Pouzolzia indica Methanolic Extract Fraction 2 and Povidone-Iodine Induced Changes in the Cyst of *Acanthamoeba* spp.: Light and Electron Microscopic Studies

Jantima Roongruangchai DDS, PhD*, Tichaporn Sookkua MS*,
Tanawan Kummalue MD, PhD**, Kosol Roongruangchai MS, MD***

** Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
*** Department of Clinical Pathology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*
**** Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand*

Objective: *To compare the minimal cysticidal concentration (MCC) between Pouzolzia indica methanolic extract fraction 2 and Povidone-Iodine (PVP-I) on the Acanthamoeba cyst and to illustrate the morphological changes of the cyst after being treated by light and electron microscopies.*
Material and Method: *Acanthamoeba spp were isolated from patients with Acanthamoeba keratitis and cultured on a non-nutrient agar plate (NNA) seeded with heat killed Escherichia coli (NNA-E.coli) at 37°C for 7 days, adjusted to a final concentration of 10^4cysts/ml. Several concentrations of PVP-I and fraction 2 of Pouzolzia indica methanolic extract were tested to find the minimal cysticidal concentrations (MCC) of both agents, at these concentrations there was no viable cyst which was confirmed by no excystment after further incubation for 7 days. The cysts were prepared for routine transmission and scanning electron microscopic studies.*
Results: *Structural damages of the treated cysts by MCC of PVP-I and fraction 2 of Pouzolzia indica methanolic extract showed a series of damages. Starting from shrinkage, destruction or rupture of the cyst walls and opercula, withdrawal of the cytoplasm or edema cyst by the outside solution passed through the damaged wall caused a decrease in wrinkle ridges of the ectocyst. Then the cyst was ripped and torn into small pieces*
Concluison: *MCC of PVP-I solution and the fraction 2 of Pouzolzia indica methanolic extract were 0.04% and 1:4, respectively. The structural damages were somewhat similar, such as the shrinkage, ruptured cyst wall and opercula, edema and end by breaking up of the cyst wall and degeneration of the inside cytosol. Pouzolzia indica may be modified as an effective disinfectant solution for a contact lens case if the active ingredients are more purified.*

Keywords: *Pouzolzia indica Benn., Acanthamoeba spp., Povidone-iodine*

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Acanthamoeba spp. are free-living amoebae which are associated with severe eye infections in contact lens wearers, the *Acanthamoeba* keratitis[1-3]. They exist principally in an active trophozoite form that feeds on bacteria and other microorganism and when environmental conditions become unfavorable, they encyst, from which the trophozoite may emerge when conditions are again favorable[2-5]. In the cyst form, the amoeba is capable of surviving up to a year and are resistant to temperature and pH. *Acanthamoeba* spp. have been isolated from several habitats, including soil, air, dust, swimming pools, air condition units, domestic tap water, dental treatment units, dialysis units, eyewash stations, contact lenses and contact

Correspondence to: Roongruangchai K, Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. E-mail: parasite52@hotmail.com

lens cases[6-10]. *Acanthamoeba* keratitis is typically associated with the use of contact lens[11,12] and seem to be the most important risk factor[13]. This becomes of great interest because of the nature of the disease and its resistance to treatment. Treatment regimen includes combination therapy with 2-3 biocides such as biguanide (Chlorhexidine and Polyhexamethylene biguanide, PHMB) or incombination of diamidines (Propamine and Hexamidine)[13-15]. Resistant to chemotherapeutic agents of cysts is probably a principal factor contributing to the increasing of cases of *Acanthamoeba* keratitis. Medicinal plant extracts, after carefully studied and modified, can be other alternative regimens for the treatment if proved to have higher efficacy and less harmful than chemicals.

Pouzolzia indica Benn, Thai name "Kob-cha-nang-dang" is a Thai medicinal plant in the family of Urticacae. It can excavate parasites in children, expel menstruation, discharge urine and treat pus. It has been used in dermatological and urological disease[16]. *Pouzolzia indica* Benn specimens were deposited in the Thai Herb Pharmacy traditional drug store named "Chao-Krom-Po" with the voucher number of BKF. No.106441 and SN 096588. The dried stems and leaves were ground into powder and macerated with ethyl alcohol and treated to yield the crude ethanolic dry extract. The dry extract was dissolved in water and chromatographed on a Diaion® HP-20 column. The column was eluted with water yielding the water fraction. The water insoluble part was dissolved in water-methanol and the soluble part was chromatographed and eluted with water-methanol. The insoluble parts in water-methanol and methanol were dissolved in methanol and ethyl acetate respectively, and the column chromatographic process was repeated. The water, water-methanol, methanol and ethyl acetate fractions or fraction 1, 2, 3 and 4 were obtained. Fraction 2, comprising sugar and several phenolic compounds, was selected and used in the present study.

Povidone-iodine (PVP-I or Betadine®) is an antiseptic against a broad spectrum of micro-organisms such as, bacteria, yeast, mold and fungi. It has been used as a topical antiseptic to prevent infections of the skin and mucous membranes during surgery with no report of toxicity to the cornea and conjunctiva when applied topically in a single dose to the ocular surface. It was recently used as a prophylaxis against neonatal conjunctivitis in protective trials in developing countries instead of treatment with silver nitrate or erythromycin because it was less toxic and less expensive[17].

Material and Method

Preparation of Acanthamoeba cyst

Acanthamoeba spp., isolated from the human eye with keratitis since 1989 and subcultured for further studies since then, were obtained from the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. They were grown on non-nutrient agar plates enriched with heat-killed *E. coli* 7 days at room temperature. Normal saline solution was added for dispersing the parasites. The suspended *Acanthamoeba* cysts were passed into normal saline solution in plastic tubes and adjusted to a final concentration of 10^4 cysts/ml.

Pouzolzia indica methanolic extract fraction 2 solution procedure

Fifty µl of dimethyl sulfoxide (DMSO) was added into the 2nd to 6th wells and another 50 µl of DMSO was added into the 7th well which was the control well.

100 µl of fraction 2 solution was added into the 1st well, and then 50 µl of fraction 2 solution of the 1st well was brought to the 2nd well and mixed. Another 50 µl of the mixture of the 2nd well was brought to the 3rd; the procedures were repeated until the dilution of the 6th well was 1:32 (by discarding the last 50 µl of the mixture of the 6th well). The six dilutions and the control wells were further tested for their amoebicidal effect in the cyst assay by adding 50 µl of the cyst suspension of 10^4 cysts/ml into each well, to make the final concentrations of 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. The well was incubated at 37°C for 24h, the wells were checked microscopically to detect the viable cysts.

Then 200 µl of normal saline was added into each wall to stop the reaction and 50 µl of living *E. coli* suspension was added. The test was performed in triplicate, one of which was transferred to the non-nutrient agar plate seeded with heat-killed *E. coli* (NNA-*E. coli*) and the viability was tested for 7 days to get the minimal cysticidal concentration (MCC) which was the concentration that resulted in no excystment and trophozoite replication[9,18], and the MCC of *Pouzolzia indica* methanolic extract fraction 2 was 1:4.

Povidone-iodine (PVP-I) or Betadine® solution experimental procedure

Fifty µl of normal saline solution was added into the 2nd to 9th wells and another 50 µl of normal

saline solution was added into the 10th well which was the control well.

One hundred µl of PVP-I was added into the 1st well, then 50 µl of 10% PVP-I of the 1st well was brought to the 2nd well and mixed. Another 50 µl of the mixture of the 2nd well was brought in to the 3rd well, the procedures were repeated until the dilution of the 9th well was 1:256 (by discarding the last 50 µl of the mixture of the 9th well).

The nine dilutions (crude, 1:2, 1:4, 1:8, 1:16, 1:32) 1:64, 1:128 and 1:256) and the control wells were tested for their amoebicidal effects of PVP-I in the cyst assay by adding 50 µl of standard cyst suspension, which was obtained by resuspending the counted cysts in the normal saline at the final concentration of 10^4 cysts/ml. The final nine dilutions of PVP-I were then, 1:2, 1:4, 1:16, 1:32, 1:64, 1:128, 1:256 and 1:152. The wells were incubated at 37°C for 24 h, the wells were checked microscopically for the viable cysts. The wells were recultured in the NNA-*E. coli* plate for 7 days to ensure the viability of the cysts. The MCC of PVP-I dilution that resulted in no excystment and trophozoite replication was 1:256 (or 0.04%) at the final concentration, at this concentration there was no viable *Acanthamoeba*.

Procedures for transmission and scanning electron microscopies

Equal volumes of treated (and untreated for control) cells suspension of about 5 ml were centrifuged at 500 g for 2 min and washed with amoeba saline solution 2 times. The pellets were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4. Then they were post-fixed with osmium tetroxide, dehydrated and transferred to propyrene oxide, embedded in araldite resin. The blocks were cut into 90 nm thin section and transferred to copper grids and stained with uranyl acetate and lead citrate for transmission electron microscopy.

For scanning electron microscopy, the pellect of *Acanthamoeba* cysts were dropped on the covered glasses which were subjected to the critical point drying, coated with gold and viewed with a JEOL scanning electron microscope.

Results

Light microscopy

Untreated Acanthamoeba cyst

The *Acanthamoeba* cell, enclosed by its plasma membrane, situates in the cyst wall which are composed of 2 layers, the ectocyst and the endocyst walls. The ectocyst lies outside appears as a scallop or wrinkle while the endocyst which lies inside appears smoother, with a space separating the two layers. The cyst wall has an opening, pore or ostiole, which is sealed by a cap known as the operculum. Inside the endocyst, the cell lies within its plasma membrane, the cytoplasm contains dominantly of several vacuoles and a nucleus (Fig. 1).

Cyst treated with fraction 2 Pouzolzia indica methanolic extract solution

With the minimum concentration of 1:4 all cysts were no longer viable. The light micrographs show the double cyst walls with wrinkle ectocyst and smooth endocyst. The cell inside is clumping, increases the space between the endocyst and the cell membrane, nucleus and other organelles can hardly be defined. Some cells are expelled as many empty cyst walls can be identified (Fig. 2).

Cyst treated with PVP-I solution

Light micrographs present morphological changes of *Acanthamoeba* cysts after being treated with 0.04% of PVP-I which is the MMC. The cysts have double cyst walls, the wrinkled ectocyst and the smooth endocyst. There are shrinkage and assembly of the cell inside the cyst walls, no typical nucleus is found while some cysts show the empty cyst walls as the cytoplasm has been expelled (Fig. 3).

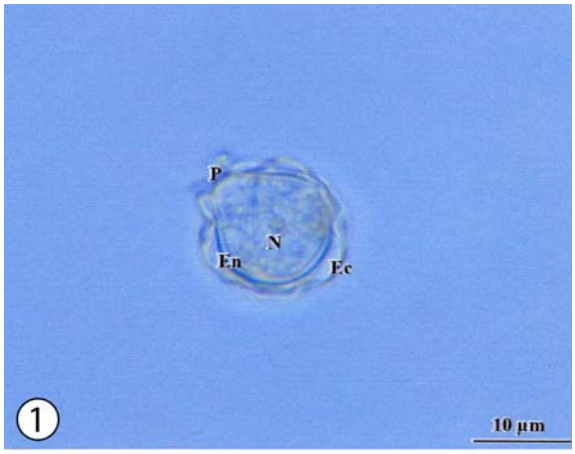

Fig. 1 Light micrographs of normal *Acanthamoeba* shows cell lies within double cyst walls. The outer (ectocyst), shows scalloped pattern while the inner (endocyst) is smooth. There is a pore or ostiole. In the cell, there is a nucleus and vacuoles at the periphery

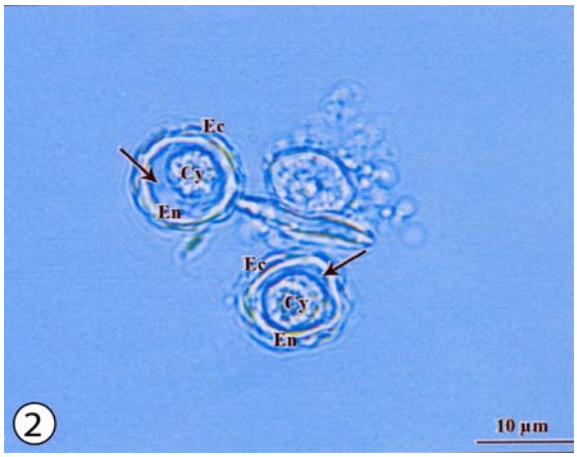

Fig. 2 Light micrograph of *Acanthamoeba* cyst treated with fraction 2 (1:4 final concentration) MCC of *Pouzolzia indica* methanolic extract solution. The ectocyst is wrinkle and the endocystic round. The cytoplasm clumps inside. Many cysts show the shrinkage of amoeba cells from the cyst wall

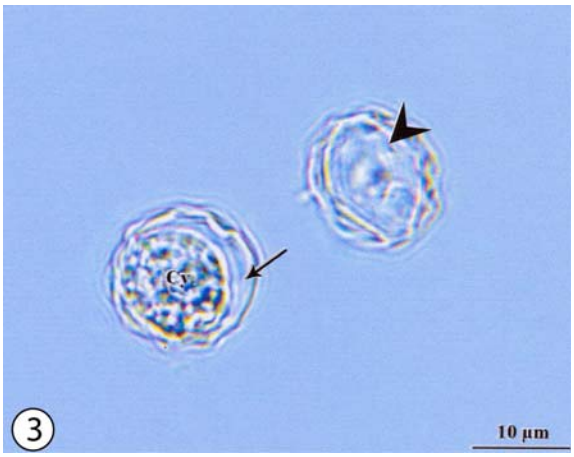

Fig. 3 Light micrograph of *Acanthamoeba* cyst treated with 0.04% PVP-I or Betadine® solution. The ectocyst is wrinkled and the endocyst is smooth, the cytoplasmic clump situates inside the endocyst. The space between the endocyst and the cytoplasm is wider

Transmission electron microscopy

Untreated Acanthamoeba cyst

The cysts have a mean diameter of about 20.75 mm. Oval or spherical cell lies within the double cyst walls, the ectocyst and endocyst. The ectocyst and endocyts appear as electron dense parallel layers separated by a space, meet and fuse at the pore or ostiole where both layers disappear and remain as another thinner electron dense layer that covers the ostiole. The ostiole is closed by a plug or operculum. The operculum is located deep to the thin electron dense layer covering the ostiole and it has the same filamentous appearance as the cyst wall. Inside the cyst wall, a space between the endocyst and the amoeba cell membrane is thin and forms an apparently empty chamber, interrupted at the attachment of the amoeba to the ostiole. The distinctive organelles in the cytoplasm are the nucleus and several clear vacuoles situated at the cell periphery (Fig. 4).

Cyst treated with fraction 2 Pouzolzia indica methonolic extract solution at the final concentration of 1:4

Transmission electron micrographs show the degeneration of the cyst walls, appearing as the stripped electron dense myelin-like structure surrounds the cytoplasm. The inside cytoplasm appears as clumping, with the unidentified plasma membrane. Some micrographs show an empty and ruptured cyst wall as the cytoplasm has been expelled to the environment (Fig. 5).

Cyst treated with 0.04% PVP-I or Betadine® solution

Transmission electron micrographs show a rough and thick electrondense filamentous structure

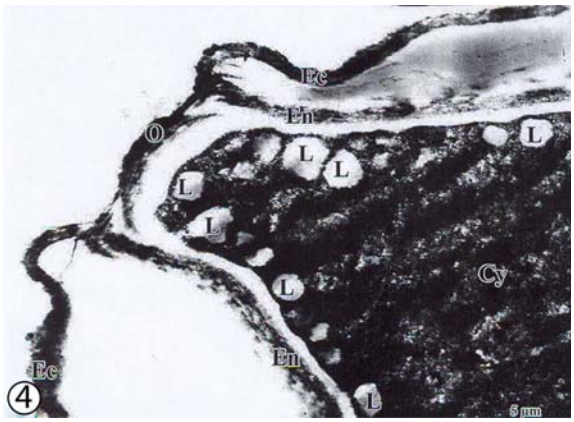

Fig. 4 Transmission electron micrographs of normal *Acanthamoeba* cysts. The walls are composed of the ectocyst and endocyst of fibrillar structures, both meet at the ostiole or pore which is closed by a thin membrane and deeper to it is an operculum of the same electrondensity as the ectocyst wall. Cytoplasmic organelles are nucleus and clear vacuoles situate at the periphery

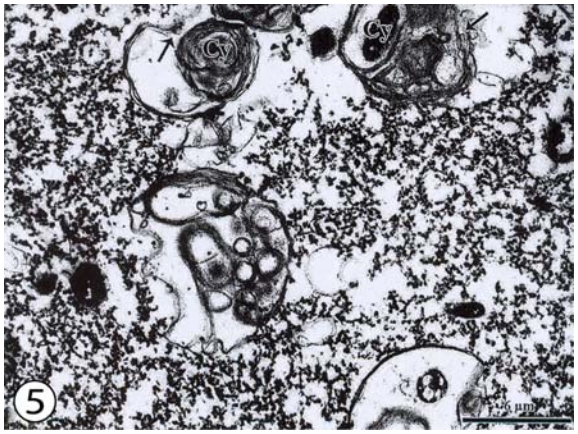

Fig. 5 The *Acanthamoeba* cyst treated with the fraction 2 *Pouzolzia indica* methanolic extract at the MCC. The cyst is on the way of degeneration, there are ruptures of ectocyst and/or endocyst, damage of the ostiole, the cytoplasm is clumping and some cyst walls are empty

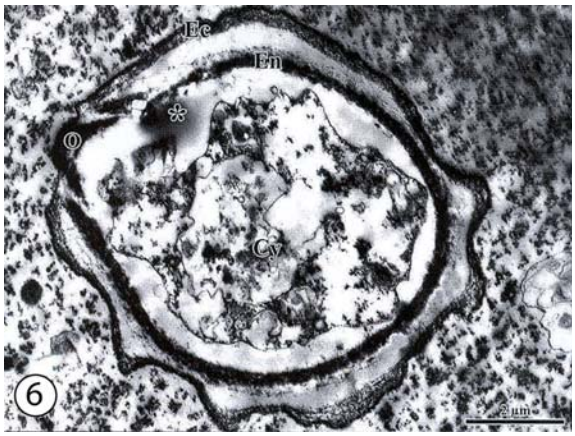

Fig. 6. The *Acanthamoeba* cyst treated with PVP-I or Betadine® at the MCC of 0.04% solution. There are damages of the cyst walls, the cytoplasm is clumping or aggregation, the opercula is degenerated and the space between the cytoplasm and the endocyst is wider

of both ectocyst and endocyst. The ectocyst still exhibits the wrinkle appearance. Some cysts still possess the operculum beneath the pore. The homogenous substance is located in the space between both cyst walls and between the endocyst and the cell. While the cytoplasm is on the way of degeneration and clumping the space between the endocyst and the cell membrane is wider. Some damaged cyto-organelles appear as membrane bound vacuoles scattered throughout the cytoplasm (Fig. 6).

Scanning electron microscopy

Untreated Acanthamoeba cyst

The scanning electron micrographs of the normal cyst show that the cyst are round or polygonal. The ectocysts are wrinkled with thin high ridges over the surface. Some micrographs show the operculum close the ostiole completely (Fig. 7).

Cyst treated with fraction 2 Pouzolzia indica methanolic extract solution

Scanning electron micrographs exhibit the characteristic features of the treated cysts, as sequences of degeneration. The oval cysts become flat, shrinkage and collapse of the ectocyst walls. Some show thick ridges on the wrinkled surfaces, some show edematous appearance and the ridges therefore decrease height. The edematous cysts occur as the opercula are damaged and the outside fluid gets inside the cyst wall, or through the damaged cyst walls. The edematous cyst walls are further ripped and torn into pieces (Fig. 8).

Cyst treated with 0.04% of PVP-I or Betadine® solution

The scanning electron micrographs show the flat and irregular shape which is caused by shrinkage and collapsed of the ectocyst walls. Some show swelling of the cyst with opening of the pores or ostioles. There are also degeneration cysts with the damaged and torn ectocyst wall (Fig. 9).

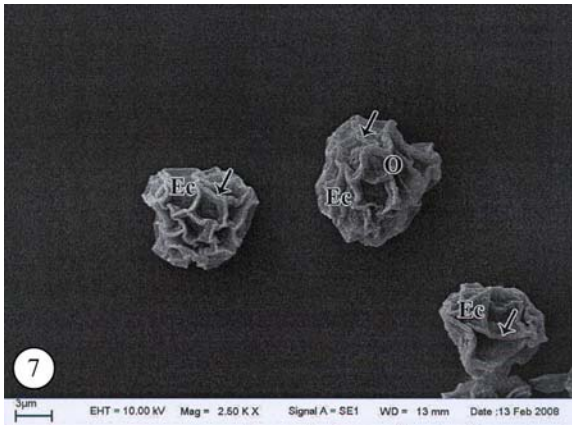

Fig. 7 Scanning electron micrograph of the normal. *Acanthamoeba* cyst. The ectocyst shows the typically wrinkle appearance. The ridges of the wrinkle are high

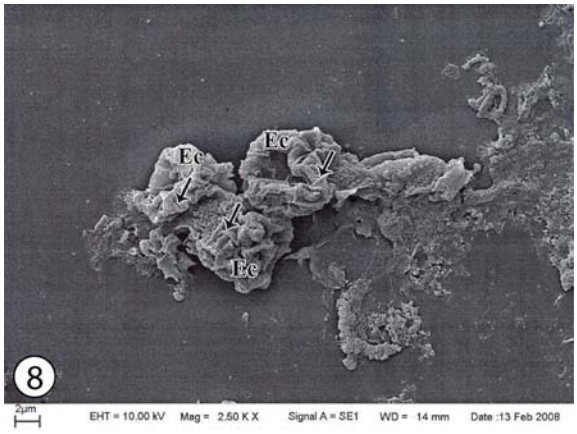

Fig. 8 The scanning electron micrograph of the *Acanthamoeba* cyst treated with fraction 2 *Pouzolzia indica* methanolic extract of MCC at 1:4. The ectocyst wall undergoes series of degeneration, start with shrinkage of the cyst wall, rupture of the cyst wall or operculum, edema of the cyst caused the low wrinkling ridges, the cytoplasm expel and shrinkage of the cyst wall with the cyst wall tearing

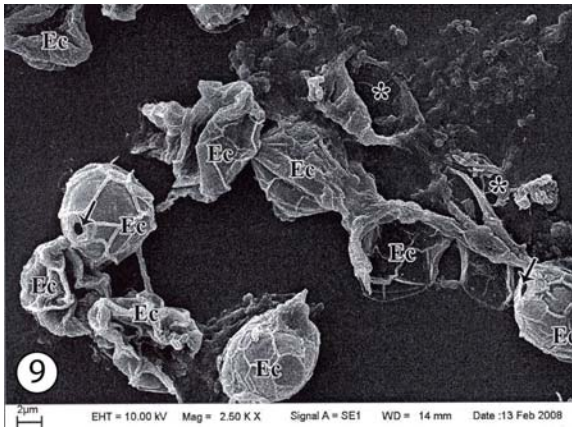

Fig. 9 The scanning electron micrograph of the *Acanthamoeba* cyst treated with PVP-I or Betadine® of MCC at 0.04% concentration. The micrograph shows the shrinkage of the cyst wall, the damaged ostiole with cyst hypertrophy, the collapsed cyst wall with cyst wall tearing

Discussion

In the present study, the antimicrobial agent, the PVP-I or Betadine®, was used to compare the cysticidal effect with the Thai medicinal plant, the *Pouzolzia indica* methanolic extract fraction 2 solution at the minimum cysticidal concentrations. The *Acanthamoeba* cysts were investigated by light and electron microscopics to demonstrate the sequences of the morphological changing from normal to degeneration. The *Acanthamoeba* cysts were killed by the PVP-I and the fraction 2 of *Pouzolzia indica* methanolic extract solutions and the MMC of 0.04% and 1:4, respectively. The light micrographs revealed double cyst walls with the cytoplasmic clumps, some showed empty walls without cytoplasm and some damaged walls. Transmission electron micrographs revealed stripped lamellated cyst walls with damaged cytoplasmic organelles, shrinkage and fragmentation of cytoplasmic components inside the cyst walls. The scanning electron micrographs showed flattened wrinkle strips, swollen and ripped torn cyst walls. These characteristics resembled each other among the cysts treated with *Pouzolzia indica* methanolic extract fraction 2 and PVP-I. These findings indicated the steps of destruction which begin with the shrinkage of the cyst, the opercula destruction, the cyst walls separation, the cytoplasmic clumping, cytoplasmic discharging from the cyst wall whether through the ostiole or through the damaged cyst wall. Then the cysts were swollen by the outside solution passed through the cyst wall, and the height of the wrinkled ridges decreased. The cyst walls were ripped and torn at the end. The fractions 2 of *Pouzolzia indica* methanolic extract was rich in the phenolic compound, which was the major cause of plasma membrane damaging[16] and resulted in leakage of intracellular substance[19]. Phenolic compounds act as an oxidizing agent by reacting at cellular proteins, lipids, nucleic acids and carbohydrates[20,21].

Povidone-iodine (PVP-I or Betadine® solution) has been demonstrated to be a broad range killing of pathogens. In the present study it can kill the *Acanthamoeba* cyst at 0.04% concentration within 24 h[17-19]. Many researchers have examined the effect of PVP-I on several wound healings, greater than 0.05% which was toxic to granulocytes, monocytes and 0.05% which was a safe concentration for fibroblast[22-24].

Although light microscope can be used to demonstrate the *Acanthamoeba* cyst effectively, the electron microscopes were aided as confirmation tools.

Conclusion

The minimal cysticidal concentrations (MCC) of the PVP-I or Betadine® and the *Pouzolzia indica* methanolic extract fraction 2 (water-methanol eluted fraction) are 0.04% and 1:4, respectively. Fifter recultured of the cysts for 7 days, it was confirmed the

MCC and ensured the non-viability of the cyst. Light microscopy aids in viewing the viable of the cyst. The detailed destruction as well as the sequences of cell death was demonstrated by transmission and scanning electron microscopies. The destruction of the cysts begin with shrinkage of the cyst walls, opercula and cyst wall disruption, cytoplasmic clumping and discharged out the cyst wall while the solution outside pass in, resulting in a swollen cyst wall and rupture and ripped into pieces. *Pouzolzia indica* Benn, as a Thai medicinal plant, needs to be further studied; the active ingredients are essential, and may be useful as an antimicrobial agent in the future.

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ขอบชะนางแดงสกัดแฟรกชั่น 2 และ โพวิโดน-ไอโอดีน เหนี่ยวนำให้เกิดการเปลี่ยนแปลงต่ออะแคนธามีบา ระยะซีสต์: ศึกษาโดยจุลทรรศน์แบบแสงและจุลทรรศน์อิเล็กตรอน

จันทิมา รุ่งเรืองชัย, ทิชาพร สุขเกื้อ, ธนวรรณ กุมมาลือ, โกศล รุ่งเรืองชัย

วัตถุประสงค์: *เพื่อเปรียบเทียบผลการทำลายซีสต์ของเชื้ออะแคนธามีบา ของน้ำยาฆ่าเชื้อโพวิโดน-ไอโอดีนกับขอบชะนางแดงสกัดแฟรกชั่น 2 โดยนำเสนอด้วยกล้องจุลทรรศน์แบบแสงและจุลทรรศน์อิเล็กตรอน*

วัตถุและวิธีการ: *เชื้ออะแคนธามีบา แยกจากผู้ป่วยกระจกตาอักเสบ นำมาเลี้ยงบนจานเลี้ยงเชื้อ จนได้ระยะซีสต์ นำมาใส่น้ำเกลือ และปรับความเข้มข้นให้ได้ 10^4 ซีสต์/มิลลิลิตร จากนั้นนำมาใส่โพวิโดน-ไอโอดีน และสารละลายขอบชะนางแดงสกัดแฟรกชั่น 2 ที่ความเข้มข้นต่างๆ เพื่อหาความเข้มข้นที่น้อยที่สุดที่สามารถฆ่าซีสต์ได้ แน่ใจได้โดยเลี้ยงเชื้อต่ออีก 7 วัน ไม่มีซีสต์ที่ยังมีชีวิตอยู่เลย นำซีสต์มาเตรียมต่อ โดยขบวนการจุลทรรศน์อิเล็กตรอนแบบส่องผ่านและแบบส่องกราด*

ผลการศึกษา: *การทำให้ซีสต์ตายด้วยการทำลายโครงสร้างของซีสต์โดยโพวิโดน-ไอโอดีนกับสารละลายขอบชะนางแดงสกัดแฟรกชั่น 2 คล้ายกัน และเป็นขั้นตอน คือ มีการหดลีบของซีสต์ มีการขาดทะลุของผนังซีสต์ หรือโอเปอร์คูลั่มของซีสต์ถูกทำลาย ไซโตพลาสมไหลออก และมีการไหลเข้าของสารละลายภายนอก ทำให้ซีสต์บวมรอยหยักที่เคยมีจึงเตี้ยลง ในที่สุดมีการฉีกขาดของผนังซีสต์เป็นชิ้นเล็กชิ้นน้อย*

สรุป: *ความเข้มข้นที่น้อยที่สุดที่สามารถฆ่าซีสต์ได้ของสารโพวิโดน-ไอโอดีน และขอบชะนางแดงสกัดแฟรกชั่น 2 คือ 0.04% และ 1: 4 ตามลำดับ ดังนั้นขอบชะนางแดงสกัดมีฤทธิ์ฆ่าเชื้อโรคได้ จึงอาจปรับปรุงเป็นน้ำยาล้างเลนส์สัมผัสได้ในอนาคต*