A COMPREHENSIVE STUDY ON THE EFFECT OF LANTHANIDE COMPLEXES ON *ACANTHAMOEBA* SP.

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INTRODUCTION

BACKGROUND OF STUDY

- *Acanthamoeba* is a free-living protozoa that can cause *Acanthamoeba* Keratitis (AK).
- Affect Contact lense wearers and non-contact lense wearers.
- Reported cases on non-contact lense wearers had been diagnosed as AK patients in Malaysia (Faridah *et al.*, 2005)
- Late treatment ⇨ Corneal transplant ⇨ Blindness

- **Current antiseptics caused side effects on human.**

- ***Acanthamoeba* will be exposed to different lanthanides complexes which are Praseodymium (Pr), Neodymium (Nd), Gadolinium(Gd), , Cerium (Ce), Dysprodium (Dy) and Samarium (Sm) with E03, EO4, EO5, and 18C6 ligands.**

PROBLEM STATEMENT

Different effect of lanthanides metal and ligands on *Acanthamoeba* sp. (Kusrini *et al.* , 2016)

Potential of Ln not yet being studied.

Different positions of lanthanides have different toxicity level.

SIGNIFICANCE OF STUDY

- **Provide knowledge and proved of suitable lanthanide complexes as antiamoebic agents with appropriate mode of cell death.**
- **Potential as effective treatment against AK.**

Presentation outline

I. Different effects of lanthanide with different ligand/chelating agent on *Acanthamoeba* sp.

II. Computational model of different ligands of Samarium (Sm) with EO5 or 18C6 interaction with profilin 1B

III. Different mode of cell death of *Acanthamoeba* sp. when exposed to different lanthanide

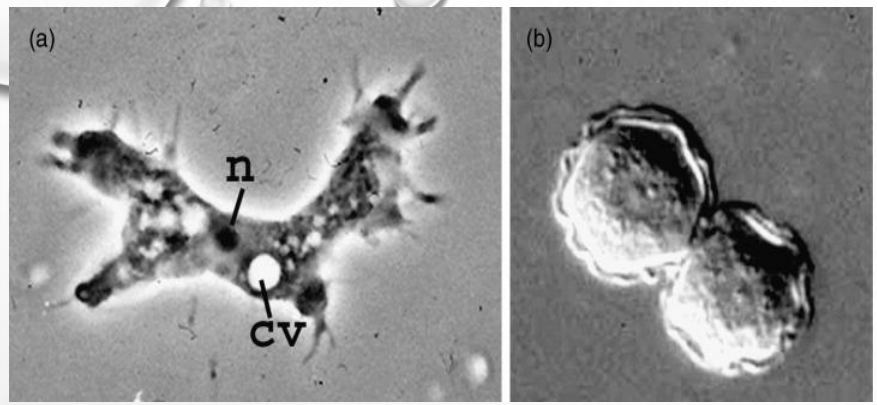
IV. level of cytoxicity of lanthanides to *Acanthamoeba* is inflected by their position in the periodic table

LITERATURE REVIEW

BACKGROUND OF STUDY

Acanthameoba sp.

- **Free-living and opportunist protozoa.**
- **Present in diverse habitat such as soils, lakes, surgical instruments.**
- **Life cycles of *Acanthamoeba***
  - **Trophozoite**
  - **Cyst**
- **Trophozoite stage can cause infections (Trevisan, 2010).**

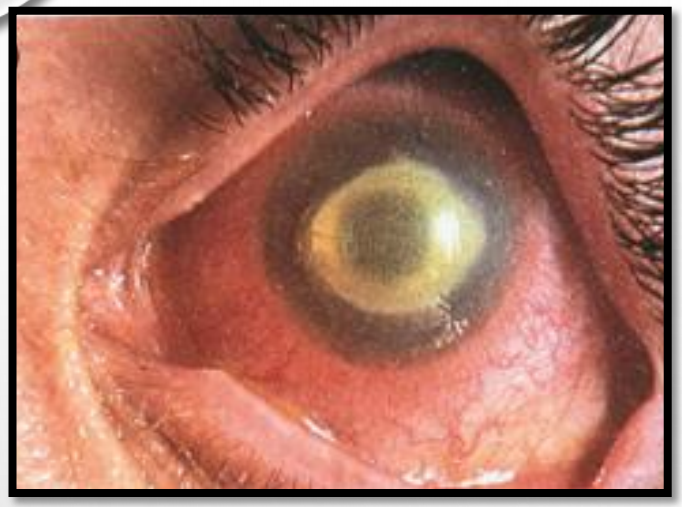


(a) The trophozoite form of *Acanthamoeba* sp. in (b) cyst form of *Acanthamoeba* sp. (n, nucleus; cv, contractile vacuole) (Visvesvara *et al.*, 2007).

	TROPHOZOITE	CYST
Activation level	Active	Dormant
Conditions	Favourable - Enough food supply - Suitable pH	Unfavourable - Food distress - High temperature
Morphological Changes	Existence of acanthapodia	Two layers created - Endocyst - Exocyst
Shape	Irregular	Round

Acanthamoeba Keratitis

- **Serious eye infections that can affect both contact lenses and non contact lens wearers.**
- **Causes**
  - **Poor personal hygiene**
  - **Wear contact lenses for long periods of time**
  - **Exposed to contaminated water (Khan, 2006)**
- **Most of cases AK related to inappropriate ways during cleaning the lense and contamination with bacteria and amoeba (Green et al, 1987)**
- **Invade the cornea.**



Acanthamoeba keratitis (Visvesvara *et al.*, 2007)

Characteristics (CDC, 2013)

- Eye pain
- Eye redness
- Vision become unclear
- Sensitive to light

Lanthanide

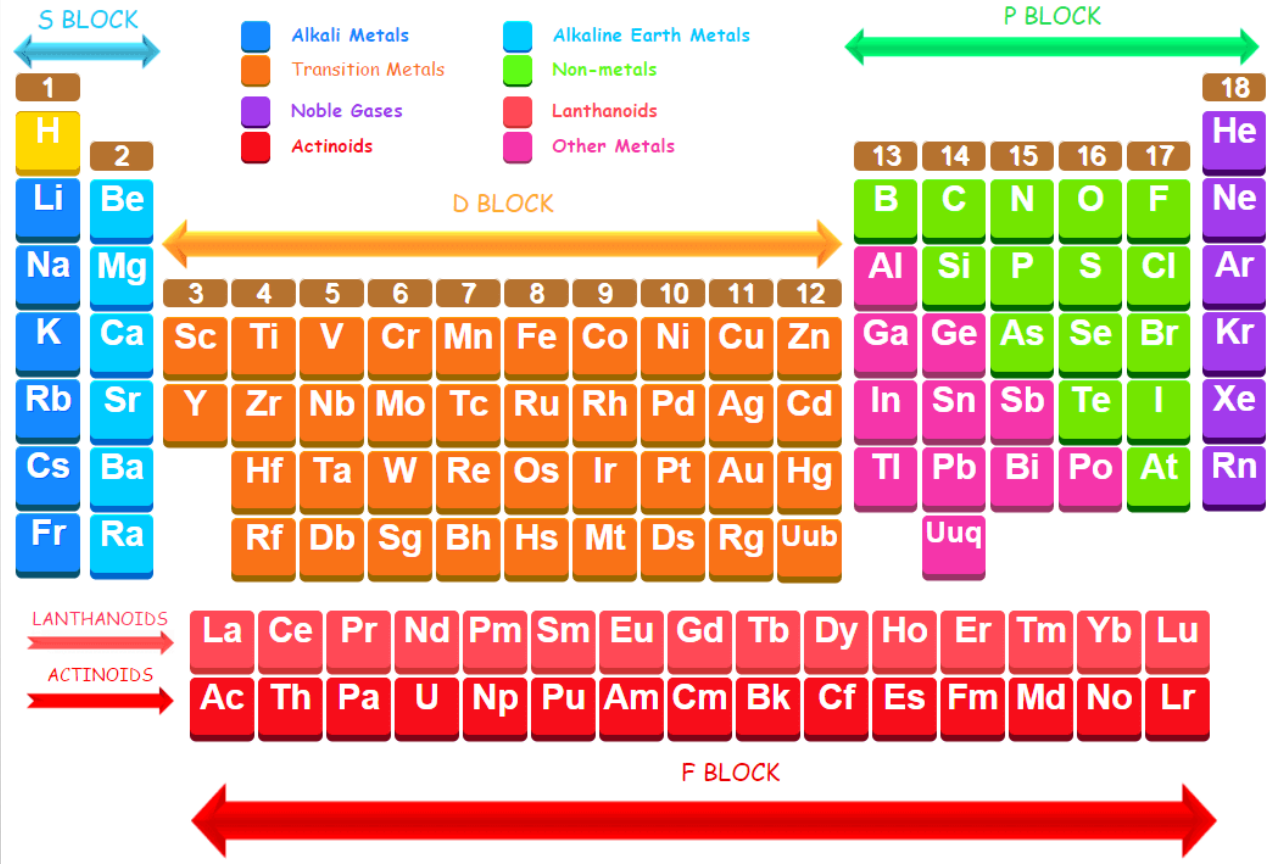
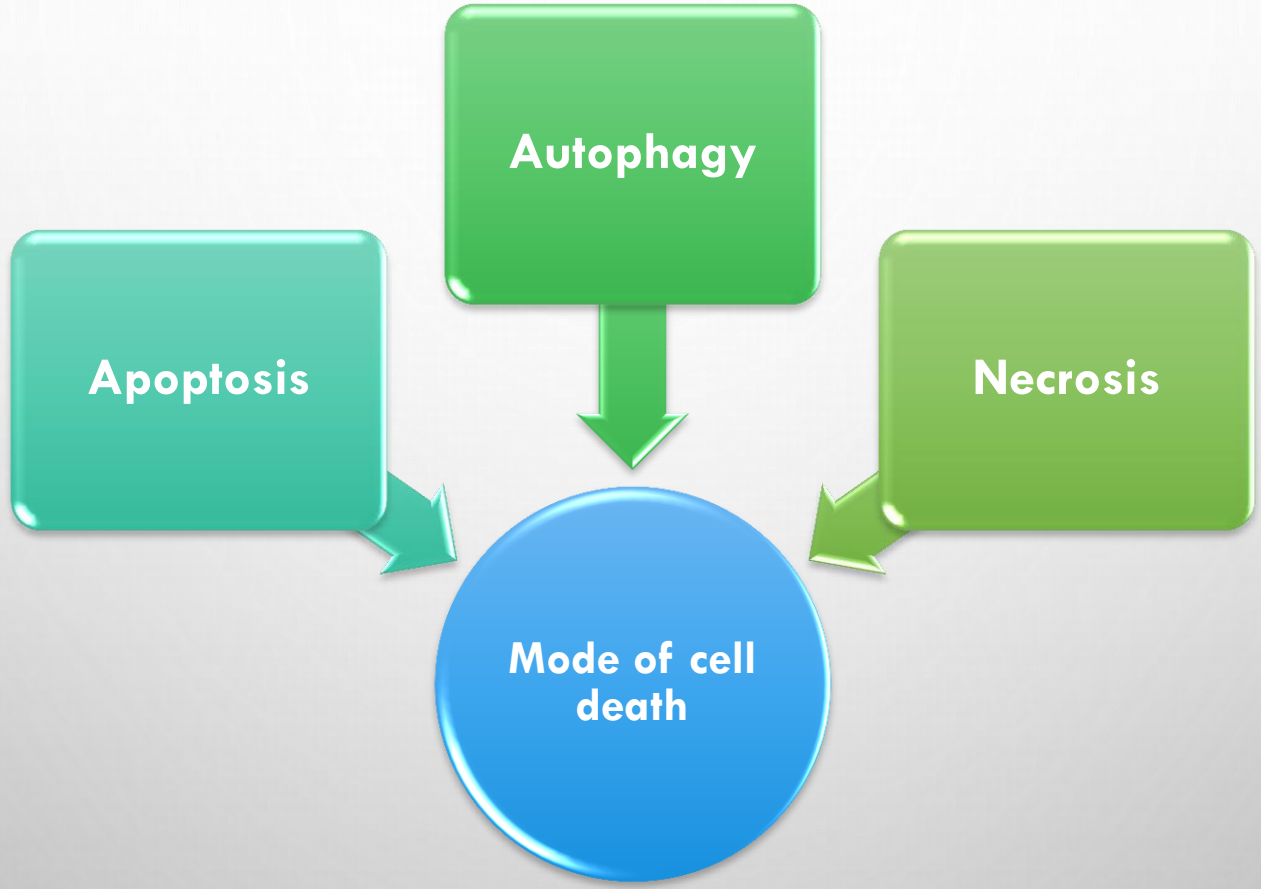
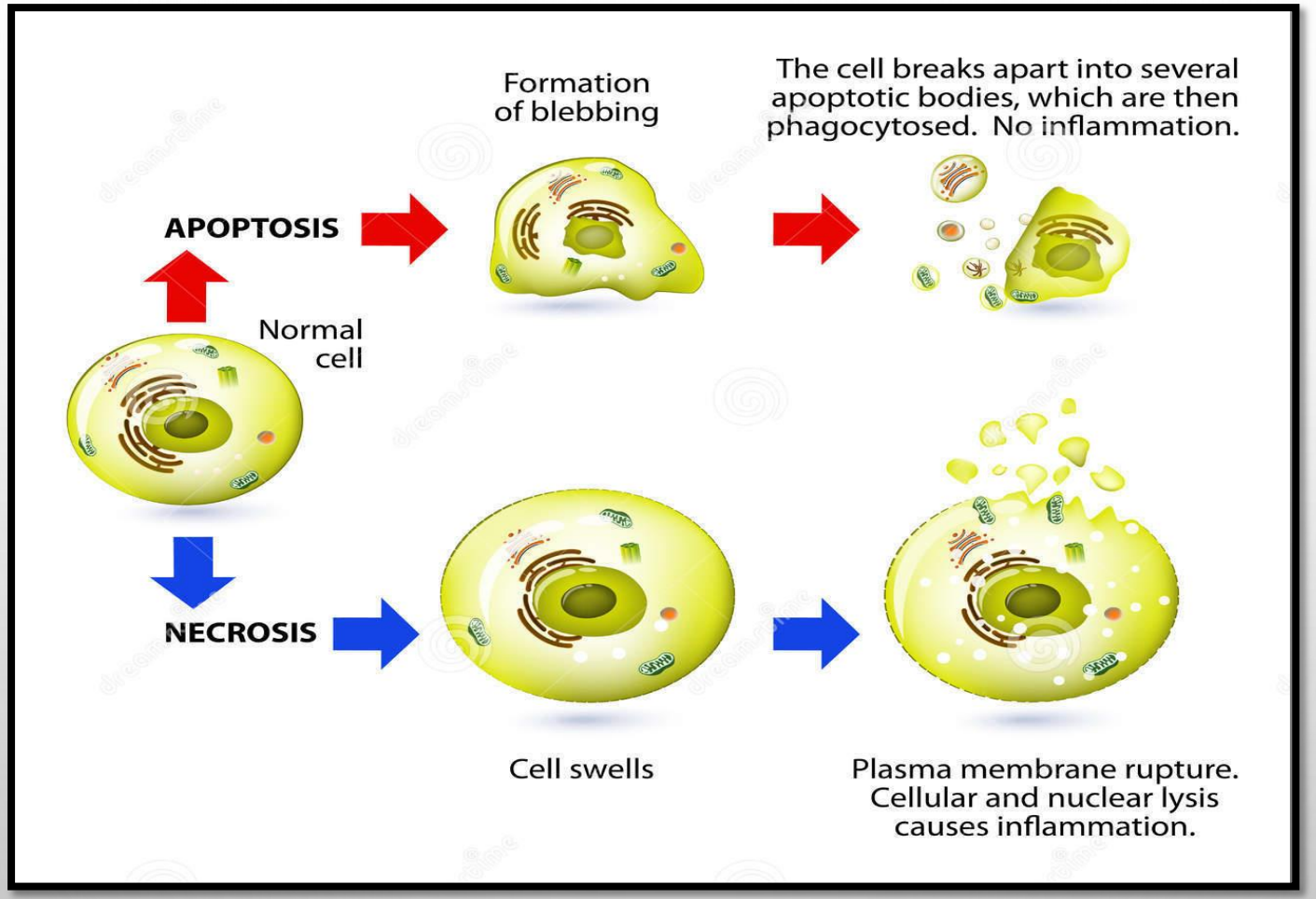


Figure 3: Positions of lanthanides on periodic table (Rajan *et al.*, n.d)

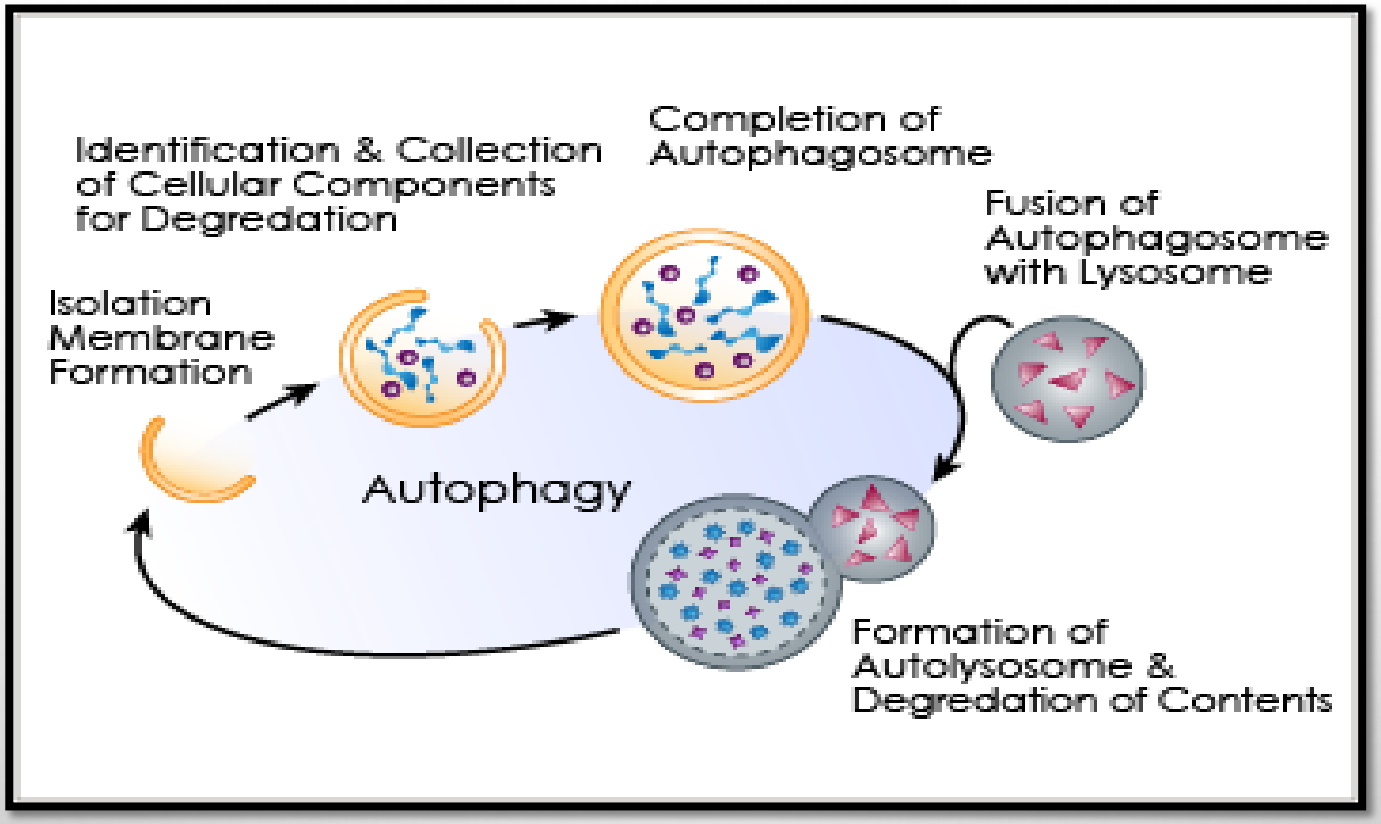
Praseodymium (Pr)	• Reactive elements. • Pr complex caused dose dependent drop in cell viability after exposed to leukemia cell (Yadav and Prevaiz, 2007).
Neodymium (Nd)	• Powerful magnet when form alloy with iron and boron (NIB). • Nd lasers able for cancer treatment (Stewart, 2012).
Gadolinium (Gd)	• High paramagnetic properties. • Gd were used in Magnetic Resonance Imaging (MRI) to get clarity images of tissue and detection of abnormalities and disease in body (Mercola, 2014).

CYTOTOXICITY BASED ON MODE OF CELL DEATH



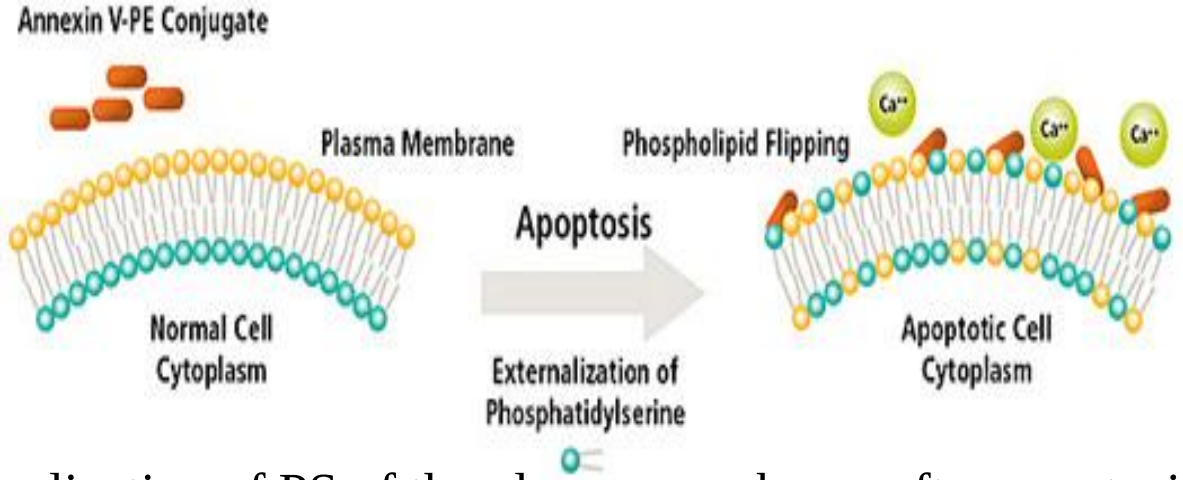


Apoptosis versus necrosis morphology. (dreamstime.com,2016)



Autophagy process (Mangan, 2015) .

Determination of apoptosis and necrosis by Annexin V-FITC

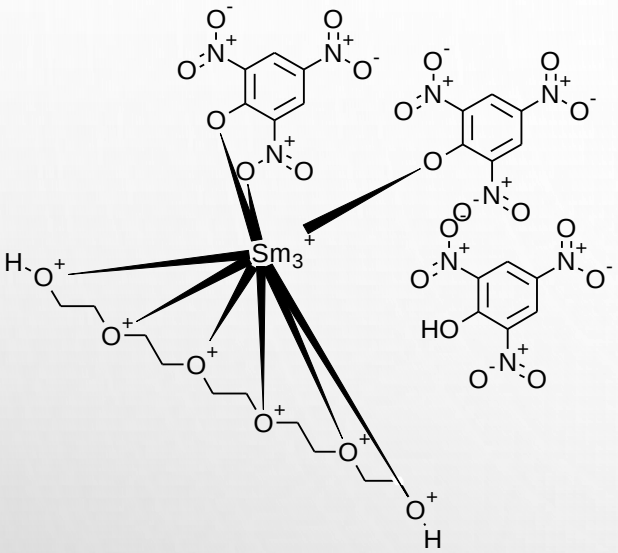


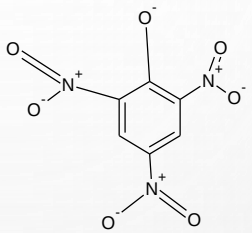
The externalization of PS of the plasma membrane after apoptosis events (Ferguson, 2015).

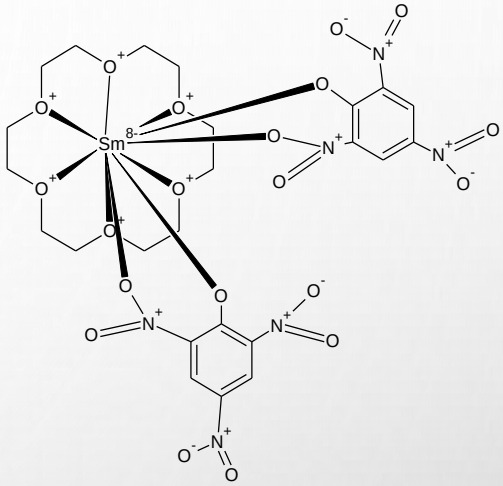
- **Phosphatydylserine (PS)** - maintain and protect the shape of cells
  - control the movement of substances that cross the cell membrane.
- PS translocate from inner to outer leaflet of membrane due to loss of membrane asymmetry.
- Annexin V bind to exposure PS.

I. DIFFERENT EFFECTS OF LANTHANIDE WITH DIFFERENT LIGAND/CHELATING AGENT ON *ACANTHAMOEBA* SP.

- A clinical isolate of A*canthamoeba* was used to observe the activity of **lanthanide ions** with **different chelating agents** based on cytotoxicity activities based on the morphological observation.
- Acyclic and cyclic samarium complexes, $[Sm(pic)_2(EO5)](pic)$ and cyclic $[Sm(pic)_2(18C6)](pic)$
- 24 hours for antiamoebic activity.







- **Scheme Of Molecular Structures Of The Acyclic [Sm(pic)$_2$(H$_2$O)(EO5)](pic) and Cyclic [Sm(pic)$_2$(18C6)](pic) Complexes**

- **The acyclic and cyclic molecular structures of both complexes are very interesting - open and closed structures as well as the fluorescence properties of both complexes in orange color due the presence of Sm^{3+} ion.**

Triethylene Glycol (EO3)

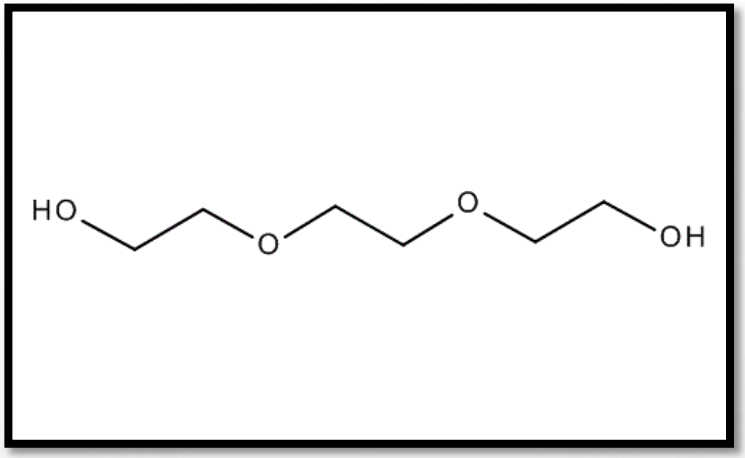
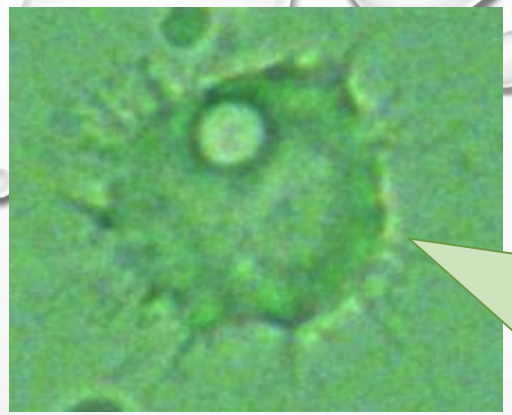
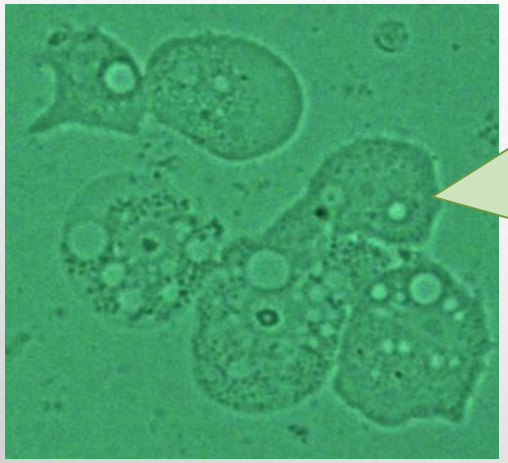


Figure 4 :Molecular structure of Triethylene Glycol (EO3) (merckmilipore.com, 2016).

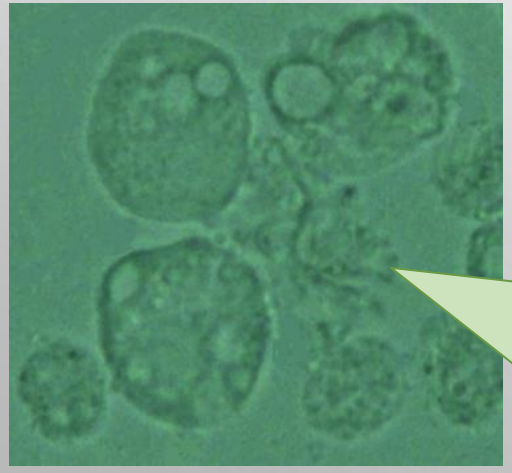
- **Molecular formula : $C_6H_{14}O_4$**
- **Stable, colourless, odourless and hygroscopic liquid.**
- **EO3 were used as ligand for Nd (III) and Sm (III) picrate to study the structural spectroscopic and photoluminescent properties of the complexes (Kusrini et al, 2012)**
- **Low acute toxicity.**



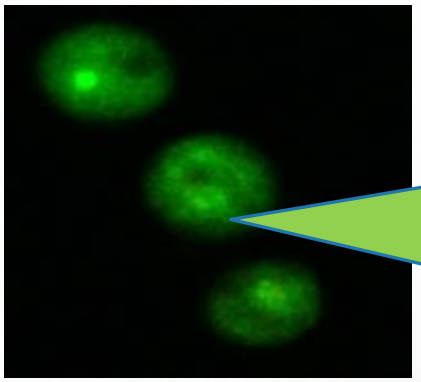
(A) the untreated *Acanthamoeba* sp., acanthapodia structure),



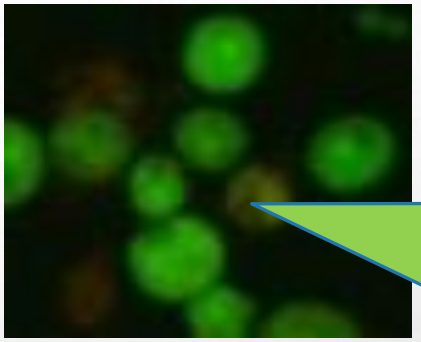
(B) acyclic sm complex-treated *acanthamoeba* , (cytoplasm aggregation)



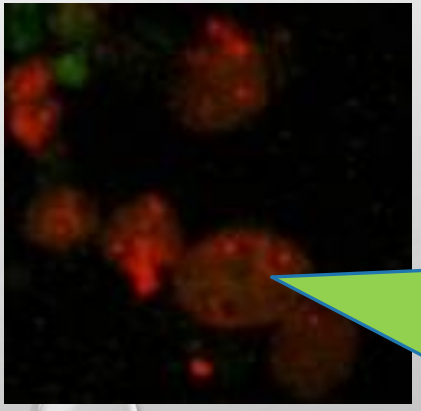
(C) cyclic-sm complex-treated *acanthamoeba* sp. (ruptured amoeba cell),



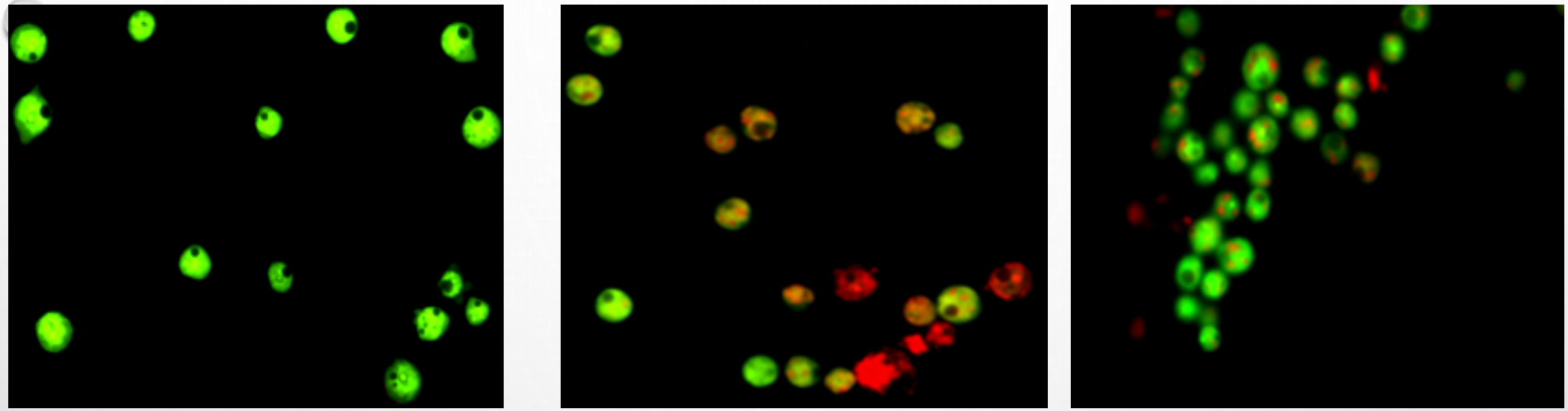
(D) the untreated *Acanthamoeba* cells showed green fluorescence cells with prominent nucleus,



(E) *Acanthamoeba* treated with acyclic Sm complex fluoresces bright yellow indicating early stage of apoptosis and



(F) *Acanthamoeba* sp. treated with cyclic Sm complex fluoresces red indicating plasma membrane leakage in necrosis



untreated

18C6.Eu(Pic)$_3$

EO3.Eu(Pic)

- AO/PI staining of A*canthamoeba* cells where treated with Eu complexes

- the variation types of cell damage are due to different types of chelating agents that bind to the sm^{3+} ions.
- The different effects are also rely on the factor of ability ligand binding to the death factor on the molecular surface of *acanthamoba* cells followed by recruitment of caspase-like series to cleave proteins and thus resulting morphological changes to occur in *acanthamoeba* cells.
- This process described the mode of action of acyclic sm complex on amoeba cells.
- However, cyclic sm complex seems to disrupt *acanthamoeba* cell membrane and damage the key organelles resulting necrosis.
- The main reason of apoptosis induction of the acyclic sm complex was observe because the covalent bonding with *acanthamoeba* protein and shape of acyclic structure of EO5 with two terminal alcohol groups.
- These findings indicated that the cyclic sm complex affected mainly on plasma membrane in parallel with simulation observation as no compatible region for the complex to fit in the profilin protein structure.
- Leakage of cytoplasmic membrane was observed after treatment with the cyclic sm complex.

- **The cytoplasmic membrane of *acanthamoeba* is unusual in the presence of lipophosphonoglycan, which is absent in mammalian cells (korn *et al.*, 1974), with sugar exposed on both side of the membrane (bowers and korn, 1974).**
- **According to xu *et al.*, (2001), changes on function or structure change of plasma membrane might also lead to the cell death.**
- **The interaction of acyclic sm complex with *acanthamoeba* on surface protein with apoptosis event was observed.**
- **It suggested that the lock and key mechanism of the EO5 ligand and membrane protein which not was not yet studied in any protozoan cells.**
- **The available *acanthamoeba* protein data in protein data bank which is profilin 1B was chosen to observe the potential interaction with sm complexes.**
- **Only acyclic sm complex was able to interact with the protein.**
- **The interaction of acyclic sm complex with *acanthamoeba* cells that cause necrosis cannot be confirmed only with accidentally cell death since necrosis also might appear with signal transduction cascade either through ROS production, ca^{+} overload, alkylating or DNA damage (galluzi et al., 2014).**
- **Both acyclic and cyclic sm complexes are cytotoxic on A*canthamoeba* sp with apoptosis and necrosis mode of cell death, respectively, indicated that different EO5 and 18C6 ligands induced the different modes of cell death in *acanthamoeba* cells.**

II. COMPUTATIONAL MODEL OF DIFFERENT LIGANDS OF SAMARIUM (SM) WITH EO5 OR 18C6 INTERACTION WITH PROFILIN 1B

- In silico and in vitro of antiamoebic activities of samarium complexes with **acyclic (pentaethylene glycol, EO5**) and **cyclic (18-crown-6, 18C6) structures.**
- To predict the mode of binding between the sm complexes with the ***Acanthamoeba* profilin 1B**, - the in silico modelling approach to observe the proper binding and its possible binding sites with the profilin 1B as selected target model as protein.

- Through the docking simulation, the **acyclic sm** complex with *acanthamoeba* profilin 1b was displayed **strong hydrogen bonds**, whereas **no interaction** was found for in silico study for **cyclic sm complex..**

- In vitro study...

✓ The Sm complexes exhibited with unique cytotoxicity characteristics on *Acanthamoeba* cells with ic_{50} of 0.8 and 6.5 μg/ml for the acyclic $[sm(pic)_2(eo5)](pic)$ and cyclic [s

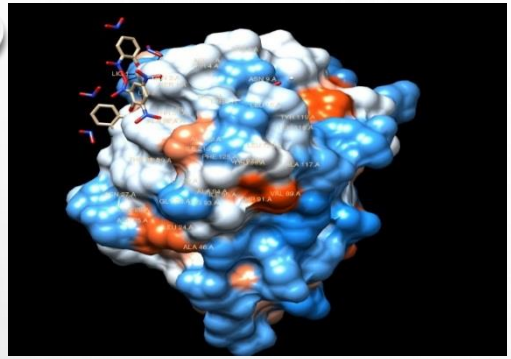
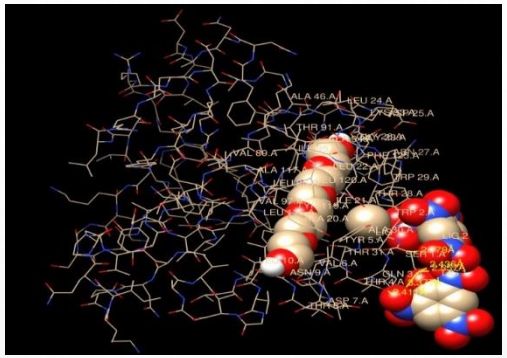
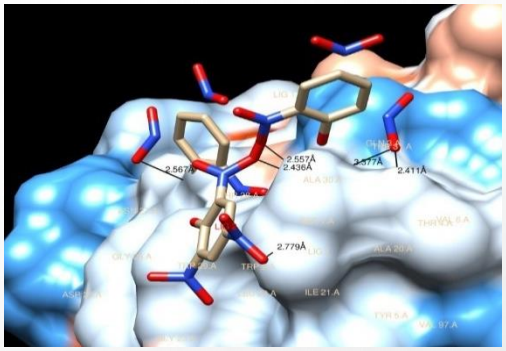
✓ $Sm(pic)_2(18C6)](pic)$ complexes, respectively.

✓ Morphological alteration in *Acanthamoeba* - significant cellular transformation for both Sm-treated *Acanthamoeba* from the native trophozoite shaped cells to the rounded form of trophozoites with loss of acanthapodia structure.

✓ Apoptotic *Acanthamoeba* cell population were observed for acyclic $[Sm(pic)_2(EO5)](pic)$ complex, while for cyclic $[Sm(pic)_2(18C6)](pic)$-treated samples, the necrotic cells was observed.

IN SILICO SCREENING BY USING AUTODOCK

- Autodock version 4 as computer screening - visualize the docking results for protein ligand and it used the latest **lamarckian genetic algorithm** (LGA).
- Docking simulations and for clustering results- **autogrid** - to create the conformational similarity, visualizing conformations, visualizing interactions between ligands and proteins and also visualizing the affinity potentials.
- Two areas of high positive potential surfaces of two *Acanthamoeba* isoforms were differing and it indicates the binding sites for **phosphatidylinositol phosphates.**
- The precision of these sites to the action binding sites gives an explanation for the **competition that occurs between actin and lipids for binding profilin**.

(a) Interactions of acyclic Sm complexes $[Sm(Pic)_2(EO5)](Pic)$ with *Acanthamoeba*'s profilin occur at hydrophilic regions of the protein (white spheres).
(b) Location of the pentathylene glycol (EO5) within the profilin structure while the Sm^{3+} located on the surface of the protein.
(c) H-bond interaction of ligand molecules based on UCFS Chimera H bond analysis

- 1B (PDB ID: 1ACF), an actin-binding protein in *Acanthamoeba* cells that functions as protein target for to observe the biological effect.
- Profilin - prevent the polymerization of actin in high concentration and vice versa in low concentration.
- The docking simulation –

❑ reveals interactions of the acyclic Sm complex with potential targeted regions of profilin.

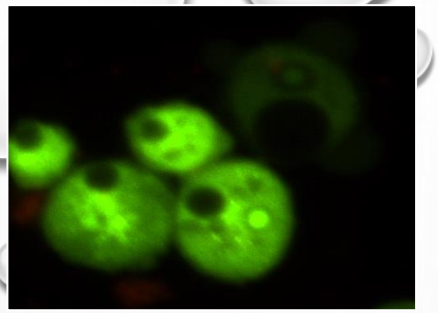
❑Not observed with the cyclic complex, it was unable to dock at any potential docking region of profilin 1B.

- Inability of the cyclic complex to form hydrogen bond with the amino acid residues due their rigid and cyclic conformation.
- The 18-crown-6 ligand only having rich electrons in the oxygen donor atoms in the ether links.
- Cyclic ligand do not have terminal alcohol groups as like in acyclic penthaethylene glycol (EO5)

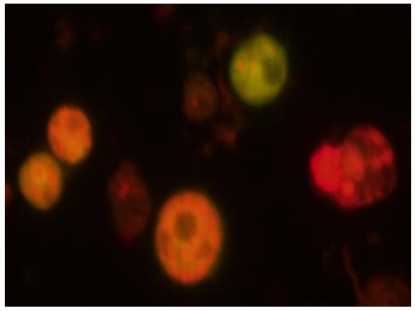
- Acyclic complex- the interactions occur **in hydrophilic pockets of the profilin**, involving the **Thr** and **Ser** amino acid residues.
- Reveal interactions - in the form of hydrogen bonding of the (acyclic) pentathylene glycol (EO5) with the embedded presence of the **Thr35, Ser1, 3, 6 residues, while the Sm^{3+} was found only on the surface of the protein** (figure 2b).
- -OH sidechains in amino acid residues such as **Ser and Thr are typical donor atom oxygen for hydrogen bonding** [Jabeen et al., 2016].
- Strong hydrogen bonds occur between the EO5 moiety with **Thr35 and Ser6** residues, with calculated bond lengths of **2.4 to 2.6 Å,** while **weak hydrogen bonds** are predicted with **Ser3** residues with bond length of **3.377 Å.**

III. DIFFERENT MODE OF CELL DEATH OF *ACANTHAMOEBA* SP. WHEN EXPOSED TO DIFFERENT LANTHANIDE

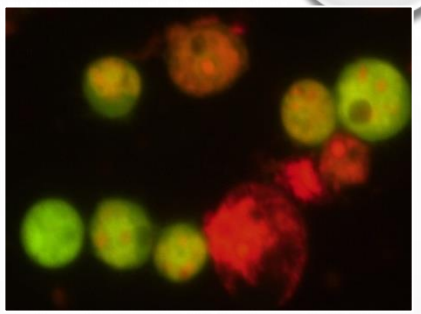
- Four different types of lanthanide with the same ligand
- MTT assay, AO/PI staining
- All lanthanide complexes are showing the cytotoxic effect toward the *acanthamoeba* sp.
- Eo4.Ce(pic) was the strongest inhibition activity with 3 µg/mlEO4.Pr(pic) -> 9.5 µg/ml, EO4.Nd(pic)-> 10.75 µg/ml, ->EO4.Dy(pic)-> 24.75 µg/ml.
- Observe the morphologic cell death of *acanthamoeba* sp.



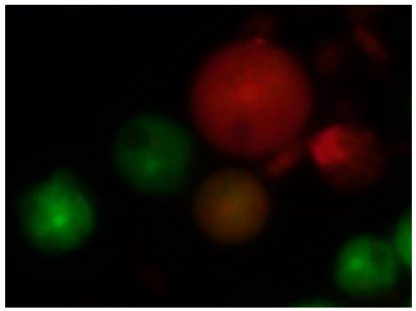
Untreated



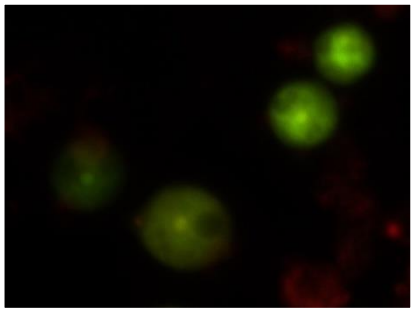
EO4.Ce(Pic)



EO4.Pr(Pic)



EO4.Nd(Pic)



EO4.Dy(Pic).

Effect of lanthanide complexes on *Acanthamoeba* sp.

- Untreated *Acanthamoeba* showed **green and intact nucleus.**
- **Cell membrane** of *Acanthamoeba* indicated healthy and viable.
- Complexes-treated *Acanthamoeba* - alteration towards the **internal organelles** of *Acanthamoeba* cells.
- t**Internal alteration-** contributes **autophagic *Acanthamoeba*** cells when orange lysosomes were observed.
- Acridine orange (AO)- **intercalating agent**, bind to the double strand structure of DNA by intercalating inside the double helix structure.
- In treated *Acanthamoeba* cells, yellow-orange granules observed in that cells - **uptake of AO dye by lysosomes**.

cont….

- Autophagy - expansion of lysosomes - resulted from sequestration and digestion of macromolecules of cytoplasmic material and cell organelles.
- The AO uptake was the result an active proton pump in lysosomes where the high proton concentration (low pH) caused AO, which could enter the lysosome in uncharged form.
- The stain becomes protonated and thus entrapped in the organelle of viable cells (Darzynkiewicz, 1997)- self digestion process results in cell death.
- No autophagy- in untreated trophozoites in the amoeba as protonated-orange-lysosomes in these *acanthamoeba* was not observed.
- AO

✓ A permeable fluorescence dye and able to enter intact plasma membrane of *Acanthamoeba* (Fatimah et al., 2013)

✓ enter internal parts of A*canthamoeba* through non-compromised membrane integrity.

✓ Bind to nucleic acids and it may fluoresce green when intercalate into double strand break of dna and intact dna.

✓ AO will fluoresce red when bind to single strand break of DNA (darzynkewick et al., 1984).

IV. LEVEL OF CYTOXICITY OF LANTHANIDES TO *ACANTHAMOEBA* IS INFLECTED BY THEIR POSITION IN THE PERIODIC TABLE

- Four different types of lanthanide with the same ligand were used to treat *Acanthamoeba* sp. - MTT assay, & AO/PI staining
- All lanthanide complexes are showing the cytotoxic effect toward the *Acanthamoeba* sp.
- EO4.Ce(pic) was the strongest inhibition activity with 3 µg/ml followed by EO4.Pr(pic), 9.5 µg/ml, EO4.Nd(pic), 10.75 µg/ml, and EO4.Dy(pic), 24.75 µg/ml.

Lanthanide Complexes	IC50 values (µg/ml)
EO4.Ce(Pic)	3
EO4.Pr(Pic)	9.5
EO4.Nd(Pic)	10.75
EO4.Dy(Pic)	24.75

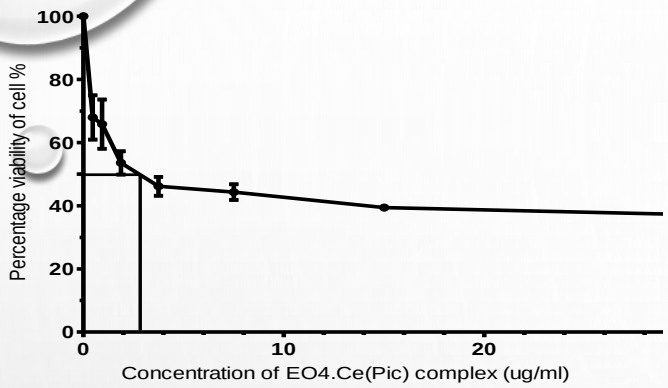


Figure 4.1 IC_{50} value of EO4.Ce(Pic) on *Acanthamoeba* sp. after 24 hours treatment

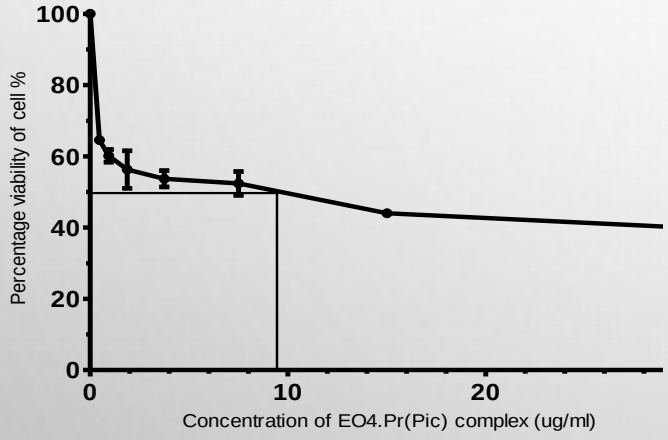


Figure 4.2 IC_{50} value of EO4.Pr(Pic) complex on *Acanthamoeba* sp 24 hours treatment

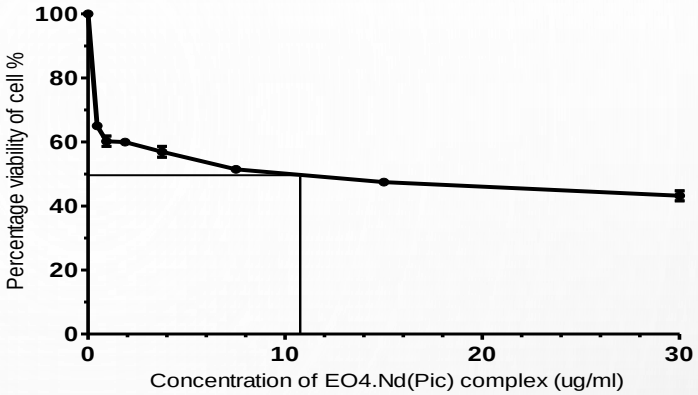


Figure 4.3 IC_{50} value of EO4.Nd(Pic) complex on *Acanthamoeba* sp. after 24 hours treatment

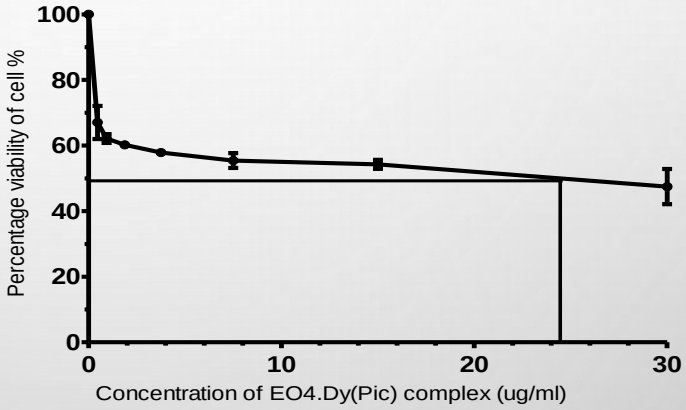
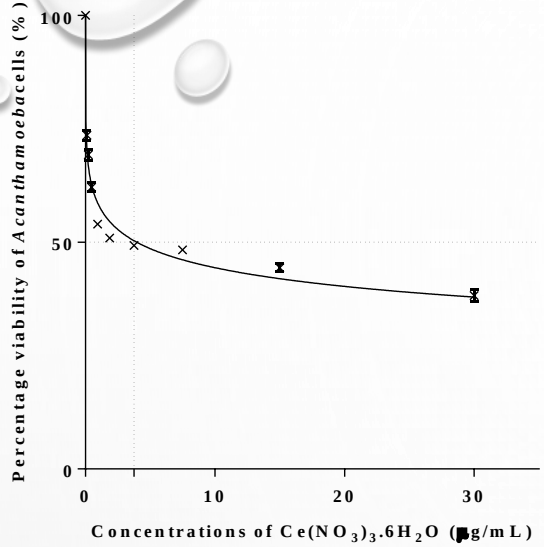
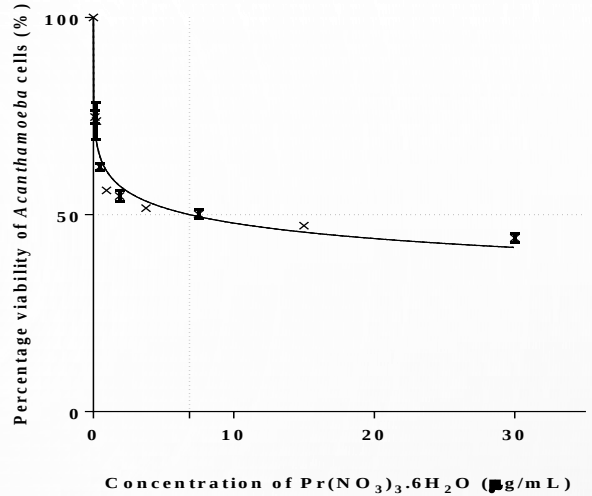
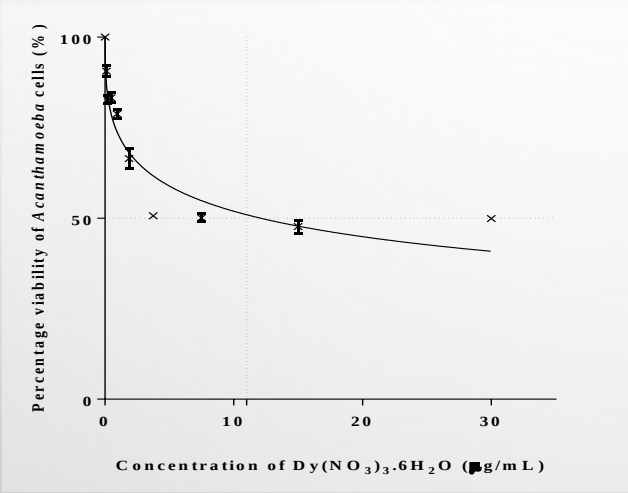
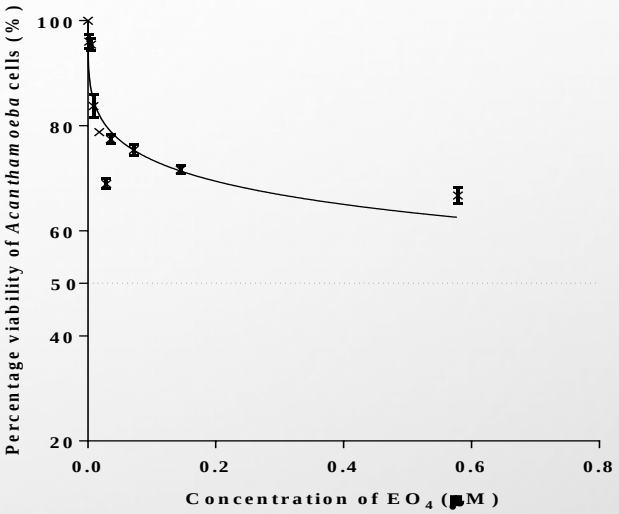


Figure 4.4 IC50 value of EO4.Dy(Pic) complex on *Acanthamoeba* sp. after 24 hours treatment









Lanthanide salts-treated *Acanthamoeba* trophozoite cells

METHODOLOGY

Culture media preparation

Acanthamoeba sp. cultivation

Lanthanide salts and ligand EO_3 stock solution preparation

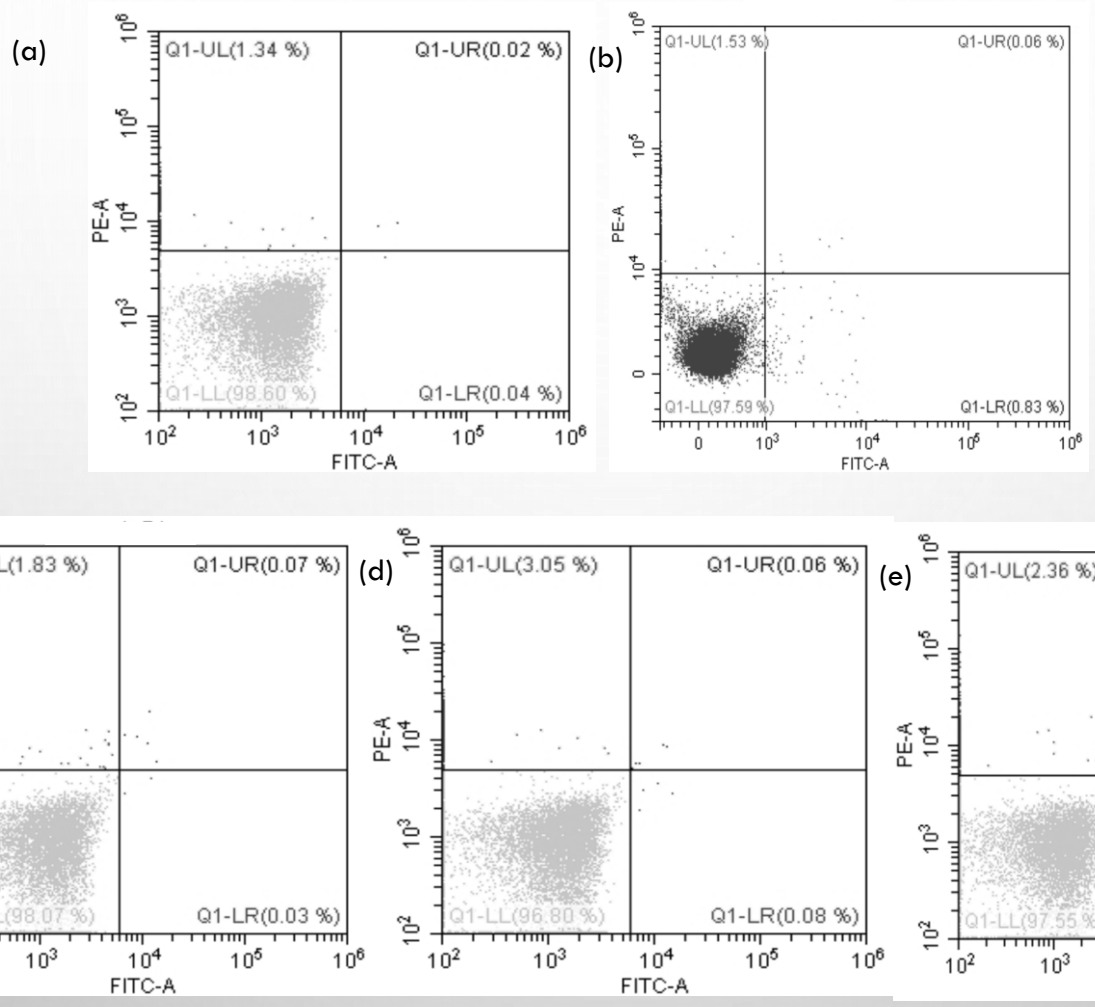
Determination of IC_{50} value by using MTT assay

Morphological observation under inverted light microscope

Mode of cell death determination by using fluorescence microscope stained by AO/PI

Detection of apoptosis and necrosis by using Annexin V-FITC

Confirmation of apoptosis and necrosis by Annexin V-FITC



The apoptosis was measured by Annexin V-FITC and flow cytometry analysis (a) untreated cells as negative control with 0.06% of apoptosis (b) chloramphenicol-treated cells 0.89% of apoptosis (c) EO_3.Pr.Pic treated cells with 0.10% of apoptosis (d) $EO_3(Nd)H_2O$.Pic treated cells with 0.14% of apoptosis (e) and EO_3.Gd.Pic treated cells with 0.08% of apoptosis.

APOPTOSIS

Chloramphenicol-treated cells	- 0.89% of apoptosis
EO_3.Pr.Pic treated cells	- 0.10% of apoptosis
EO_3(Nd)H_2O.Pic treated cells	- 0.14% of apoptosis
EO_3.Gd.Pic treated cells	- 0.08% of apoptosis.

Apoptosis

- Indicated externalization of PS.
- Annexin V-FITC bind to PS.

Autophagy – lanthanide complexes treated cells

- Intercellular cell death
- No externalization of PS occurred.
- Annexin V-FITC unable to detect due to no exposure of PS.

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