

# COMPARISON OF *POUZOLZIA INDICA* METHANOLIC EXTRACT AND VIRKON® AGAINST CYSTS OF *ACANTHAMOEBA* SPP

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**Abstract.** The present study was conducted to investigate the morphological and structural changes of *Acanthamoeba* cysts after being treated with various concentrations of *Pouzolzia indica* methanolic extract fraction 3 (methanol eluted) and Virkon® solution. Changes in the *Acanthamoeba* cysts were detected by light microscopy, scanning electron microscopy and transmission electron microscopy. The results show *Acanthamoeba* cysts were killed by *Pouzolzia indica* methanolic extract fraction 3 at a concentration of 1:8 and by Virkon® solution at a concentration of 0.25%, with a minimal cysticidal concentration (MCC) by 24 hours. Both agents caused similar structural damage to *Acanthamoeba* cysts in the same sequence. Step by step structural alterations occurred within the cyst. First, the cyst shrank, collapsed and had clumping of cytoplasmic structures inside the cyst walls. Second, the cysts began to bulge, swell, have a decrease in wrinkles in the cyst walls and spill the cytoplasmic contents into the environment. Finally, the cyst walls broke into small pieces. This study may be beneficial to compare with future studies of pharmaceutical agents against *Acanthamoeba* keratitis.

**Key words:** *Acanthamoeba* spp, *Pouzolzia indica* Benn, Virkon®, TEM, SEM

## INTRODUCTION

*Acanthamoeba*, is a group of widely distributed free-living protozoa that are opportunistic pathogens in humans (Khan, 2003; Clarke and Neiderkorn, 2006). There are two stages in the life cycle of *Acanthamoeba*, an infective trophozoite and a resilient cyst (Murti and Shukla, 1984; Marciano-Cabral and Cabral, 2003).

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Trophozoites live on variety of bacteria; when environmental conditions become unfavorable, the organism encysts. In cyst form, the ameba is capable of surviving up to a year and is resistant to temperature and pH changes (Neiderkorn *et al*, 1999). *Acanthamoeba* spp have been isolated from several habitats, including soil, air, dust, natural and treated water, seawater, swimming pools, sewage, sediment, air conditioning units, domestic tap water, drinking water, dental treatment units, hospitals, dialysis units, eyewash stations, contact lenses and lens cases (Rivera *et al*, 1987; Kilvington *et al*, 1990; Paszko-kolva *et al*, 1991; Marciano-Cabral and Cabral, 2003;

Wynter-Allison *et al*, 2005).

*Acanthamoeba* spp commonly causes *Acanthamoeba* keratitis, a painful eye infection, with inflammation, epithelial defects, photophobia, epithelial loss and stromal abscesses (Khan, 2003). The most characteristic clinical feature is the presence of a ring-like stromal infiltrate, thought to be composed of infiltrating inflammatory cells (Marciano-Cabral and Cabral, 2003). *Acanthamoeba* keratitis is typically associated with the use of contact lenses (Moreira and Prajna, 2003; Gagnon and Walter, 2006) which is the most important risk factor for developing infection (Preechawat *et al*, 2007). The treatment of *Acanthamoeba* keratitis includes combination therapy with 2-3 biocides, such as biguanide (chlorhexidine and polyhexamethylene biguanide, PHMB) or diamidines (propamidine and hexamidine) (Kosrirukvongs *et al*, 1987; Tien and Sheu, 1999; Sun *et al*, 2006). The resistance of the cysts to chemotherapeutic agents is probably the principal factor contributing to the increasing number of cases of *Acanthamoeba* keratitis. Medicinal plant extracts may be an alternative for treatment.

*Pouzolzia indica* Benn., known in Thai is "Kob-cha-nang-dang", is a Thai medicinal plant in the family Urticaceae. It has been used to treat parasites in children, treat menstrual disorders, help to pass urine and help treat infections with pus (Trakulsomboon *et al*, 2006). Dried stems and leaves of *Pouzolzia indica* Benn. were purchased from a Thai Herbal Pharmacy. The stems and leaves were then cut into small pieces and ground into powder. The powdered drug was macerated with ethylalcohol and concentrated under reduced pressure to yield a crude ethanolic dry extract. The extract was dissolved in water and the water soluble part was chromatographed on a Diaion® HP-20 col-

umn. The thin-layer chromatography were carried at repeatedly until the water eluted fraction (fraction 1), the water-methanol eluted fraction (fraction 2), the methanol eluted fraction (fraction 3) and the ethyl acetate eluted fraction (fraction 4) were obtained. Fraction 3 (methanol elute) containing some phenolic components, polar triterpenes, phytosteryl glycoside and aglycones, was selected for this study.

Virkon® has been widely studied regarding its antimicrobial activity and rapid killing of up to 99.999% of microorganisms in less than 10 minutes (Gasparini *et al*, 1995). The active ingredient, potassium peroxymonosulphate, has an exceptional safety profile being non-toxic, non-sensitizing and is fully biodegradable over time and hence is environmentally friendly. Virkon® is most often sold as pink tablets or powder which is dissolved in water. Virkon® was the antimicrobial control used in this study to compare with fraction 3 of the medicinal plant, *Pouzolzia indica* methanolic extract against *Acanthamoeba* cysts, using the light, scanning electron and transmission electron microscopic studies.

## MATERIALS AND METHODS

*Acanthamoeba*, isolated from the eye of a person with keratitis, was cultured at the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The cysts were obtained by culturing ameba on a non-nutrient agar plate seeded with heat killed *Escherichia coli* (NNA-*E.coli*), incubated at 37°C for 7-12 days. The cysts were harvested, washed in normal saline solution, and adjusted to a final concentration of 10<sup>4</sup> cysts/ml.

### Fraction 3 testing (Fig 1a-c)

Fifty microliters of dimethyl sulfoxide

*Pouzolzia indica* preparation

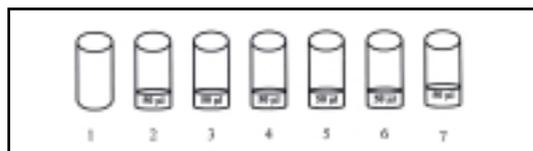


Fig 1a—Fifty microliters of DMSO were added to wells 2-7, the 7<sup>th</sup> well was the control well.

Virkon® preparation

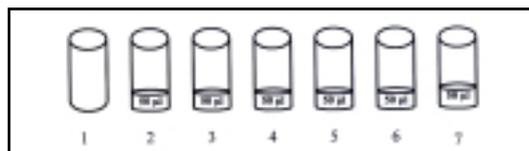


Fig 2a—Fifty microliters of normal saline solution was added each to the 2<sup>nd</sup> - 7<sup>th</sup> wells; the 7<sup>th</sup> well was the control well.

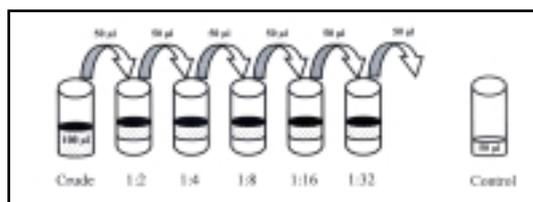


Fig 1b—100 microliters of fraction 3 was added to the 1<sup>st</sup> well, then 50 µl of the 2<sup>nd</sup> well was added to the 3<sup>rd</sup> well then 50 µl of the 3<sup>rd</sup> well was then added to the 4<sup>th</sup> well until all 6 wells were completed.

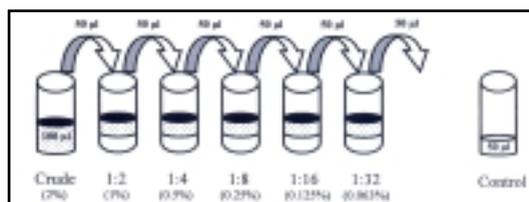


Fig 2b—100 microliters of 2% Virkon® was added to the 1<sup>st</sup> well, the 50 µl was removed from the 1<sup>st</sup> well and added 2<sup>nd</sup> well, then 50 µl of the 2<sup>nd</sup> well was added to the third well and so on until all 6 wells were completed.

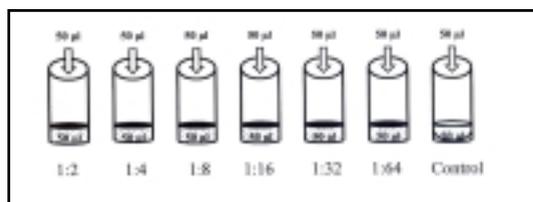


Fig 1c—Each of the six dilutions and control were tested for the amebicidal effects of fraction 3 against 50 µl of cyst suspension.

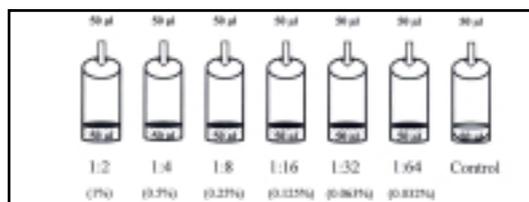


Fig 2c—Six dilutions and control were tested for the amebicidal effects of Virkon® against 50 µl of cyst suspensions.

(DMSO) was added to the 2<sup>nd</sup> through 6<sup>th</sup> wells and another 50 µl of DMSO was added to the 7<sup>th</sup> well as a control. One hundred microliters of fraction 3 solution (50 mg/ml) was added to the 1<sup>st</sup> well, then 50 µl of the solution in the 1<sup>st</sup> well was added to the 2<sup>nd</sup> well and mixed. Fifty microliters from the 2<sup>nd</sup> well was then added to the 3<sup>rd</sup> well and mixed. The procedure was repeated until the 6<sup>th</sup> well had a dilu-

tion of 1:32; 50 µl from the 6<sup>th</sup> well was then discarded. The six dilutions (crude, 1:2, 1:4, 1:8, 1:16 and 1:32) and the control well were tested for their amebicidal effects against acanthamoeba cysts by adding 50 µl of the standardized cyst suspension containing  $1 \times 10^4$  cysts/ml to each well. The fraction 3 dilutions 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 and the control well were incubated at 37°C for 24 hours and the wells

were checked microscopically to detect viable cysts.

#### **Virkon® solution testing (Fig 2a-c)**

Fifty microliters of normal saline solution was added to the 2<sup>nd</sup> through 6<sup>th</sup> wells and another 50 µl of normal saline solution was added to the 7<sup>th</sup> well, which was the control well. One hundred microliters of 2% Virkon® was added to the 1<sup>st</sup> well, then 50 µl from the 1<sup>st</sup> well was moved to the second well, then 50 µl from the second well was moved to the 3<sup>rd</sup> well and so on until all 6 wells were completed, with the 6<sup>th</sup> well having a dilution of 1:32; 50 µl of the 6<sup>th</sup> well was then discarded.

The the six dilutions and control well were tested for their amebicidal effects against a cyst assay containing 1x10<sup>4</sup> cysts/ml, which was added to each well. The final concentrations of Virkon® were 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 and the control. These were incubated at 37°C for 24 hours, then checked microscopically to detect viable cysts.

Two hundred microliters of normal saline was added to each well, both for fraction 3 and Virkon® to stop the reaction. The wells were aspirated and washed twice with 200 µl of normal saline. The minimum cysticidal concentration (MCC) was defined as the lowest concentration of fraction 3 or Virkon® that resulted in no excystment or trophozoite replication after repeated reculture for 7 days of incubation (Kilvington *et al*, 1990; Galti *et al*, 1998). In this experiment, the minimum cysticidal concentration of fraction 3 was 1:8 and Virkon® was 1:8 at a final concentration of 0.25%. Examination of the structural changes of the nonviable cysts were performed by light and electron microscopy.

Light microscopy was used to observe the morphologies of treated and untreated specimens, using an Olympus inverted

research microscope; photographs were obtained using a Zeiss AxioCam MRc digital camera.

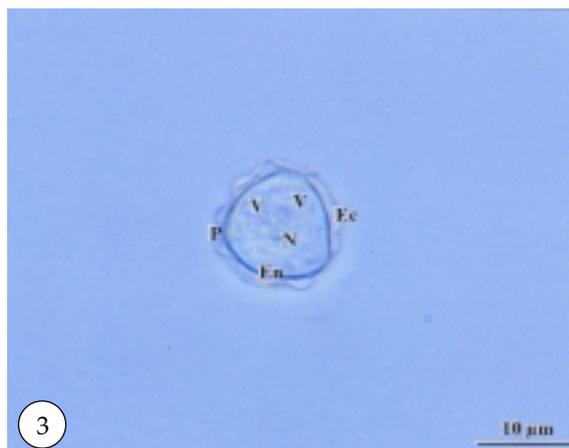
For scanning electron microscopy, treated and untreated *Acanthamoeba* cyst were placed in eppendolffs and centrifuged. The sediment was fixed in 2.5% glutaraldehyde in 0.1 M PBS buffer for 1 hour at room temperature then postfixed in 1% OsO<sub>4</sub> for 1 hour. A pellet of the *Acanthamoeba* cysts was placed on the cover glass and subjected to critical point drying, coated with gold and viewed with a JEOL scanning electron microscope.

For transmission electron microscopy, the treated and untreated specimens were fixed in 2.5% glutaraldehyde in 0.1 M PBS for 1 hour and postfixed in 1% OsO<sub>4</sub> for 1 hour and processed further for routinely transmission electron microscopy, cut with an ultramicrotome (LEICA EMUC6, France) deposited on a copper grid, then stained for 45 minutes with uranyl acetate and lead citrate. The grids were observed under a transmission electron microscope.

## RESULTS

### **Light microscopy**

**Untreated *Acanthamoeba* cysts (Fig 3).** Light microscopy revealed the untreated *Acanthamoeba* cyst contained an *Acanthamoeba* cell lying within double cyst walls: the ectocyst and endocyst walls. The ectocyst appeared wrinkled and a clear space separated it from the flat, smooth endocyst. The walls protected the cell and cytoplasmic organelles from harsh environmental conditions. *Acanthamoeba* cysts possess pores known as ostioles at the angles of the polygonal cysts which are sealed internally by caps or opercula. The cysts have a mean diameter of approximately 23.75 ± 2.59 µm and are ovoid, spherical or polygonal in shape. In the



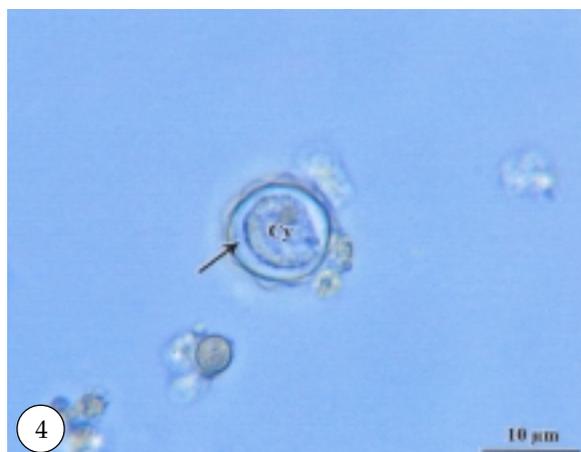
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Fig 3—Light micrograph of a normal *Acanthamoeba* cyst. The cyst is round with double cyst walls. The outer cyst wall is called the ectocyst (Ec), inner cyst wall is called endocyst (En) and the pore (P) or ostiole. In the cytoplasm there is one nucleus (N) and several vacuoles (V).



5

Fig 5—Light micrograph of an *Acanthamoeba* cyst after being treated with 0.25% Virkon®. There are cytoplasmic clumps (Cy) and empty cyst walls (arrowheads).



4

Fig 4—Light micrograph of an *Acanthamoeba* cyst after treatment with fraction 3 (1:8 MCC). The micrograph shows round cyst wall with a cytoplasmic clump (Cy). The arrowhead indicates an empty cyst. Many cysts have cytoplasmic clumps (Cy) and shrinkage of ameba cells away from the cyst wall (arrows).

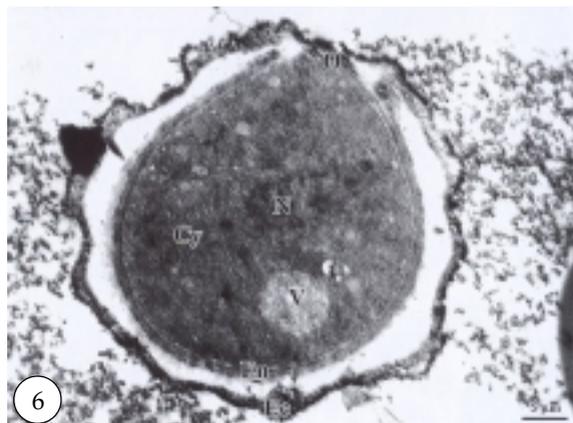
cells, there are one or several vacuoles per cell, one nucleus and other unidentified cytoorganelles.

**Cyst treated with fraction 3** (Fig 4). The cysts had a mean diameter of  $21.96 \pm 3.55 \mu\text{m}$ . All exhibited double cyst walls composed of wrinkled ectocyst and smooth endocyst walls. Most showed shrinkage of the cytosol, or cytoplasmic clumps while no typical nuclei were visible. Some cysts had empty double-walled cysts with a wrinkled ectocyst and a round endocyst.

**Cysts treated with Virkon® solution** (Fig 5). The cysts had a mean diameter of  $24.75 + 3.64 \mu\text{m}$ . The cysts had double cyst walls with a wrinkled ectocyst wall and a smooth round endocyst wall with shrinkage or clumping of the cytosol. Some cysts had empty walls because the cytosol were discharged out.

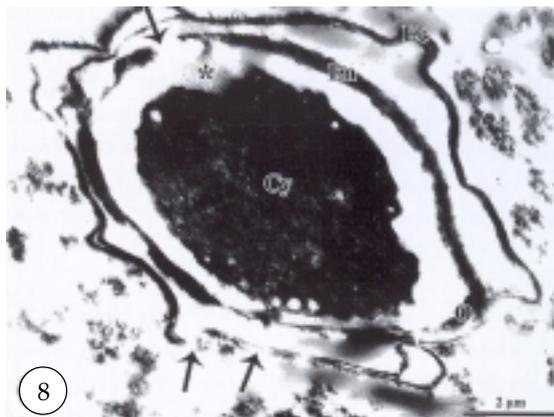
**Transmission electron microscopy**

**Untreated *Acanthamoeba* cysts** (Fig 6). The cysts had a mean diameter of  $20.75 + 0.94 \mu\text{m}$ . These had a spherical or polyhedral shape within a double-walled cyst. The



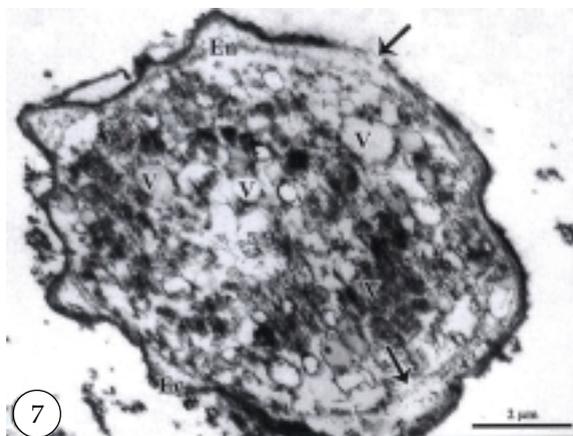
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Fig 6—Transmission electron micrograph of a normal *Acanthamoeba* cyst. The ectocyst wall (Ec), the endocyst wall (En) fused at the pore or ostiole closed by the operculum (O). Inside the cell are a nucleus (N), clear vacuole (V), and many lipid droplets (L).



8

Fig 8—Transmission electron micrograph of an *Acanthamoeba* cyst after treatment with 0.25% Virkon® solution. There was thickening of the ectocyst and endocyst walls and discontinuous or ruptured walls (arrows). The cytoplasm is clumped (Cy) so the space between the endocyst and the cytoplasm is wide.



7

Fig 7—Transmission electron micrograph of an *Acanthamoeba* cyst after treatment with fraction 3 of a concentration of 1:8. There are ruptured ectocyst and endocyst wall (arrows) with damage cyto-organelles which appear as membrane bound vacuoles (V).

ectocyst appeared as an electron dense layer parallel to the cell surface. The endocyst was separated from the ectocyst by a space. Both walls were parallel and were

fused at the pores or ostiole regions where both layers disappeared and remained as a thin electron dense layer that covered the ostiole. The ostiole was closed by a plug called an operculum. The operculum was located under a thinner layer which covered the ostiole and had a fibrinous appearance and an electron dense cyst wall. Inside the cyst, the space between the endocyst and the plasma membrane was thin and interrupted where the ameba was attached to the ostiole. Inside the cell, there was a nucleus with organelles essential for excystment, such as mitochondria and several clear vacuoles similar to lipid droplets situated at the periphery of the cell.

**Cysts treated with fraction 3 (Fig 7).** The cysts had a mean diameter of  $20.08 \pm 0.66 \mu\text{m}$ . A transmission electron micrograph showed round, thicker walls and discontinuous cyst walls. Inside the cyst wall, there were clumps, with shrinkage of the

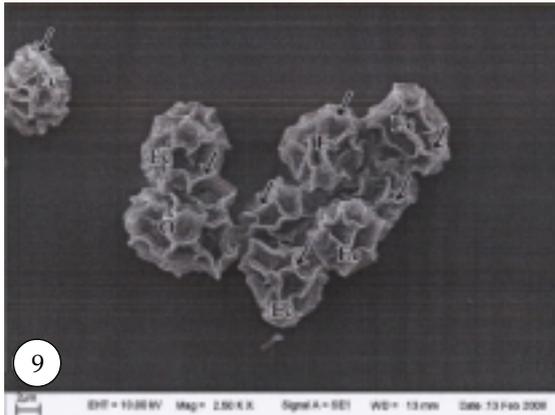


Fig 9—Scanning electron micrograph of a normal *Acanthamoeba* cyst. The cyst is oval with an ectocyst wall (Ec) folded as high ridges (arrow).

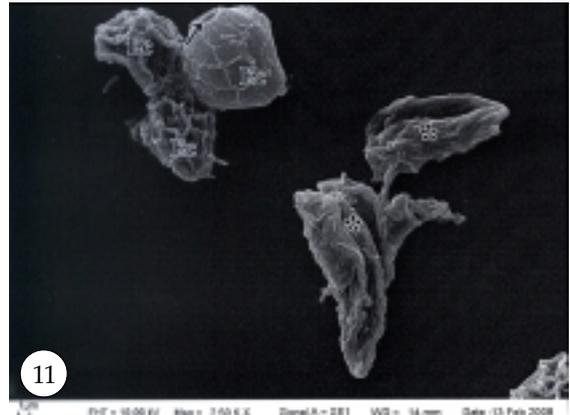


Fig 11—Scanning electron micrograph of an *Acanthamoeba* cyst after treatment with 0.25% Virkon® solution. The cysts had shrinkage, wall rupture, edema, decreased wrinkles, were ripped or torn (asterisks), arrow = ostiole.

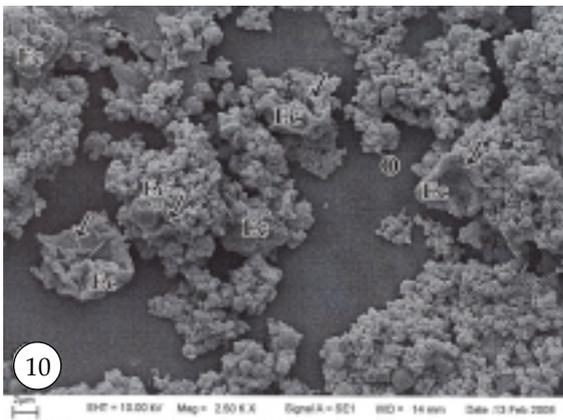


Fig 10—Scanning electron micrograph of an *Acanthamoeba* cyst after treatment with a 1:8 concentration of fraction 3. The cysts (Ec) are more difficult to identify because they are somewhat obscured by *Pouzolzia* sediment. Some cysts were collapsed, had shrinkage or decreased wrinkles.

cytoplasm and the membrane bound vacuoles.

**Cysts treated with Virkon® solution** (Fig 8). The cysts had a mean diameter of  $22.26 \pm 0.25 \mu\text{m}$ . Most had rough, thick, discontinuous cyst walls. Some cysts had rup-

tured walls at the ridges of the cyst. Inside the cyst wall, there were clumps and shrinkage of the cytoplasm with lipid droplets lining the border of the cell. A homogenous substance filled in the space between the inner endocyst wall and the plasma membrane.

#### Scanning electron microscopy

**Untreated *Acanthamoeba* cyst.** On a scanning electron micrograph of the *Acanthamoeba* cysts, they were round or polygonal shaped. The ectocysts were wrinkled with thin ridges over the surface (Fig 9).

**Cyst treated with fraction 3** (Fig 10). Micrographs exhibited the characteristic feature of the treated cyst. The oval cysts became flat, and there was shrinkage and collapse of the ectocyst walls. Some had thick ridges on the wrinkled surface, and some had a decreased wrinkled surface cause by edema of the cyst.

**Cyst treated with Virkon®** (Fig 11). Scanning electron micrographs show morpho-

logical changes of the walls of *Acanthamoeba* cysts after treatment with 0.25% Virkon® solution. These were flat, irregularly shaped causing collapse of the ectocyst walls. Some had swelling of the cyst with openings at the pores or ostioles. These were degenerated cysts which had splitting cyst walls.

## DISCUSSION

*Pouzolzia indica* methanolic extract solution (Fraction 3) having a Thai name "Kob-cha-nang-dang", have been used to treat parasites in children, expelle menstrual fluid, assist with passing urine and treat pus (Trakulsomboon *et al*, 2006). In this study, *Acanthamoeba* cysts were killed by the fraction 3 and Virkon® at MCCs of 1:8 and 0.25%, respectively. Light micrographs revealed many cytoplasmic clumps, empty double-walled cysts and damaged cyst walls. Transmission electron micrographs revealed the stripped and ruptured cyst walls with damaged cytoplasmic organelles and shrinkage and fragmentation of the cytoplasmic components inside the cyst wall. Scanning electron micrographs revealed flatten wrinkled strips with swollen and segregated cyst walls. These characteristics were similar between cysts treated with *Pouzolzia indica* (fraction 3) and Virkon®. These findings show the steps of a degenerating cyst until it dies and is destroyed. First, the cyst walls shrink, the opercula are destroyed and the cyst wall separates, along with cytoplasmic clumping and expelling of the cyst wall. Then, the cysts become edematous due to passing of the solution into the cell, causing decreased wrinkling of the cyst wall. As was seen in the scanning electron micrograph, the cysts were swollen and the wrinkles were

decreasing. In the last step, the cyst walls tear and break into small pieces. Trakulsomboon *et al* (2006) reported there is a large amount of sugar and phenolic substances in extracted *Pouzolzia indica* Benn. The phenolic compounds can cause damage to the plasma membrane resulting in leakage of intracellular constituents of the cell (McDonnell and Russell, 1999). Phenolic compounds act as oxidizing agents causing cell membrane damage by reacting with cellular proteins, lipids, nucleic acids and carbohydrates (Pacifici and Davies, 1991; Roufogalis *et al*, 1999).

Many researchers have examined the mechanisms of Virkon® on the cell walls of bacteria and fungi, revealing potassium peroxymonosulphate as an active ingredient of Virkon®, which is a strong oxidizing agent and has a high level of surfactant which reacts with some proteins of the cell wall or cell cytoplasm (Gasparini *et al*, 1995; Moreton *et al*, 1999).

In summary, the minimal cysticidal concentrations (MCC) of the *Pouzolzia indica* methanolic extract fraction 3 (methanol eluted) and Virkon® were 1:8 and 0.25%, respectively. Recultures of the cysts for 7 days confirm the non-viability and the MCC of both antimicrobial agents. Light microscopy also aided in viewing the viability of cysts, but the detailed destruction of the cysts were most reliably seen by transmission and scanning electron microscopy. In this study, we demonstrated order of cyst destruction by light and electron microscopy, beginning with shrinkage of the cyst walls, opercula and wall disruption, cytoplasmic clumping and discharging out the ruptured cyst wall. The outside solution passed through the ruptured wall resulting in cyst edema and decreasing the wrinkle ridges. During the stage the cell ruptured.

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