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# A novel antiamoebic agent against *Acanthamoeba* sp. — A causative agent for eye keratitis infection

Eny Kusrini [a,*], Fatimah Hashim [b], Wan Nor Nadhirah Wan Noor Azmi [b], Nakisah Mat Amin [b], Ari Estuningtyas [c]

[a] Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Kampus Baru UI, 16424, Depok, Indonesia
[b] School of Fundamental Science, Universiti Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia
[c] Faculty of Medical, Universitas Indonesia, Kampus baru UI, Depok 16424, Indonesia

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ABSTRACT

The terbium trinitrate.trihydrate.18-crown ether-6, $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex has been characterized by elemental analysis, photoluminescence and single X-ray diffraction. The $IC_{50}$ values were determined based on MTT assay while light and fluorescence microscopy imaging were employed to evaluate the cellular morphological changes. Alkaline comet assay was performed to analyze the DNA damage. The photoluminescence spectrum of the Tb complex excited at 325 nm displayed seven luminescence peaks corresponding to the $^5D_4 \rightarrow {}^7F_{0,\,1,\,2,\,3,\,4,\,5,\,6}$ transitions. The cytotoxicity and genotoxicity studies indicated that the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex and its salt form as well as the 18C6 molecule have excellent anti-amoebic activity with very low $IC_{50}$ values are 7, 2.6 and 1.2 μg/mL, respectively, with significant decrease ($p < 0.05$) in *Acanthamoeba* viability when the concentration was increased from 0 to 30 μg/mL. The mode of cell death in *Acanthamoeba* cells following treatment with the Tb complex was apoptosis. This is in contrast to the $Tb(NO_3)_3.6H_2O$ salt- and 18C6 molecule-treated *Acanthamoeba*, which exhibited necrotic type cells. The percentage of DNA damage following treatment with all the compounds at the $IC_{25}$ values showed high percentage of type 1 with the % nuclei damage are 14.15 ± 2.4; 46.00 ± 4.2; 36.36 ± 2.4; 45.16 ± 0.6%, respectively for untreated, treated with Tb complex, Tb salt and 18C6 molecule. The work features promising potential of $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex as anti-amoebic agent, representing a therapeutic option for *Acanthamoeba* keratitis infection.



## 1. Introduction

Lanthanide ions exhibit unique spectroscopic and luminescent properties that are useful for bioimaging and bioassay applications. We can modify the physico-chemical characteristics of the lanthanide complexes for certain or more suitable for some applications by complexation method between the lanthanide ions with some ligands. Lanthanide complexes have received considerable attention in recent years as bioimaging and drug delivery system. The activity and toxicity of lanthanide ions have influenced from their efficacy at certain concentration [12]. For the high concentration is most toxic depended on the application or treatment. When lanthanide ions are coordinated with organic ligands, it will produce metal-ion complexes, which have also been used in toxicological studies.

Recently, terbium complexes received increasing attention in therapeutic and medicines fields. It is reported that terbium is able to increase the cytotoxicity of cisplatin as one of the cancer drug in human breast cancer, ovarian cancer, human head and neck cancer cells [1].

In this study, a terbium complex with 18-crown-6 in the presence of nitrate anions was synthesized and analyzed for anti-amoebic activity determination. In this study, we used the 18-crown-6 molecule as ligand because the unique properties of the cyclic molecule and the rigid structure that having six oxygen donor atoms that be able to coordinate with the metal central ions. Ligand is the compound that having donor electron with neutral or negative charge as well as rich electrons. The nitrate anions for charge balancing in the complex and the nitrate anion acts as bidentate mode to coordinate with the central metal ion. The $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex, 18C6 molecule and Tb salt were investigated for their individual cytotoxic and genotoxic potential on *Acanthamoeba* sp. We have successfully synthesized $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex, of which crystal structure is in agreement to that reported by Rogers and dan Rollins [11]. To the best of our knowledge, the study presents the first investigation of the terbium complex as antiamoebic agent, with earlier studies mainly focussed on the novel development of the crystal structure. The motivation for development of the new crystal structure are to obtain the physical, chemical and biological properties are different with the pristine compound or precursor. Moreover, a comprehensive knowledge regarding the cytotoxicity and genotoxicity activities based on morphological changes observation of the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex and its potential

* Corresponding author.
*E-mail address:* ekusrini@che.ui.ac.id (E. Kusrini).

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for metallo-drug discovery to treat *Acanthamoeba* keratitis infection is essential to be studied as well as the 18-crown-6 molecule and $Tb(NO_3)_3.6H_2O$ salt.

Antiamoebic agent to fully treat *Acanthamoeba* keratitis has yet to be discovered with the current available treatments give rise to unwanted side effects [9,10]. This study is important as drugs recommended and used for *Acanthamoeba* keratitis infection treatment such as polyhexamethylene biguanide (0.02%) or chlorhexidine (0.02%) along with a diamidine [4] only show efficacy at early stages of *Acanthamoeba* infection.

Further focus of this study is observation on *Acanthamoeba* sp. following treatment with the $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule at varying concentrations. The study is to determine the effective concentrations of the candidate materials hat are lethal to 50% of the *Acanthamoeba* population. The mode of cytotoxicity of $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule were investigated by MTT assay, in conjunction with observation on the alteration of *Acanthamoeba* cells with light and fluorescence microscopy. Cytotoxicity is a cell death mode occurs in three different events which are apoptosis, necrosis and autophagic cell death and all these events were distinguished by using acridine orange and propidium iodide (AO/PI) staining on the *Acanthamoeba* cells after exposed to all compounds. Observation under fluorescence microscopy indicated that all the compounds tested including the $Tb(NO_3)_3(OH_2)_3.$(18C6) complex induced apoptosis, necrosis and autophagic cell death in the amoeba cells in different treatment. The genotoxicity activity of the $Tb(NO_3)_3(OH_2)_3.$(18C6) complex on the *Acanthamoeba* cell was evaluated by alkaline comet assay and showed the genotoxic effect on the amoeba cells with different types of comet were observed. This study is very important to find the material candidate as antiamoebic agents for treatment of the *Acanthamoeba* keratitis infection.

## 2. Experimental

### 2.1. Preparation of $Tb(NO_3)_3(OH_2)_3.$(18C6) complex

To prepare 1.0 mmol $Tb(NO_3)_3.6H_2O$ and 1 mmol 18C6 is dissolved in $CH_3CN$. The $[Tb(NO_3)_3(OH_2)_3]$.(18C6) complex was isolated after one day with yield 86%. Anal. Calc. For $Tb(NO_3)_3(OH_2)_3.$(18C6): C, 21.71; H, 4.52; N, 6.33. Found: C, 21.82; H, 4.13; N, 6.23%. Decomposition point: 192.2–269.4 °C

### 2.2. PYG Culture media preparation

Protease yeast glucose (PYG) media was prepared by adding 3.75 g Protease, 3.75 g of yeast and 7.5 g $D^+$ glucose in 500 μL of Page's amoeba saline (PAS). Distilled water was added until the volume reached 1000 mL.

#### 2.2.1. Acanthamoeba sp. cultivation

The *Acanthamoeba* sp. (Hospital Kuala Lumpur isolate) was isolated from corneal scrapping of keratitis patient and then were grown axenically in 10 mL of PYG media in T-25 tissue culture flask. The *Acanthamoeba* culture was kept in 30 °C incubator and was subcultured in every 4 days.

#### 2.2.2. Lanthanide stock solution preparation

Each of 1 mg of the $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, Tb salt and 18C6 ligand were dissolved in 20 μL of DMSO, followed by adding PYG media to the final volume of 1000 μL. The final concentration for the stock solution was 1000 μg/mL. Then, the solution was homogenized by vortex and kept at 4 °C prior to use.

### 2.3. $IC_{50}$ value determination by MTT assay

*Acanthamoeba* cells were seeded in a 96-well microplate at $1 \times 10^5$ cells/well and were incubated at 30 °C. After 8 h, the medium was removed and replaced with medium containing the $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, Tb salt and 18C6 ligand over a range of doubling dilutions of 0–30 μg/mL. Triplicate cultures were confirmed for each treatment. Twenty-four hours (24 h) later, 20 μl of 4,5-dimethylthiazol-2-yl-,5 diphenyltetrazolium bromide (MTT) (5 mg/mL) in PBS solution was added to each well and the plate was further incubated for 4 h in $CO_2$ incubators. All the remaining supernatants were removed and 150 μl of DMSO was added to each well and mixed thoroughly to dissolve the formed formazan crystal. After a few minutes of incubation to ensure that all crystals were dissolved, the cytotoxic activity of all the compounds on *Acanthamoeba* cells were examined by measuring the absorbance of each well at 570 nm using microplate reader (Dynatech MR580 microElisa, USA). $IC_{50}$ values represent the test agent concentration that reduced the mean of cell viability of untreated wells to 50%. Assays were repeated to obtain standard deviations (S.D.) and standard error of means (S.E.M.) as well as to produce reproducibility. The independent sample ANOVA as well as Duncan test from SPSS v11.5 Windows statistical package was used at 95% confidence interval (CI) to compare the significant difference between various concentrations in the treatments.

### 2.4. Observations under light microscopy

The amoeba treatments with $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, Tb salt and 18C6 molecule were carried out in 6-well plates containing 3 mL culture medium with $10^4$ cells/ml and $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, Tb salt and 18C6 molecule at their $IC_{50}$ concentration at 30 °C. After 24 h, the effects of all the compounds on the morphology of *Acanthamoeba* sp. were assessed and compared with that observed in control cultures. Observation were made directly from the culture flask using an inverted microscope (Leica Dmire Microscope, Germany) which can be seen with particular alteration on the acanthapodia structure and shape of the cell as well as decreasing cell sizes indicated the changes in internal cell's activity affected by the compounds.

### 2.5. Fluorescence microscopy by acridine orange and propidium iodide staining

The amoeba treatments with $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, Tb salt and 18C6 molecule were carried out in 6-well plates containing 3 mL of culture medium with $10^4$ cells/ml, the $Tb(NO_3)_3(OH_2)_3.$(18C6) complex, Tb salt and 18C6 ligand at their respective $IC_{50}$ concentration at 30 °C. After 24 h incubation time, untreated and all the compounds-treated *Acanthamoeba* were harvested by centrifugation at 3000 rpm for 15 min. The supernatant then were discarded and the pellets were washed once with PBS and recentrifuged at 1000 rpm for 5 min. The pellets obtained were resuspended in 100 μL AO/PI solution. The solution was prepared by adding 2 μL of acridine orange (1 mg/mL) and 2 μL propidium iodide (1 mg/mL) in 996 μL PBS. The cell suspensions were then incubated for 10 min in the dark since both dyes were light sensitive. The *Acanthamoeba* cell suspension was placed onto a slide and was carefully covered with a cover slip. The slide was then viewed under fluorescent microscopy under UV filter (Leica DMire Microscope, Germany).

### 2.6. Analysis of DNA damage by alkaline comet assay

Analysis of DNA damage by alkaline comet assay for *Acanthamoeba* spp. was performed after 2 h treatment in 6-well plate containing $10^4$ of trophozoites per mL and all the compounds at their $IC_{25}$

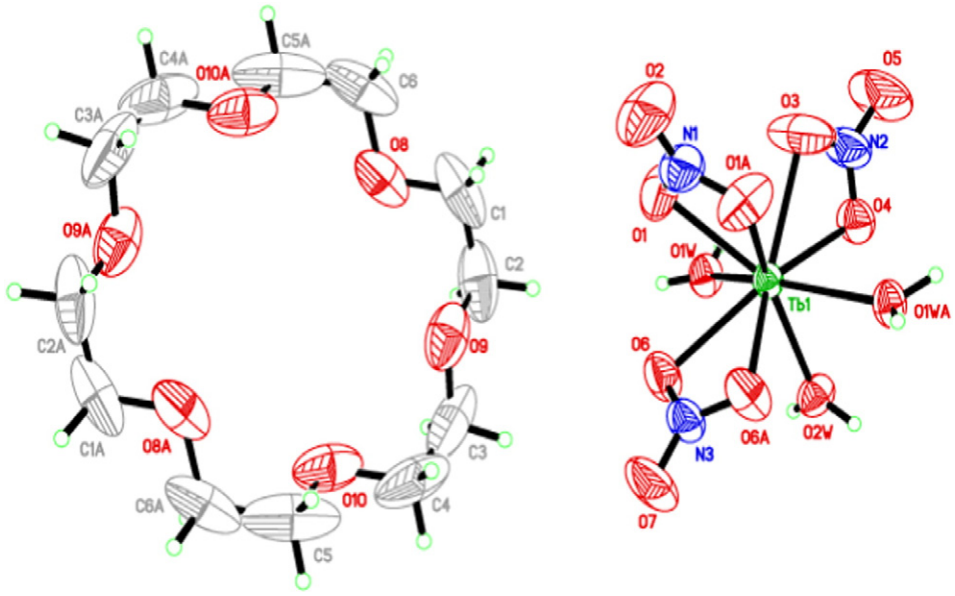


**Fig. 1.** Molecular structure of $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex with 50% thermal-ellipsoids probability showing the numbering scheme of each atoms. The H-atoms is omitted for clarity.

concentration. The alkaline comet assay protocol described by Lah et al. [6] was followed. In this procedure, trophozoites were harvested and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the pellet was washed once with $Ca^{2+}$ and $Mg^{2+}$ free PBS and recentrifuged. Following that, the pellet formed was mixed thoroughly with 80 μl 0.7% low melting agarose (LMA) and spread over the hardened 0.6% normal melting agarose (NMA) that was prepared earlier as the first layer gel on the slide. Cover slip was placed to spread the cell-0.6% LMA suspension and the slide was left on ice to solidify the LMA in approximately 5 min. The cover slip then was removed and the gel was covered with 200 μL of 0.5% LMPA in order to prevent nuclear DNA from escaping during the cell lysing process and electrophoresis. Three layered slides incorporated with cells were first incubated in alkaline lysis buffer for one hour at 4 °C. The slides then were submerged in cold electrophoretic buffer (pH > 13) to unwind the nuclear DNA for another one hour and then subjected to electrophoresis in the same buffer. The electrophoresis was carried out at 1 V/cm and 300 mA, for 5 min. After electrophoresis, the slide was neutralized with 400 nM Tris-HCl (pH 7.5) for three times, five minutes each. The slide then was stained with ethidium bromide (20 μg/mL) and was left overnight at 4 °C before analyzing with fluorescence microscopy equipped with 590 nm filter (Leica Dmire Microscope, Germany). An image of comet tail observed, resulting from the damaged DNA that traveled towards the anode during the electrophoresis. One hundred cells (with three replicates of slides) were viewed, classified and quantified based on Collins [2].

**Table 1**
Crystallographic Data of $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex.

| Parameter | $Tb(NO_3)_3(OH_2)_3.(18C6)$ |
|---|---|
| Empirical formula | $C_{12}H_{30}N_3O_{18}Tb$ |
| Formula weight | 663.31 |
| Crystal system | Orthorombic |
| Space group | Pnma |
| Volume ($Å^3$) | 2415.65(5) |
| Unit cell dimensions (Å, °) | $a = 15.268(2)$, $b = 14.275(2)$, $c = 11.084(1)$, $\alpha = \beta = \gamma = 90$ |
| Z | 4 |
| Density (calculated) ($gcm^{-3}$) | 1.824 |
| Absorption coefficient ($mm^{-1}$) | 3.014 |
| Crystal size (mm) | 0.22 × 0.27 × 0.56 |
| Index ranges | −27 ≤ h ≤ 27<br>−24 ≤ k ≤ 24<br>−19 ≤ l ≤ 19 |
| F(000) | 1328 |
| θ range for data collection, ° | 2.27 − 39.69 |
| Tot., uniq data, R(int) | 57021/7488 [R(int) = 0.030] |
| Observed data [I > 2.0σ(I)] | Least square matrix on $F^2$ |
| Data/restraints/parameter | 7488/0/172 |
| Final R indices [I > 2σ(I)] | R1 = 0.032, wR2 = 0.079 |
| R indices (all data) | R1 = 0.053, wR2 = 0.091 |
| Goodness-of-fit on $F^2$ | 1.005 |
| Largest diff. peak, hole ($eÅ^{-3}$) | 1.027 and −0.913 |

### 2.7. Physical measurements

The elemental analysis was performed with a PerkinElmer 2400 Series II elemental analyzer. Photoluminescence (PL) measurement was made at room temperature with a Jobin Yvon HR800UV system, and the data were collected and processed with LabSpec version 4 software. An HeCd laser was used for excitation at 325 nm, and the emission spectra were scanned from 330 to 1000 nm.

### 2.8. X-ray crystallography

X-ray diffraction data were collected in School of Physics, USM, Malaysia. X-ray data was collected from a single crystal using a Bruker APEX II CCD diffractometer with graphite monochromatic Mo-$K_\alpha$ radiation at a detector distance of 5 cm with APEX2 software [15]. Collected data were reduced by the SAINT program, and the empirical absorption corrections were performed with the SADABS program [15]. The

**Table 2**
Selected bond lengths and bond angles in the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex.

| Atom | Bond lengths (Å) | Atom | Bond angles (°) |
|---|---|---|---|
| Tb1–O1 | 2.412(2) | O1–Tb1–O3 | 51.3(1) |
| Tb1–O3 | 2.445(3) | O5–Tb1–O5A | 51.6(1) |
| Tb1–O5 | 2.429(2) | O6–Tb1–O6A | 52.2(9) |
| Tb1–O1A | 2.429(2) | O1–N1–O3 | 114.6(3) |
| Tb1–O6 | 2.446(2) | O5–N2–O5A | 116.0(3) |
| Tb1–O6A | 2.446(2) | O6–N3–O6A | 115.4(3) |
| Tb1–O2W | 2.393(2) | | |
| Tb1–O1W | 2.371(2) | | |
| tb1–O1WA | 2.371(2) | | |

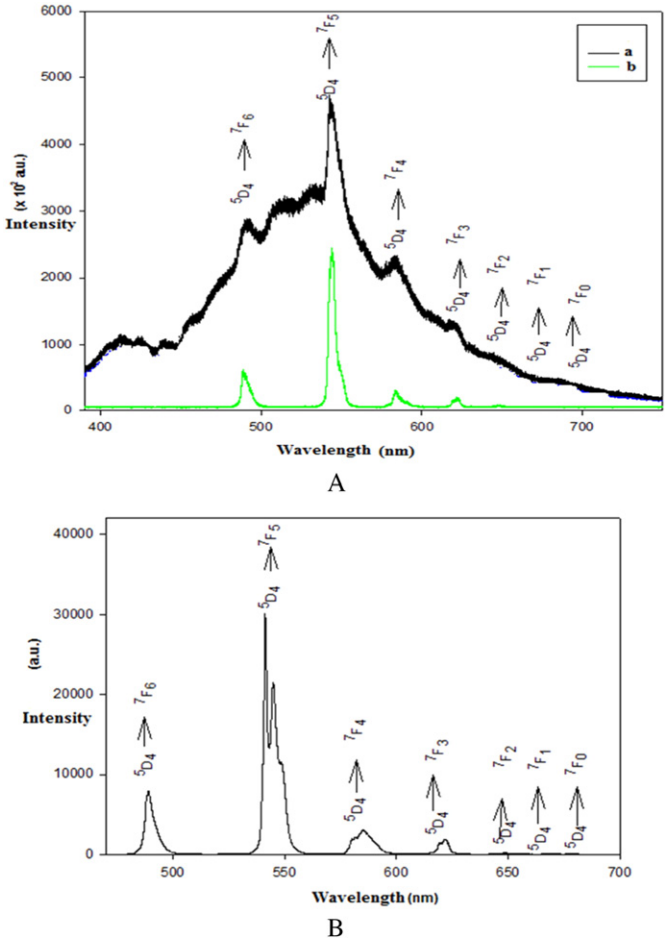


**Fig. 2.** Comparison of photoluminescence spectra of A: (a) $Tb(Pic)_2(EO5).(Pic)$ complex [14], and (b) $Tb(NO_3)_3(OH_2)_3.(18C6)$ (present study); B: $Tb(NO_3)_3.6H_2O$ salt.

structure of the complex was determined by direct methods; it was refined using the full-matrix least-squares method on $F^2$ with the SHELXTL program [16]. All non-hydrogen atoms were refined anisotropically. Hydrogen atoms were placed in calculated positions, and they were refined isotropically in riding model. The final refinement converged well. Data for publication were prepared with SHELXTL [16] and PLATON [17].

## 3. Results and discussion

### 3.1. Characterization of $[Tb(NO_3)_3(OH_2)_3].(18C6)$ complex

The elemental composition and structure properties of terbium trinitrate.trihydrate.18-crown ether-6 complex, $Tb(NO_3)_3(OH_2)_3.(18C6)$, was characterized. The asymmetric unit of $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex contains one terbium atom, three nitrate anions and three water molecules. Another one of the stand-alone 18C6 molecule was generated by the inversion center symmetry operation. The terbium ($Tb^{3+}$) ion was coordinated by six oxygen atoms from three of nitrate anions in bidentate mode, and three oxygen atoms from three water molecules, forming a nine-coordination number (Fig. 1). The 18-crown-6 molecule as solvated molecule, it is not acting as a ligand in this $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex. The average bond lengths of Tb-$O_{nitrato}$ and Tb–$O_{water}$ were about 2.435(2) and 2.378(2)Å, respectively. The coordination geometry of the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex was monocap square antiprismatic with the O1 atom in capping position. This structure of $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex is similar with the structure has been previously reported by [11]. Crystallographic data of the complex studied is shown in Table 1. Selected bond lengths and bond angles of the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex is listed in Table 2.

The rare earth metal ion ($Tb^{3+}$) did not coordinated with 18-crown-6 molecule. The $Tb^{3+}$ ion did not coordinate to the 18C6 molecule because the competition between the nitrato anions and water molecules. In addition to that, we assumed that the 18C6 molecule is rigid and cyclic molecule, thus the $Tb^{3+}$ ion as a heavy metal ions give rise that it cannot encapsulate and coordinate to the oxygen atom from the 18C6 molecule. The torsion angles C–O–C–C for the Tb complex is all in anti conformation with average angle of 174.4(3)°, while the torsion angles O–C–C–O referring to the *gauche* conformation by a pattern take turns ±g with average angle is more than 60°, that is 68.9(5)°.

### 3.2. Photoluminescence studies of $[Tb(NO_3)_3(OH_2)_3].(18C6)$ complex

The photoluminescence spectrum of the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex in solid state is shown in Fig. 2. The emission spectrum of the complex excited at 325 nm displayed seven peaks sharp luminescence corresponding to the $^5D_4 \rightarrow {}^7F_{0,\,1,\,2,\,3,\,4,\,5,\,6}$ transitions (see Table 3). From the Fig. 2a and b, we observed the $^5D_4 \rightarrow {}^7F_5$ transition is

**Table 3**
Photoluminescence data of the $[Tb(NO_3)_3(OH_2)_3].(18C6)$ complex in solid state.

| Emission transition | Compounds | | | | | |
|---|---|---|---|---|---|---|
| | $Tb(Pic)_2(EO5).(Pic)$ complex [14] | | $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex | | $Tb(NO_3)_3.6H_2O$ salt | |
| | λ (nm) | Intensity × $10^2$ (a.u.) | λ (nm) | Intensity × $10^2$ (a.u.) | λ (nm) | Intensity (a.u.) |
| 18C6 molecule | 414.7 | 1123 | – | – | – | – |
| $^5D_4 \rightarrow {}^7F_6$ | 493.1 | 2873.6 | 488.9 | 596 | 489.4 | 7739 |
| | – | – | 490.2 | 570 | – | – |
| $^5D_4 \rightarrow {}^7F_5$ | 542.9 | 4692 | 542.5 | 2140 | 541.4 | 29868.2 |
| | – | – | 544.2 | 2427 | 545.1 | 21307 |
| | – | – | 549.1 | 666.6 | 548.8 | 11413.4 |
| $^5D_4 \rightarrow {}^7F_4$ | 584.3 | 2335 | 584.1 | 297 | 581.5 | 2137.2 |
| | – | – | 590.9 | 121 | 582.5 | 2029.2 |
| | – | – | – | – | 585.9 | 2908 |
| $^5D_4 \rightarrow {}^7F_3$ | 620.4 | 1350 | 619.5 | 166 | 619.4 | 1491 |
| | – | – | 621.3 | 209 | 621.7 | 1930 |
| | – | – | 623.3 | 186 | – | – |
| $^5D_4 \rightarrow {}^7F_2$ | 645.5 | 841 | 647.2 | 88 | 649.5 | 191.2 |
| | – | – | 649.0 | 87 | – | – |
| $^5D_4 \rightarrow {}^7F_1$ | – | – | 669.4 | 68 | 669.5 | 77 |
| $^5D_4 \rightarrow {}^7F_0$ | 687.1 | 468 | 682.7 | 69 | 679.2 | 76.8 |

hypersensitive peak at 542 nm regarding in green emission. These luminescence characteristics $Tb^{3+}$ ions are often used as a luminescent reagent and label in various biological activity [18,19]. So, the $Tb(NO_3)_3(OH_2)_3$.(18C6) complex is used as reagent for biological activity as anti-amoebic. Comparison of photoluminescence properties of the $Tb(Pic)_2(EO5).(Pic)$ complex [14], and $Tb(NO_3)_3(OH_2)_3$.(18C6) complex is shown in Fig. 2a. In this $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, the 18-crown-6 molecule was not coordinated to the $Tb^{3+}$ ion in the inner-coordination sphere, thus the presence of this crown molecule is not significantly effect, even though the intensity for the hypersensitive peak in the $Tb(NO_3)_3(OH_2)_3$.(18C6) complex is higher than those found in its salt (see Fig. 2b and Table 3). The $^5D_4 \rightarrow {}^7F_6$, $^5D_4 \rightarrow {}^7F_4$ and $^5D_4 \rightarrow {}^7F_2$ transitions are sensitive to the environment around the $Tb^{3+}$ ion. While, the $^5D_4 \rightarrow {}^7F_5$, $^5D_4 \rightarrow {}^7F_4$ and $^5D_4 \rightarrow {}^7F_3$ transitions having a role in optic activity.

### 3.3. Acanthamoeba cell viability and $IC_{50}$ determination using MTT assay

Optimization of MTT assay method on *Acanthamoeba* cells was performed in order to determine the appropriate number of *Acanthamoeba* cells needed in each well to react with MTT reagents for 4 h in 96-well plate. This duration of treatment were chosen due according to population doubling time of this isolate of *Acanthamoeba* sp. that took 8 h for population doubling time. The MTT assay used in this study was modified some steps regarding the previous method reported by Mossman [7] that rinses for each well of 96-well plate for two times with PBS to remove all the media. Because the MTT reagent is sensitive to glucose and it is the main components in *Acanthamoeba* sp. PYG culture media. For this study, $1 \times 10^4$ of *Acanthamoeba* cells were seeded in 96-well plate and followed for further treatments with all the compounds. The $IC_{50}$ were determined based on the cell viability versus concentration after treatment (see Fig. 3a, b and c).

All the compounds inhibit the growth of *Acanthamoeba* sp. as shown by the viability of cell decrease with increasing concentration for all of the compounds. The $IC_{50}$ values derived from the plotted graphs were 7, 2.6 and 1.2 μg/mL for $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, Tb salt and 18C6 molecule, respectively. The $IC_{50}$ value obtained from this study is classified as very low with $IC_{50}$ value is less than 30 μg/mL [20]. This $IC_{50}$ value is acceptable and considered very active against *Acanthamoeba* cells. Synthesized $Tb(NO_3)_3(OH_2)_3$.(18C6) complex with the 18C6 molecule exhibit higher $IC_{50}$ value compared to the $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule. This indicated that the combination between the $Tb^{3+}$ ions and 18C6 molecule would increase the volume of compound in order to inhibit the growth of *Acanthamoeba* cells. It has been proved that only 18C6 molecule and $Tb(NO_3)_3.6H_2O$ salt have highly activity of water interaction. The stability of the complex that decrease the 18C6 molecule and protein receptor interaction on the membrane of amoeba, thus the 18C6 molecule and $Tb(NO_3)_3.6H_2O$ salt which have more freely electron donor and electron acceptor that create higher activity towards *Acanthamoeba* cells even though at very low concentration. This observation also has been reported in antimicrobial activity as it is influenced by the nature of the ligand, the total charge of the complex, the nature of counter ion in case of the ionic complex and the nuclearity of the central metal ion [21].

Further study, the $Tb(NO_3)_3.6H_2O$ salt or 18C6 molecule can be combined with the other ligand or salt that might also would introduce an active effect on pathogenic microorganisms. It is believed that, the relationship between a stability of structure and toxicity of compound could ensure the compound to perform its toxin effects much better than less stable structures. However, based on the $IC_{50}$ value obtained, the complexation between 18-crown-6 molecule and $Tb(NO_3)_3.6H_2O$ salt reduced the toxicity of the $Tb(NO_3)_3.6H_2O$ salt which are contradict result was found in this macrocylic compound when it bind with potassium (K) [22]. We suggested that the chemical, physical and biological properties of $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, and 18-crown-6 molecule are different due to the presence of free electron donor and electron acceptor, solvated or coordinated water, primary sphere coordination, secondary sphere coordination, the presence or position of the ligand in the coordination sphere as well as the coordinated metal ion that may varies the result in this study.

### 3.4. Light microscopy observation

The treated and untreated *Acanthamoeba* cells by using the $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule were observed under inverted microscope using phase contrast filter. These indicated that after treatment with all the compounds, different morphology of amoeba cells were observed and compared with the untreated *Acanthamoeba*. Incubation of untreated *Acanthamoeba* demonstrated irregular shape of *Acanthamoeba* trophozoite with numerous acanthapodia on the surface of *Acanthamoeba* for 24 h (see Fig. 4a). *Acanthamoeba* exposed to the $[Tb(NO_3)_3(OH_2)_3]$.(18C6) complex, Tb salt and 18C6 molecule underwent encystment process with

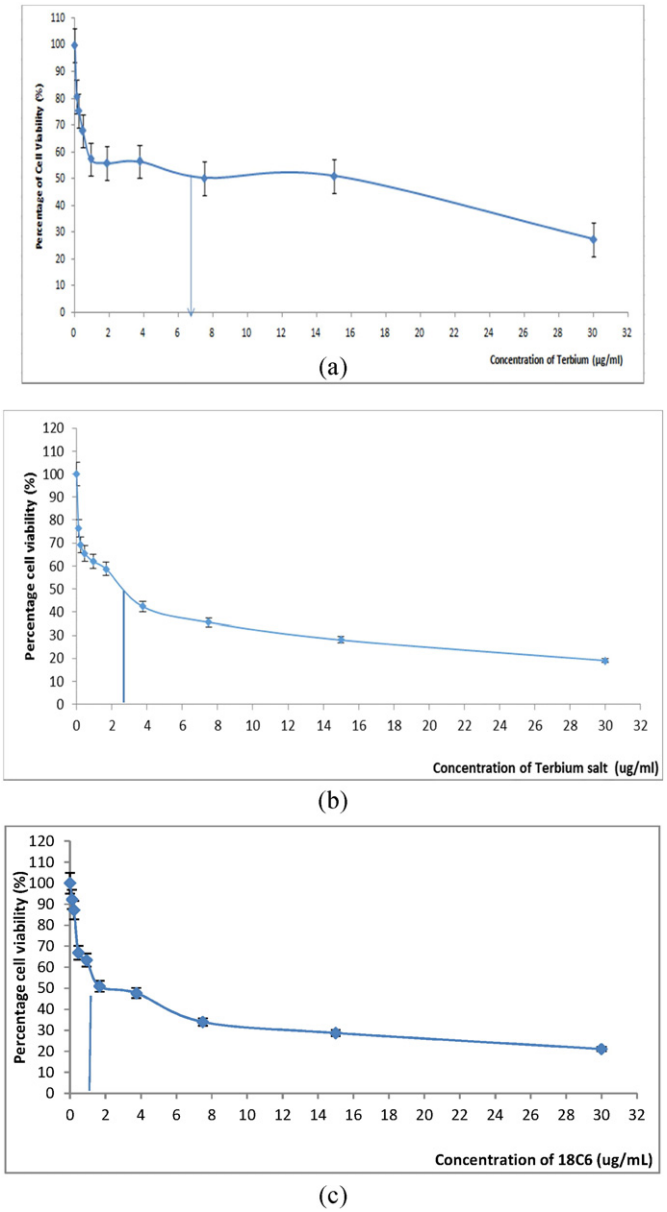


**Fig. 3.** (a) Cytotoxicity of $Tb(NO_3)_3(OH_2)_3$.(18C6), (b) Tb Salt and (c) 18C6 molecule on *Acanthamoeba* sp. after 24 h treatment, assessed based on percentage of cell viability by MTT assay. The data represent the mean (± S.E.M.) of three separates experiments.

loss of acanthapodia structure (see Fig. 4b, c and d). In this study, all of the compounds are able to modify the shape of *Acanthamoeba* sp. and alteration towards the amoeba morphology.

Acanthapodia structure is very important in *Acanthamoeba* pathogenesis since this structure are used to capture prey, attach to any substance including animal tissue and any surface for its survival [5]. The ability of $Tb(NO_3)_3(OH_2)_3$.(18C6) complex to change the surface morphology of *Acanthamoeba* cell structures contribute to the alteration of the amoeba, thus decrease the pathogenic potential of this genus of protozoa during infection. The accumulation of dark precipitates in the treated *Acanthamoeba* cells cytoplasm were observed. Because of the deformation of organelles and ruptured of vacuoles. Based on the morphological changes of treated *Acanthamoeba* cells, it indicated that the treatments given by using $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule have significant inhibition affects towards active stage of *Acanthamoeba* cells.

### 3.5. Mode of cell death in Acanthamoeba cells determination by fluorescence microscopy observation

In mammalian cell systems, apoptosis cell death mode has been described based on morphological and biochemical criteria [13]. Apoptosis is a potent mechanism to remove unicellular parasitized cells from the host as this process is also essential in multicellular organisms for the development, homeostasis and defense systems. The internal alteration of morphology of *Acanthamoeba* cells after treatment with $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, Tb salt and 18C6 molecule cannot be confirmed only based on observation by light microscopy. Therefore, observation by fluorescence microscopy and two fluorescence dyes involved acridine orange (AO) and propidium iodide (PI) were conducted to discriminate the mode of cell death induced by the all of the compounds (see in Fig. 5a, b, c and d).

Based on the Fig. 5(a), untreated *Acanthamoeba* showed green and intact nucleus and cell membrane indicated healthy and viable *Acanthamoeba* cells. However, the treated *Acanthamoeba* sp. by using the $Tb(NO_3)_3(OH_2)_3$.(18C6) complex, Tb salt and 18C6 molecule demonstrated alteration towards the internal organelle of *Acanthamoeba* cells as well as its membrane integrity. *Acanthamoeba* treated with $Tb(NO_3)_3(OH_2)_3$.(18C6) complex appear with green cytoplasm and orange granules dispersed in the cytoplasm (Fig. 5(b)). It is suggested that the internal alteration was due to apoptosis event. Apoptosis can be seen in *Acanthamoeba* cells by the condensation of cytoplasm and chromatin, cell shrinkage and smaller in size of *Acanthamoeba* cells [8]. Meanwhile, the autophagic activity of lysosome in *Acanthamoeba* cells was also observed. Yellow–orange granules in the range 0.1–0.2 μm lysosomes in treated *Acanthamoeba* cells were resulted from the uptake of AO dye by the organelle. Expansion of lysosomes was actually resulted from sequestration and digestion of macromolecules of cytoplasmic material and cell organelles in the autophagy process.

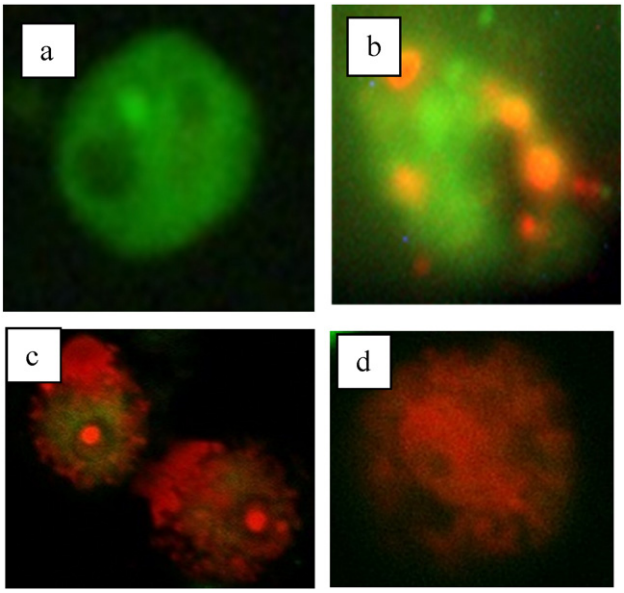


**Fig. 5.** *Acanthamoeba* sp. stain with AO and PI dyes: (a) Untreated cells; (b) Cells treated with the $[Tb(NO_3)_3(OH_2)_3]$.(18C6) complex (c) Acanthamoeba incubated with 18C6 ligand and (d) with Tb salt. Both (c) and (d) images showed necrotic type of *Acanthamoeba* cells. (Magnification was 400×).

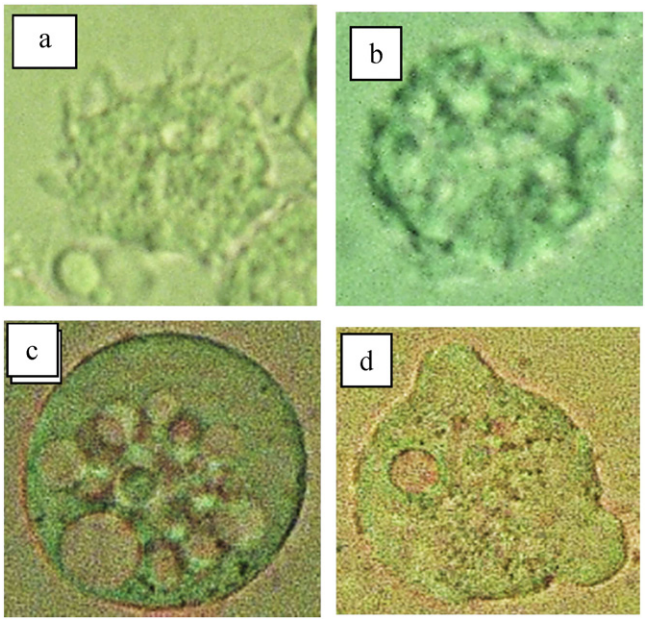


**Fig. 4.** The *Acanthamoeba* sp. under observed under phase contrast light microscopy: (a) untreated *Acanthamoeba* cells with numerous acanthapodia and vacuole. (b) *Acanthamoeba* cells treated with the $Tb(NO_3)_3(OH_2)_3$.(18C6) complex with rounded form of amoeba cell and loss of acanthapodia structure, (c) and (d) *Acanthamoeba* cells treated with 18C6 molecule and Tb salt appear with large vacuole and loss of acanthaphodia structure. Magnification was 400×.

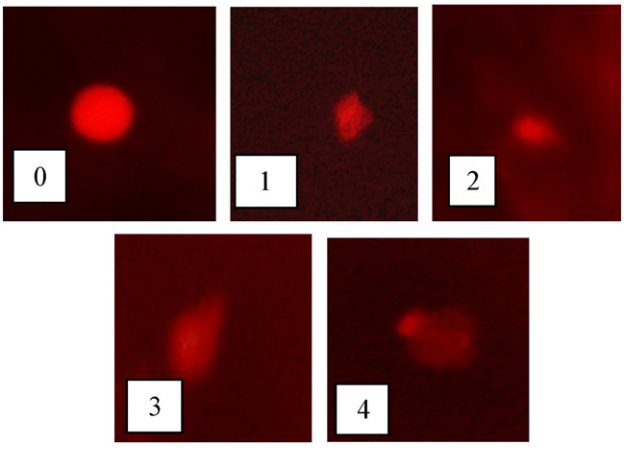


**Fig. 6.** Photomicrographs of single cell gel electrophoresis (SCGE) on *Acanthamoeba* sp. showing type 0 to 4 stage of DNA damage. Type 1 with less than 25% DNA at tail and Type 2, the proportion of DNA at tail is between 25 and 50% while Type 3, DNA at tail is between 50% and 75%. Type 4, the proportion of DNA is more than 75% of DNA is at tail. Dye: ethidium bromide. (Magnification was 200×).

**Table 4**
The percentage of type of comet observed microscopically in *Acanthamoeba* spp. treated with Tb complex, Tb salt and 18C6 molecule.

| Treatment | % of nuclei | | | | |
|---|---|---|---|---|---|
| | Type 0 | Type 1 | Type 2 | Type 3 | Type 4 |
| Untreated | 84.91 ± 0.9 | 14.15 ± 2.4 | 0.94 ± 1.5 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| $[Tb(NO_3)_3(OH_2)_3].(18C6)$ | 36.00 ± 4.5 | 46.00 ± 4.2 | 14.00 ± 3.7 | 4.00 ± 4.24 | 4.00 ± 0.75 |
| $[Tb(NO_3)_3.6H_2O]$ salt | 56.06 ± 1.1 | 36.36 ± 2.4 | 7.58 ± 2.0 | 0.00 ± 0.00 | 0.00 ± 0.00 |
| 18C6 molecule | 37.10 ± 1.3 | 45.16 ± 0.6 | 11.29 ± 0.8 | 6.45 ± 1.32 | 0.00 ± 0.00 |

The AO uptake was the result of an active proton pump in lysosomes; the high proton concentration (low pH) caused AO dye, which could enter the lysosome in uncharged form, to become protonated and thus entrapped in the organelle when viewed under fluorescence microscope. The orange precipitates are due to the high activity of hydrolytic enzymes of lysosomes which caused the pH inside the lysosomes is low and the self-digestion process is finally results in cell death. This event was absent in untreated trophozoites. No autophagy process occurred in the amoeba because of the protonated-orange-lysosomes in these *Acanthamoeba* was not observed.

As we know that the AO dye is permeable fluorescence dye and able to enter intact plasma membrane of *Acanthamoeba* cells [3]. This dye also is able to bind to nucleic acids and it is green fluorescence when intercalate into double strand break of DNA and intact DNA, but it fluorescence in red when bind to single strand break of DNA. In addition, the PI dye only can enter damage plasma membrane or by dead cells which forming red or orange fluorescence cell. Healthy cells remain green due to the intact membrane and do not allow the PI dye entering the *Acanthamoeba* cells. In this study, nucleus for all the compounds-treated cells also becomes small compared to untreated cell of *Acanthamoeba*. These indicated that the *Acanthamoeba* sp. treated with all the compounds were unable to maintain its homeostasis because all of the compounds probable inhibit the cellular activity for cells growth and moving process.

Most of the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex treated cells showed green-yellow and bright-orange nuclei (see Fig. 5(b)). According to Fatimah et al. [3] cells damaged with yellow nuclei fluorescence indicated early apoptosis type of *Acanthamoeba* cells while cells that fluorescence with bright orange nuclei showed late apoptosis type of cells. It is suggested that the different fluorescence given by the nuclei cells was due to the different in pH of the nuclei where the amount of $H^+$ in late apoptosis were higher compared to early apoptosis. Basically, DNA structures in the nuclei were hold by the hydrogen bonds and when there were damaged occurred or breaks in the backbone of DNA structures (single strand breaks) it cause the high accumulation of $H^+$ which then made the condition to become acidic that eventually causing AO to stained the nuclei with yellow and bright orange fluorescence and therefore, not fluoresced green as can be seen in the untreated *Acanthamoeba* cells. The necrosis type of cells which fluorescent in the red-orange nuclei by using treated *Acanthamoeba* cells with 18C6 molecule and Tb salt (see Fig. 5(c) and (d)). Necrosis type of cells indicated the completely damaged of cells that is more serious than apoptosis which was considered as unnatural death process of cells and this form of cell death were stained by the propidium iodide (PI) dye.

PI is a dye that impermeable to intact membranes but readily penetrates the membrane of non-viable cells and binds to DNA or RNA, causing orange or red fluorescence. Therefore, by using these two dyes simultaneously, the mode of cell death of *Acanthamoeba* cells after exposed to Tb complex, 18-crown-6 molecule and Tb salt could be determined and distinguished based on the fluorescence results. In this study, the mode of cell death observation indicated that apoptosis phenomena which are important in disease control and treatment as compared to necrosis type of cell death only occurred after the *Acanthamoeba* cells were treated with $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex. Because of the stability of the $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex during interaction with the *Acanthamoeba* biological system such as its death receptor on the cell membrane that this complex able to induce *Acanthamoeba* cell suicide.

### 3.6. Genotoxicity or DNA damage analysis using alkaline comet assay

Among the techniques available that have been established for detecting the effects of genotoxins on DNA is comet assay or single cell gel electrophoresis, a sensitive, simple, economic and reliable method that has been developed by Singh et al. [23]. This method was later adapted to use for unicellular eukaryotic cells, specifically for *Tetrahymena thermophila* and yeast, *Saccharomyces cerevisiae* [6]. This technique was performed widely in genetic toxicology, radiation biology and medical as well as environmental research for the detection of single-strand breaks, double strand breaks and alkali-labile sites in DNA and also able to visualize DNA degradation due to necrosis or apoptosis.

Genotoxicity activity on *Acanthamoeba* sp. after treated with the $[Tb(NO_3)_3(OH_2)_3].(18C6)$ complex, Tb salt and 18C6 molecule were performed by using alkaline comet assay. This technique involved a separation of two DNA strands by alkaline denaturation which is similar to alkaline unwinding, alkaline elution and alkaline sucrose sedimentation. The use of high concentration of alkaline solution does not affect the DNA damage. It only makes the tail more clear and increase the resolving power of the assay when viewed under fluorescent microscopy at 20× magnification equipped with an excitation filter of 515–560 nm and barrier filter of 590 nm. A total of 100 cells with minimum three replicates were scored for each treatment of compounds on *Acanthamoeba* cells. The comet formed from the nuclei then was observed using fluorescence microscope and there are five types of comet from 0 to 4, where zero (0) indicates no tails form and 4 indicate almost DNA lyse and form tail [2]. The comets formed after observation under fluorescence microscopy are shown in Fig. 6.

The percentage number of DNA damage after treated with $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex at $IC_{25}$ for each compound was presented in Table 4. Among types are different distribution based on the statistical analysis with $p > 0.05$ by using Kruskall–Wallis test. The percentage of nuclei damage with type 0 for untreated *Acanthamoeba* was 84.91 ± 0.9%, while *Acanthamoeba* treated with Tb complex, $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule are 36.00 ± 4.5, 56.06 ± 1.1 and 37.10 ± 1.3%, respectively. In the present study, the comet type 0 for untreated *Acanthamoeba* population are obtained. Increasing the tail length or percentage of DNA at tail were observed after treatment for all of the compounds (see Table 4). For alkaline comet assay, the percentage of viable cells at 75% and above was recommended to be used in order to avoid the occurrence of false positive as previously suggested by Henderson et al. [24]. However, Collins [2] concluded that the satisfactory condition of cells for comet assay analysis in untreated cells should give comets background level of breaks mostly type 0 or around 10% DNA in the tail.

Low DNA damage after two hour exposure time by using 18C6 molecule. The DNA was stained with ethidium bromide, which expanded the unwind single-strand DNA [2]. The $Tb(NO_3)_3(OH_2)_3.(18C6)$ complex showed a potent genotoxic activity on *Acanthamoeba* sp. because it can alter the nucleus structure of *Acanthamoeba*. These results

supported the morphological observation under fluorescent microscopy observation when interaction of the $Tb(NO_3)_3.6H_2O$ salt and 18-crown-6 molecule are minimal after two hour incubation for alkaline comet assay as it only produced low level of DNA damage.

No endogenous damage, such as attack by reactive oxygen species produced from normal metabolic products by all of the compounds, especially the process of oxidative deamination or endogenous cellular processes; oxidation, alkylation, hydrolysis. The DNA damage for $Tb(NO_3)_3(OH_2)_3.$(18C6) complex is higher than those found in the treatment by $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule. These indicated that the complexation with terbium ion are very potential for application in metal-based drugs. The given data are promising and significant contributed for biological activity as antiamoebic agent.

## 4. Conclusion

The $Tb(NO_3)_3(OH_2)_3.$(18C6) complex has been synthesized and characterization for cytotoxic and genotoxic activities on *Acanthamoeba* sp. The $^5D_4 \rightarrow {}^7F_5$ transition is hypersensitive peak at 542 nm regarding in green emission was observed in the Tb complex. The DNA damage for $Tb(NO_3)_3(OH_2)_3.$(18C6) complex is higher than those found in the treatment by $Tb(NO_3)_3.6H_2O$ salt and 18C6 molecule. We identified the potential of $[Tb(NO_3)_3(OH_2)_3].$(18C6) complex as anti-amoebic agent for *Acanthamoeba* sp., representing a therapeutic option and potent for treatment of *Acanthamoeba* keratitis infection.

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## Appendix A. Supplementary data

Crystallographic data for the structural analyses of the complex was deposited with the Cambridge Crystallographic Data Center, CCDC 1405494. Copies of this information may be obtained free of charge from: The Director, CCDC, 12 Union Road, Cambridge, CB2 1EZ, UK (Fax: +44-1223-336-033; e-mail: deposit@ccdc.cam.ac.uk or www: http://www.ccdc.cam.ac.uk).

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