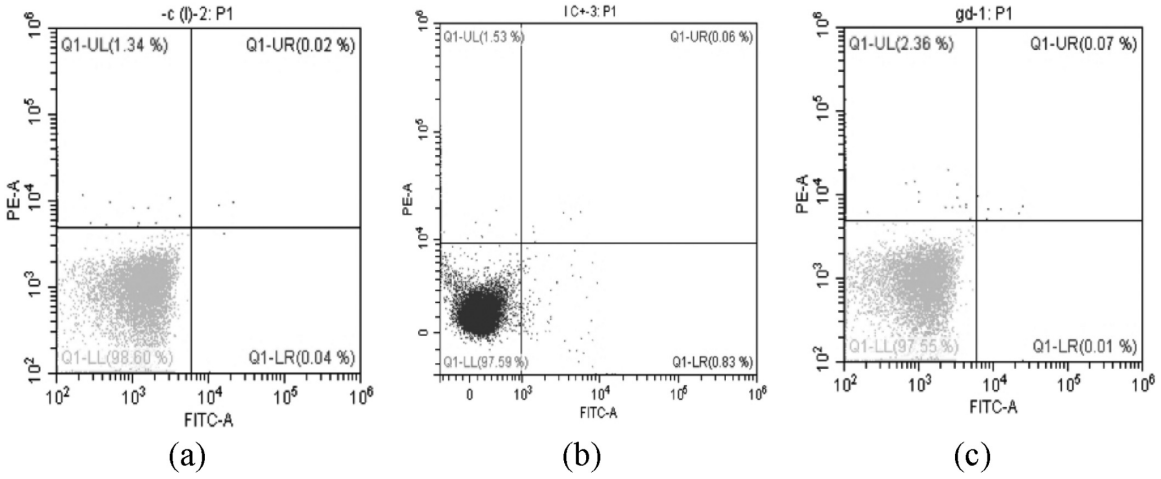

Fig. 4. The effects of exposure time on the apoptosis and necrosis using the Annexin V-FITC and flow cytometry analysis for (a) untreated cells with 0.06% of apoptosis, (b) chloramphenicol-treated cells 0.89% of apoptosis, and (c) treated cells with 0.08% of apoptosis.

isolated from the membrane, usually the endoplasmic reticulum membrane, plays a key role in the engulfment of damaged cells or disturbance substances in cells.

On the other literature, Agha et al. [34] reported the correlation between biodiversity and food security, which an agricultural biodiversity has an immediate impact on economic growth and quality of life. Thus, this system needs a wide variety of plant and animal organisms that can use for better of life and accelerate the societal progress to increase healthy of life and their ecosystem. In addition, our research group are deeply exploring about the potential of lanthanide compounds such as cerium oxide (CeO_2) from Bangka tin slag II as the biofouling and antibacterial agents. The use of lanthanides [35] and transition metal compounds [36] as an antimicrobial agent are of interesting to investigate for many applications in industries, to support for climate change mitigation, and contributing in development of advanced materials.

4. Conclusion

The unique structure of the Gd complex and its performance activity, including biological, medical and drug, are of interesting subject to study. Alterations and modification of morphology of treated *Acanthamoeba* cells from active to inactive stage showed that Gd complex was less-toxic and had potential as an anti-amoebic agent. The flow cytometry result showed a low percentage of apoptosis (0.08%) of the Gd-treated cells, which indicated a mild occurrence of externalization of the internal membrane. This finding confirmed that the series of methods to identify the mechanisms of cell death of *Acanthamoeba* after exposure with the Gd complex is accurate and rapid.

Even though the Annexin V-FITC technique was unable to detect the autophagic cell death, however, this method only detected the apoptosis and necrosis cell modes. Furthermore, studies of autophagic cell death in *Acanthamoeba* sp. are important to deep explore to find a new anti-amoebic drug. The invention of the lanthanide complex as an anti-amoebic agent can contribute to finding the potential drug to treat of AK and also support sustainable development goals, especially good health and well-being in Indonesia and other countries.

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