บทคัดย่อ

รหัสโครงการ: MRG6080065

ชื่อโครงการ: ศึกษาเปรียบเทียบการต้านของซีสต์ของเชื้ออะแคนทามีบาต่อน้ำยาฆ่าเชื้อที่ใช้ในสถานพยาบาลและประสิทธิภาพของ Contact lens solutions ในการทำลายเชื้ออะแคนทามีบาจีโนไทม์ T3, T4 และ T5

หมายเหตุ งานวิจัยได้ทำการศึกษาเพิ่มเติม นอกเหนือจากการทดสอบประสิทธิภาพของ contact lens solution ตามที่ได้ขอทุน โดยแบ่งการศึกษาวิจัยเป็น 5 ส่วน คือ

1. ศึกษาประสิทธิภาพของ contact lens solutions ในการทำลายเชื้ออะแคนทามีบา 3 genotypes **(status - manuscript preparation)**
2. ศึกษาประสิทธิภาพของ eye drop solutions (gatifloxacin, levofloxacin และ gentamicin) ในการฆ่าเชื้ออะแคนทามีบา 3 genotypes
   (submitted to International ophthalmology journal-status: under review)
3. ศึกษาประสิทธิภาพของยา miltefosine ในการฆ่าเชื้ออะแคนทามีบา 3 genotypes
   (status - manuscript preparation)
4. ศึกษาประสิทธิภาพของยา antibiotic และ disinfectant ในการทำลายเชื้ออะแคนทามีบา 3 genotypes **(status - manuscript preparation)**
5. ศึกษาประสิทธิภาพของสมุนไพร 3 ชนิด ในการฆ่าเชื้ออะแคนทามีบา 3 genotypes
   (status - manuscript preparation)

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การศึกษาส่วนที่ 1

The efficacy of commercial contact lens solutions against cysts of *Acanthamoeba* spp.

บทคัดย่อ:

เชื้ออะแคนทามีบาคือเชื้อโปรโตซัวพบในสิ่งแวดล้อมทั่วไป ได้แก่ ในน้ำ ในดิน และในอากาศ ทำให้เกิดโรคเยื่อสมองอักเสบและโรคกระจกตาอักเสบ การศึกษานี้คือการทดสอบประสิทธิภาพของน้ำยา contact lens ต่อการต้านเชื้ออะแคนทามีบาซึ่งน้ำยาแช่ contact lens ที่มีสามารถฆ่าเชื้อได้จะช่วยลดความเสี่ยงในการทำให้เกิดโรคกระจกตาอักเสบ ตัวอย่างที่ใช้คือเชื้ออะแคนทามีบา 3 สายพันธุ์ T3, T4 และ T5 การทดสอบทำโดยการบ่มเชื้อในน้ำยา contact lens ที่เวลา 2 4 6 8 และ 10 ชั่วโมงที่อุณหภูมิ 28 องศา หลังจากนั้นทำการตรวจสอบการมีชีวิตของเชื้อโดยการย้อมด้วยสี 0.4 % trypan blue ซึ่งเชื้อที่ตายแล้วจะติดสีฟ้า ส่วนเชื้อที่ยังไม่ตายจะไม่ติดเชื้อ และทำการคำนวณ เปอร์เซ็นการตายของเชื้อ ผลการทดสอบพบว่า น้ำยา Duna สามารถฆ่าเชื้อ T3 ได้ 100% , ฆ่าเชื้อ T4 ได้ 92.22% และ ฆ่าเชื้อ T5 ได้ 96% หลังจากแช่ไปได้ในเวลา 2 ชั่วโมง แต่เมื่อเพิ่มเวลาเป็น 4 ชั่วโมง สามารถฆ่าเชื้อ T3, T4 ได้ 100% สามารถน้ำยา Q eye multi-purpose และ Renu fresh™ multi-purpose ไม่สามารถฆ่าเชื้อได้แม้จะแช่ในน้ำยาเป็นเวลา 24 ชั่วโมง สรุปว่าน้ำยา Duna ซึ่งมีสารสำคัญคือ 0.001% Polidronium Chloride และ 0.0005% Myristamidopropyl Dimethylamine มีประสิทธิภาพในการฆ่าเชื้อได้ เหมาะที่จะใช้เป็นน้ำยารักษาสภาพ contact lens

คำหลัก: Contact lens solutions; *Acanthamoeba* genotypes, Resistance, Keratitis

Abstract

Acanthamoeba are free-living amoebae found in the environment, including soil, freshwater, brackish water, seawater and tap water. *Acanthamoeba* species can cause keratitis and fatal granulomatous encephalitis in humans. *Acanthamoeba* contaminated contact lens care is the risk factor of AK. To study the effects of multipurpose contact lens solutions against the cysts of the three genotypes of *Acanthamoeba* spp. (T3, T4, and T5). *Acanthamoeba* spp. cysts were collected after culture on 1.5% non-nutrient agar plates for 3 weeks. Three different multipurpose contact lens solutions were tested. Each sample was incubated at 28 °C for 2 h, 4 h, 6 h, 8 h, 10 h and 24 h (according to the manufacturers' contact lens soaking time recommendations)**.** After incubation, 0.4 % trypan blue was transferred into the sample both experimental tube and control tube, then counted the percentage of viable reduction**.** The results showed 100% reduction of T3, 96.22% of T4 and 96% of T5 after testing in Duna solution for 2 hours, while T3 and T4 showed 100% reduction after incubation for 4 hours. By contrast, all of three genotypes have resisted to the Renu fresh™ multi-purpose solution and Q eye multi-purpose solution for all incubation times which were 0% reduction. The data demonstrated that the contact lens care solutions which composted of active ingredients of 0.001% Polidronium Chloride and 0.0005% Myristamidopropyl Dimethylamine had the greatest inactivity of three genotypes at 2 hours for T3 and at 4 hours for all genotypes.

Keywords: Contact lens solutions; *Acanthamoeba* genotypes, Resistance, Keratitis

Introduction

Acanthamoeba is a Free-living amoeba, found in the soil, air, in either salt or fresh water, as well as in air-conditioning units, in water mains, swam pools, showers, sanitary and dental equipment and fluids for contact lenses. *Acanthamoeba* spp. is responsible for a more chronic infections, termed granulomatous amoebic encephalitis (GAE) and also a major group of amoebae found to cause keratitis (AK) (Khan, 2006; Marciano-Cabral et al., 2000; Siddiqui and Khan, 2012). First discovered by Nagington et al. (1974) in the UK, *Acanthamoeba* keratitis has been recognized as a significant ocular microbial infection (Khan, 2006). Most cases of *Acanthamoeba* keratitis develop in immunocompetent persons when amoebae infect the corneal surface, either by trauma or by an improperly cared for contact lens or lens case (Schuster and Visvesvara, 2004). It can occur in non-contact lens wearers, it is mostly associated with the use of contact lenses. In Thailand, the first documented case reports of acanthamoebic keratitis

However, the lack of knowledge regarding about amoebae against contract lens solution. To evaluate the efficacy of commercially contract lens solution were against to three genotypes of *Acanthamoeba*. However, Information which greatly helped regarding the susceptibility of *Acanthamoeba* to commercial contract lens solution is essential for reducing and prevention the risk of infection in contact lens wearers.

Materials and methods

Preparation of *Acanthamoeba*.

Acanthamoeba genotypes (T3, T4 and T5) were isolated from natural water samples in the Northeastern provinces of Thailand and cultured on 1.5% non-nutrient agar plates and identification of *Acanthamoeba* was based on morphological and

molecular techniques using PCR and DNA sequencing, as described previously (Thammaratana et al., 2016).

Contact lens solutions.

Three different multipurpose contact lens solutions were tested; Renu fresh™ multipurpose solution, Duna no rub multipurpose solution and Q eye multipurpose solution. These 3 solutions were selected for this study because it was commonly brands used by Thai people. The solutions used in this study, along with their active ingredients are listed in Table 1.

Table 1. Contact lens solutions used in this study and their ingredients.

Contact lens solution	Active ingredient	Other ingredients	Recommended time
Renu fresh™ multi-purpose solution	Polyaminopropyl biguanide 0.0001% (DYMED™)	Hydroxyalkylphosphonate (Hydranate), boric acid,edetate disodium,Poloxamine, sodium borate, sodium chloride	≥ 4 h
Duna no rub multi-purpose solution	POLYQUAD® (Polidronium Chloride) 0.001% and ALDOX® (Myristamidopropyl Dimethylamine) 0.0005%	Aminomethylpropanol, Sodium Chloride, Boric Acid, Sorbitol, Aminomethylpropanol and Edetate Disodium	≥ 6 h
Q eye multi-purpose solution	Polyhexamethylene Biguanide Hydrochloride 0.0001%	Poloxamer, Sorbitol, Dexpanthenol, Trometamol, Disodium Edetate, Sodium Dihydrogen Phosphate Dihydrate.	≥ 6 h

Experimental design and susceptibility assay.

To study the efficacy of multipurpose contact lens solutions against cysts of three genotypes of *Acanthamoeba* spp. (T3, T4, and T5), cysts of each genotype were collected after 3 weeks culture on 1.5% non-nutrition agar. Cysts were harvested from the agar plates by scraped with an inoculating loop, washed two times with 15 ml of phosphate buffer saline (PBS), the cysts were centrifuged at 2,000 rpm at 28 ℃ for 5 min after that the number of cysts were counted by using a hemocytometer and adjusted to yield 10^4 cysts per 1 ml. Using a 15 ml centrifuge tube, fifty microliters (50 µl) of the calibrated cyst suspension was inoculated into each tube, the cysts were centrifuged at 2,000 rpm at 28 ℃ for 5 min to avoid disturbance of adherence of amoebae onto the tube' surface. The supernatant PBS solution was removed around 30 µl and 100 µl of each multipurpose contact lens solutions was added into the tube. The tube was incubated at 28 ℃ for 2 h, 4 h, 6 h, 8 h, 10 h and 24 h (according to the manufacturers' contact lens soaking time recommendations). In addition, controls containing only the parasite in PBS as a non-treated. Each experiment was performed in duplicate. After incubation times period, 50 µl of 0.4 % tryphan blue was transferred into test and control tube, left for 10 min then counted by simple smear under light microscope. Each examination was performed in triplicate. Unstained with dye cysts were viable and stained cysts were nonviable.

Counting the number of viable cyst and nonviable cysts after testing and staining with 0.4% tryphan blue was done using the simple smear under light microscope (Degerli et al., 2012). The percent of growth reduction according to the equation: Percent of growth reduction (A−B/A×100), that A is the mean number of cysts in control and B is the mean number of cysts in contract lens solution treated

was calculated (Palmas et al., 1984). Non-viable cysts, an additional test was performed to confirm the results obtained. After incubation, the non-viable cysts were washed by PBS and then centrifugation at 2,000 rpm at 28 °C for 5 min, inoculated on non-nutrition agar plates 1.5% (NNA) with *Escherichia coli*, and incubated at 30°C. The plates were examined after 3 days under light microscope for the presence of trophozoites stage and the efficacies of the contact lens solutions were recorded as growth or not (Ezz Eldin and Sarhan, 2014).

Statistical analysis.

Statistical analysis was performed using the percentage of growth reduction to compare the control of three genotype of *Acanthamoeba* cysts.

Results

Of the three contact lens solutions that were examined for their efficacies in inactivating cysts of the three genotype of *Acanthamoeba* species, one of these solutions that contained POLYQUAD® (Polidronium Chloride) 0.001% and ALDOX® (Myristamidopropyl Dimethylamine) 0.0005% (Duna) demonstrated the greatest inactivation of cysts of all three genotype of *Acanthamoeba* (Table 2). These contact lens solution indicated that decreasing numbers of amoeba with increasing exposure times at 2 h until 24 h (Figs 1-3), the percentage of reduction in genotype T3 was reduced 100%, T4 was reduced 96.22% and T5 was reduced 96.0% after 2 hours. While after 4 to 24 h, the greatest percentage of reduction in genotype T3, T4 and T5 were reduced 100% completely inactive cyst of three genotyping compared with the similar times of control. By contrast all of three genotype were the most against to the Renu fresh™ multi-purpose solution and Q eye multi-purpose solution after incubation 2 to 24 h , the percentage reduction in T3, T4, T5 were not reduced.

After collected the band of contact lens solution at the time that completely inactivated cyst and then the cyst were inoculated on non-nutrition agar plates 1.5% (NNA) with *Escherichia coli*, and incubated at 30°C for 3 days indicated that the most of all time (2, 4 and 6 h) of three genotyping cannot growth on the agar plate.

Table 2. Percentage of viable reduction and Mean count of three genotypes of *Acanthamoeba* spp. after exposure to different contact lens solution.

Band	Time	T3		T4		T5	
		Mean	%	Mean	%	Mean	%
Renu fresh™ multi-purpose solution	2 h	101.33	0.00	110.67	0.00	34.33	0.00
	4 h	90.67	0.00	109.67	2.00	33.33	2.00
	6 h	93.67	0.00	93.33	1.11	30.00	1.11
	8 h	90.67	0.00	104.67	7.97	46.00	7.97
	10 h	79.67	0.00	122.33	0.00	32.33	0.00
	24 h	98.00	0.00	117.00	8.65	34.67	8.65
Duna no rub multi-purpose solution	2 h	150.67	100	123.33	96.22	35.33	96.00
	4 h	137.33	100	97.00	100	44.67	100
	6 h	121.33	100	103.67	100	34.67	100
	8 h	130.33	100	144.67	100	40.33	100
	10 h	127.00	100	145.00	100	33.67	100
	24 h	121.33	100	119.67	100	38.67	100
Q eye multi-purpose solution	2 h	91.67	0.00	108.67	0.00	39.00	0.00
	4 h	79.67	0.00	114.33	0.00	30.33	0.00
	6 h	94.00	0.00	100.33	0.00	27.00	0.00
	8 h	89.00	0.00	115.00	0.00	26.67	0.00
	10 h	111.67	0.00	74.67	0.00	32.33	0.00
	24 h	100.00	0.00	118.67	0.00	37.33	0.00
Control	2 h	151.67	0.00	163.67	0.00	44.00	0.00
	4 h	138.33	0.00	147.67	0.00	33.33	0.00
	6 h	122.67	0.00	108.00	0.00	34.67	0.00
	8 h	107.00	0.00	121.33	0.00	30.67	0.00
	10 h	144.67	0.00	133.67	0.00	35.00	0.00
	24 h	122.33	0.00	132.33	0.00	39.67	0.00

Figure 1. Effect of contact lens solution to Genotype T3

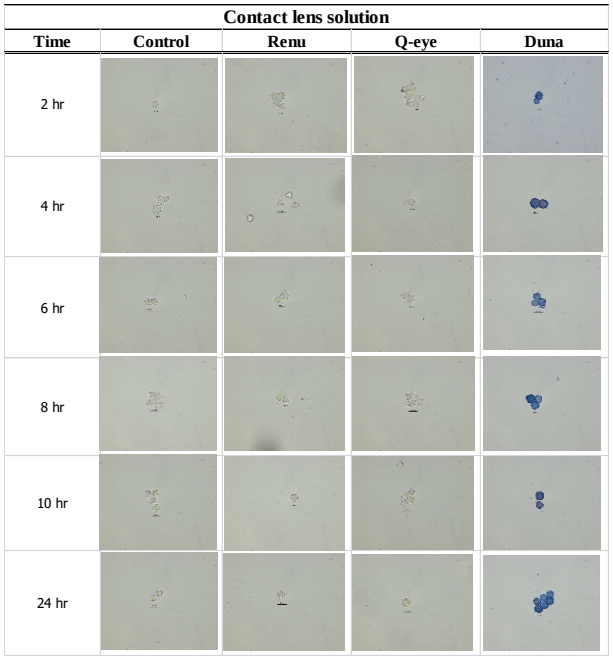


Figure 2. Effect of contact lens solution to Genotype T4

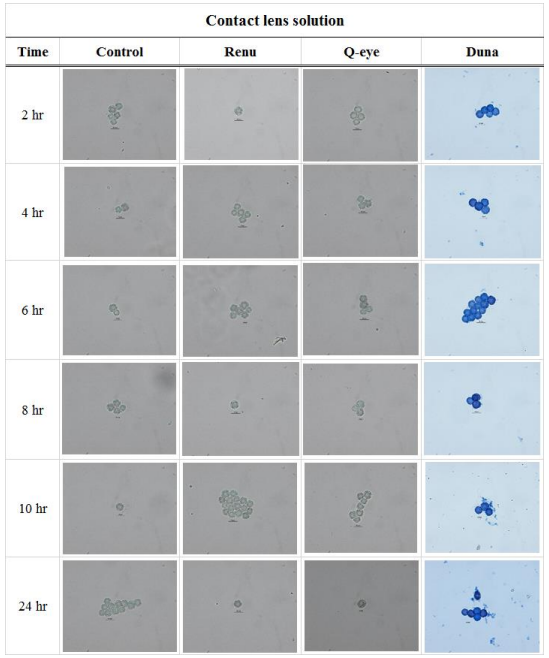
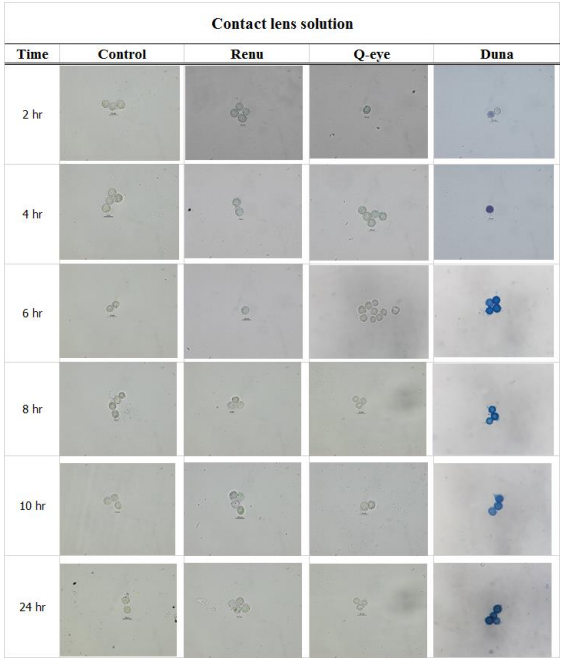


Figure 3. Effect of contact lens solution to Genotype T5



Discussion

In this study, genotype T3 of *Acanthamoeba* cysts were more sensitive to Duna solution which has the ingredients of 0.001% Polidronium Chloride and 0.0005% Myristamidopropyl Dimethylamine after incubation for 2 hours when compared with genotype T4 and T5 which were sensitive at 4 hours. On the other hand, all three genotypes were resist to other two contact lens solutions, Renu fresh™ multi-purpose solution and Q eye multi-purpose solution which composed of Polyaminopropyl 0.0001% biguanide and Polyhexamethylene Biguanide, 0.0001% Hydrochloride, respectively. In the prior study, showed that ReNu with MoistureLoc successfully killed both *A. castellanii* and *A. polyphaga* cysts within the MMRDT, but Complete Moisture Plus, Solo-care Plus, and Optifree Express did not success (Borazjani and Kilvington, 2005). ReNu with MoistureLoc contains Alexidine 0.00045%, whereas ReNu and MultiPlus contain polyaminopropyl biguanide 0.0001%. Although the relevant ingredients in contact lens solution in this study were not exactly the same as those used in the previous studies, they contained the same active agents against *Acanthamoeba*. The concentration of active ingredients of the contact lens solution (Except Duna) was lower than the effective concentration against three genotype of *Acanthamoeba* cysts. The cysticidal activity of contact lens solutions was directly proportional to the soaking time of the organisms in the solution.

Acanthamoebic keratitis is one of the most threatening diseases associated with contact lens use. Diagnosis and treatment of the disease is difficult because the clinical manifestations are similar to other kinds of microbial keratitis, as well as unfamiliarity of clinicians due to the low prevalence of the disease, and the limitations of therapeutic alternatives. Therefore, good lens hygiene is extremely important for contact lens users to prevent ocular infections which can lead to serious microbial keratitis. Contact lens wearers should strictly follow the proper lens

care guidelines and wearing instructions provided by their eye care professional. The efficacy of commonly available CLS in use should be communicated to users to help prevent *Acanthamoeba* infection of the cornea. The manufacturers' minimum recommended disinfection times for the products for killing *Acanthamoeba* must be reconsidered. CLS effective against *Acanthamoeba* should be investigated and adequate exposure times determined and communicated to contact lens users.

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การศึกษาที่ 2

Evaluating the *in vitro* efficacy of gatifloxacin, levofloxacin and gentamicin against *Acanthamoeba* cysts

บทคัดย่อ:

ทดสอบประสิทธิภาพของยาหยอดตา 3 ชนิด ที่มีตัวยา gatifloxacin, levofloxacin และ gentamicin ในการฆ่าเชื้ออะแคนทามีบา 3 สายพันธุ์ คือ T3, T4 และ T5 โดยบ่มเชื้อในยาหยอดตาแต่ละชนิดที่เวลา 1, 24, 48 และ 72 ชั่วโมง ที่อุณหภูมิ 37 องศา หลังจากนั้นทดสอบการตายของเชื้อด้วยวิธีย้อมสี 0.4% trypan blue, เพาะเลี้ยงในอาหารเลี้ยงเชื้อ NNA และ การเลี้ยงเชื้อใน PYG medium และการทดสอบพบว่า ยาหยอดตาที่มีตัวยา gentamicin ฆ่าเชื้อทั้ง 3 สายพันธุ์เมื่อบ่มที่ 1 ชั่วโมง และ ตัวยา gatifloxacin ฆ่าเชื้อทั้ง 3 สายพันธุ์เมื่อบ่มที่ 24 ชั่วโมง สำหรับตัวยา levofloxacin ไม่สามารถฆ่าเชื้อทั้ง 3 ชนิดได้ถึงแม้จะทำการบ่มเป็นเวลา 72 ชั่วโมง สรุปได้ว่าตัวยา gentamicin และ gatifloxacin เหมาะที่จะใช้ในการรักษาอาการของผู้ที่ตาอักเสบที่สงสัยว่าอาจจะเกิดจากการติดเชื้ออะแคนทามีบาโดยเฉพาะถ้าใช้รักษาในระยะการติดเชื้อเริ่มแรกจะช่วยป้องกันการดำเนินของโรคไม่ให้รุนแรงได้

คำหลัก: Gatifloxacin • Gentamicin • levofloxacin • *Acanthamoeba* cysts • Ophthalmic drugs

Abstract

Purpose To evaluate the *in vitro* efficacy of three commercial ophthalmic solutions (gatifloxacin, levofloxacin and gentamicin) against cysts of *Acanthamoeba* species.

Design Experimental study *Methods Acanthamoeba* cysts belonging to genotypes T3, T4 and T5 were incubated with three ophthalmic solutions for different periods of time; 1, 24, 48 and 72 h at 37 °C. After incubation, treated cysts were stained with trypan blue and counted to express the percent of growth inhibition. Additionally, the viability of treated cysts was assessed by culturing them in PYG medium at 30 °C for 72 h as well as on non-nutrient agar plates at 30 °C for 1 month.

Results Acanthamoeba cysts of all genotypes were susceptible to gentamicin and gatifloxacin after exposure for 1 h and 24 h, respectively. No genotypes were susceptible to levofloxacin even after 72 h of incubation.

Conclusions Two out of three ophthalmic drugs were effective against *Acanthamoeba* cysts. These were gentamicin and gatifloxacin. Although our results need to confirm in animal models, this result will guide the choice of the appropriate ophthalmic drugs for early treatment of eye infection caused by *Acanthamoeba* spp.

Keywords Gatifloxacin · Gentamicin · levofloxacin · *Acanthamoeba* cysts · Ophthalmic drugs

Introduction

Free-living amoebae of the genus *Acanthamoeba* are the worldwide distribution in a variety of habitats such as soil, water and even in the air [1]. There are two forms in their life cycle, a vegetative trophozoite stage, and a highly resistant cyst stage. Trophozoite can develop into protective cyst form when environmental conditions become adverse. Based on the variation of nucleotide sequences of the Diagnostic fragment 3 (DF3) region of the 18S rRNA gene, *Acanthamoeba* spp. have been classified into 20 genotypes, T1-T20 [2]. *Acanthamoeba* species can cause serious infections, granulomatous amoebic encephalitis (GAE) which is rare but high fatality rate in immunocompromised persons and amoebic keratitis (AK) in immunocompetent persons, which can result in poor vision or blindness if delay in diagnosis and inadequate treatment. In developing countries, AK has been reported in people previously suffering from corneal ulcers and having contact with contaminated water and soil, even without wearing contact lenses [3]. In Thailand, AK cases have been reported among both contact lens users and non-contact lens users. Twenty-four AK cases occurred in Thailand between 1996 and 2006 [4, 5], of which five were reported in 1999 [6]. Misdiagnosis of AK is common because signs and symptoms are not specific and can mimic bacteria, fungal, or viral keratitis [1] together with lacking of consensus of AK diagnosis, these lead to AK remains significant. Therefore, AK treatment depends on clinicians' experience. At present, the first-line drugs for AK are biguanides, such as chlorhexidine and PHMB, and diamidines such as propamidine isethionate and neomycin. Although the treatment of AK often requires a combination of biguanides and diamidines [7], not all AK cases are cured with these [8]. Trophozoites are usually susceptible to biocides including therapeutic drugs. The majority problem for AK treatment is therefore, unable to eliminate cyst stage completely [9]. Various therapeutic drugs have been studied *in vitro* by

focusing on cysticidal efficacy, as effectiveness is essential for subsequent studies *in vivo* therapy of *Acanthamoeba* keratitis [10-12]. However, their protocols have not been standardized for using and no eye drops preparations are commercially available. Moreover, there are no fully effective against all strains in the available treatments. Therefore, it is necessary to study new therapeutic agents to be the alternative drugs for AK treatment.

The aim of this study was to evaluate the *in vitro* efficacy of three commercially available as ophthalmic solutions (gatifloxacin, levofloxacin and gentamicin) against cysts of three *Acanthamoeba* strains, Thailand isolates. These ophthalmic drugs are usually used to treat keratitis caused by bacteria. Chlorhexidine, first-line AK treatment, was used as a reference drug. This study will guide the choice of drug to treat early stages of eye disease caused by *Acanthamoeba* spp.

Methods

Acanthamoeba strains and cysts preparation

Three environmental strains of *Acanthamoeba* were examined: GenBank accession numbers KT897271 (T3), KT897265 (T4) and KT897268 (T5) [13]. Each strain was cultured onto non-nutrient agar plates which were seeded with 5 µl of heat-killed *Escherichia coli* and incubated at 30 °C for 3 weeks. The cysts were harvested by washing plates with phosphate buffered saline (PBS) and decanted into 15 ml tubes which were centrifuged at 3,000 rpm for 5 min. The supernatant was discarded and 0.5% sodium dodecyl sulfate (SDS) added for 10 min to lyse immature cysts and then the tubes were centrifuged at 3000 rpm for 5 min. The SDS was washed out twice using PBS. Finally, cysts were counted with a hemocytometer and standardized to a concentration of 20×10^4 cysts/ml.

Chemicals

Four antimicrobial agents were tested: chlorhexidine (Sigma-Aldrich, USA) was prepared at the commercially-available concentrations (0.02%) and used as reference drug, and three ophthalmic solutions, 0.3% gentamicin (3 mg/ml) (Seng Thai Pharmaceutical Laboratory, Bangkok, Thailand), 0.3% gatifloxacin (3 mg/ml) (Allergan Sales, Texas, USA), and 0.5% levofloxacin (5 mg/ml) (Santen Pharmaceutical Laboratory, Osaka, Japan).

Evaluation of the cysticidal activity

Qualification assays

Experiments for eye drops testing on cysts were performed in the sterile microtube. Briefly, 100 µl of each of calibrated *Acanthamoeba* strain (20×10^4 cysts/ml) was incubated with 100 µl of each eye drop for 1, 24, 48 and 72 h at 37 °C, each in duplicate. After incubation, the suspension was washed twice with PBS to eliminate residual drugs and resuspended with 100 µl PBS. The cyst viability was examined by taking 20 µl of sample into NNA medium coated with heated *Escherichia coli* and incubated at 30 °C, examining the samples every day by microscope for 1 month. The remaining content was then cultured in 500 µl axenically PYG medium containing 50 µg/ml enrofloxacin (General Drugs House, Bangkok, Thailand) and incubated at 30 °C for 72 h in 96-well microtiter plate and examined by inverted microscope. The presence of trophozoite was considered as drug-resistant strain.

Quantification assays

For growth inhibition evaluation, 100 µl of treated cysts of each eye drop were stained with 100 ul 0.4% trypan blue for 10 min, unstained cysts were viable and stained cysts were nonviable [14]. Viable and nonviable cysts were then counted using the hemocytometer at 1, 24, 48 and 72

h. The percent of growth inhibition was calculated: Percent of growth inhibition = a−b/a×100 (a; mean number of a non-treated cysts, b is the mean number of treated cysts) [15].

All assays, both qualification and quantification were repeated five times, each in duplicate, for each strain. In addition, control containing cysts treated with 0.02% chlorhexidine gluconate as a reference drug control and cysts in PBS as non-treated control, were performed to the same procedure. The effect of eye-drops on cysts for morphological observation was examined by a light microscope (×40).

Data analysis

Data management and analysis were made using SPSS version 19.0 for Windows. Cyst number include viable and nonviable cysts were calculated as percent of growth inhibition. The mean numbers were compared using Student's t test. *P* values<0.05 were considered as statistically significant, *P* values<0.001 were considered as statistically highly significant.

Results

Cysticidal activity assays

All *Acanthamoeba* strains were susceptible to 0.3% gentamicin and 0.3% gatifloxacin. In contrast, there were no completely killed even after 72 h of exposure to 0.5% levofloxacin. The percent of growth inhibition of *Acanthamoeba* species after incubation to each eye drop for different exposure time was shown in Fig 1; both gatifloxacin and gentamicin showed highly significant difference (*P*<0.001) and levofloxacin showed no statistically significant difference as compared with non-treated control.

In addition, treated cysts were inoculated into PYG medium and on NNA medium overlaid with heated *Escherichia coli* for qualification viability tests. The results from NNA culture were correspond to PYG culture; gentamicin and gatifloxacin were highly effective against *Acanthamoeba* cysts of all genotypes after exposure 1 h and 24 h, respectively. In contrast, levofloxacin had the lowest effectiveness against all three genotypes. Even after incubation with this drug for 72 h, cysts of all three genotypes were capable of yielding trophozoites in the viability test. Figure 2 showed the results of *Acanthamoeba* cysts of genotype T4 treated with each eye drop for 72 h and then cultured in PYG medium. After observation by inverted microscope (×20), the results revealed there were no excysted trophozoites in samples treated with 0.02% chlorhexidine, 0.3% gentamicin and 0.3% gatifloxacin, but presented in sample treated with 0.5% levofloxacin. The other two strains, T3 and T5 genotypes, also revealed the same results but data not shown. The altered morphology with cytoplasm destruction after 24 h of incubation with each effective eye drop, chlorhexidine, gatifloxacin and gentamicin, were shown in Fig 3.

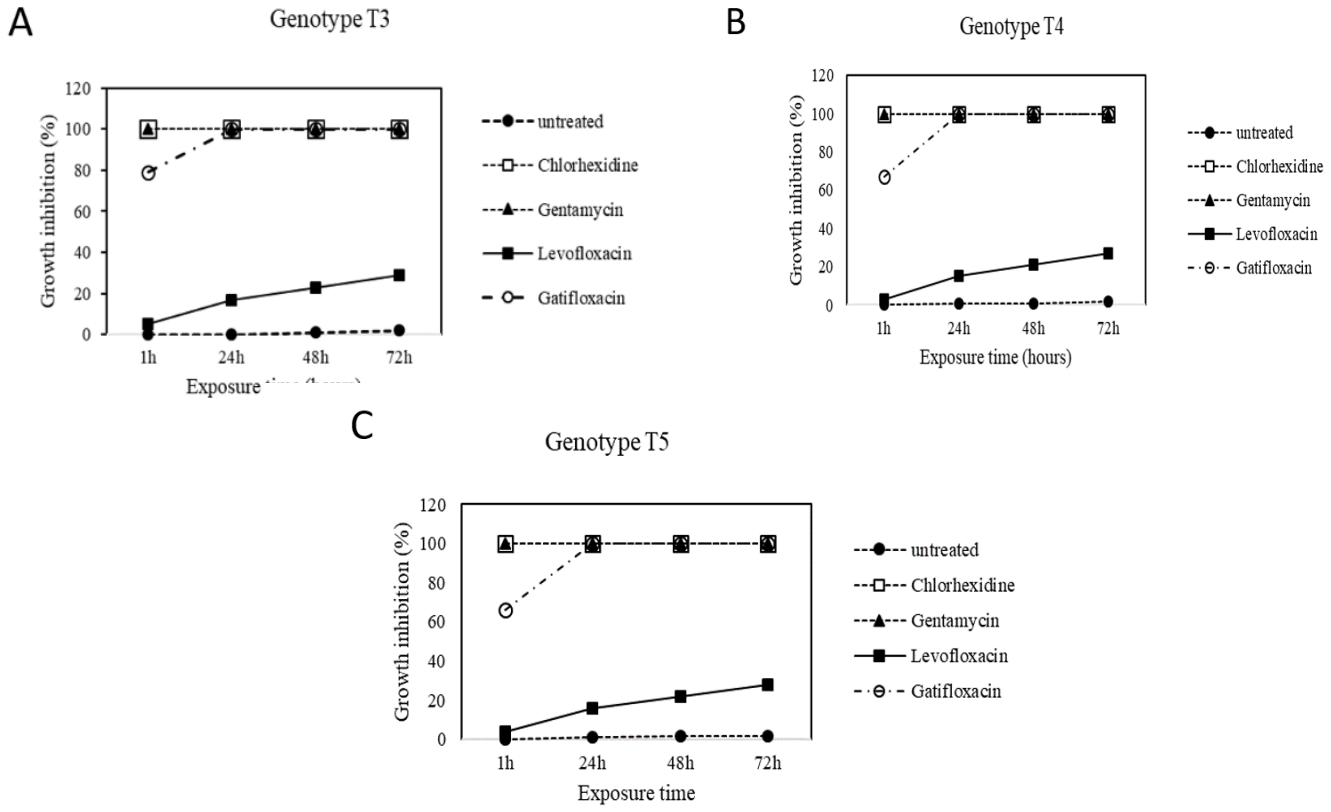

Fig. 1 Percent of growth inhibition of *Acanthamoeba* species after exposure to 0.02% chlorhexidine, 0.3% gentamicin, 0.3% gatifloxacin and 0.5% levofloxacin for different periods of time. A. *Acanthamoeba* genotype T3; B. *Acanthamoeba* genotype T4; and C. *Acanthamoeba* genotype T5

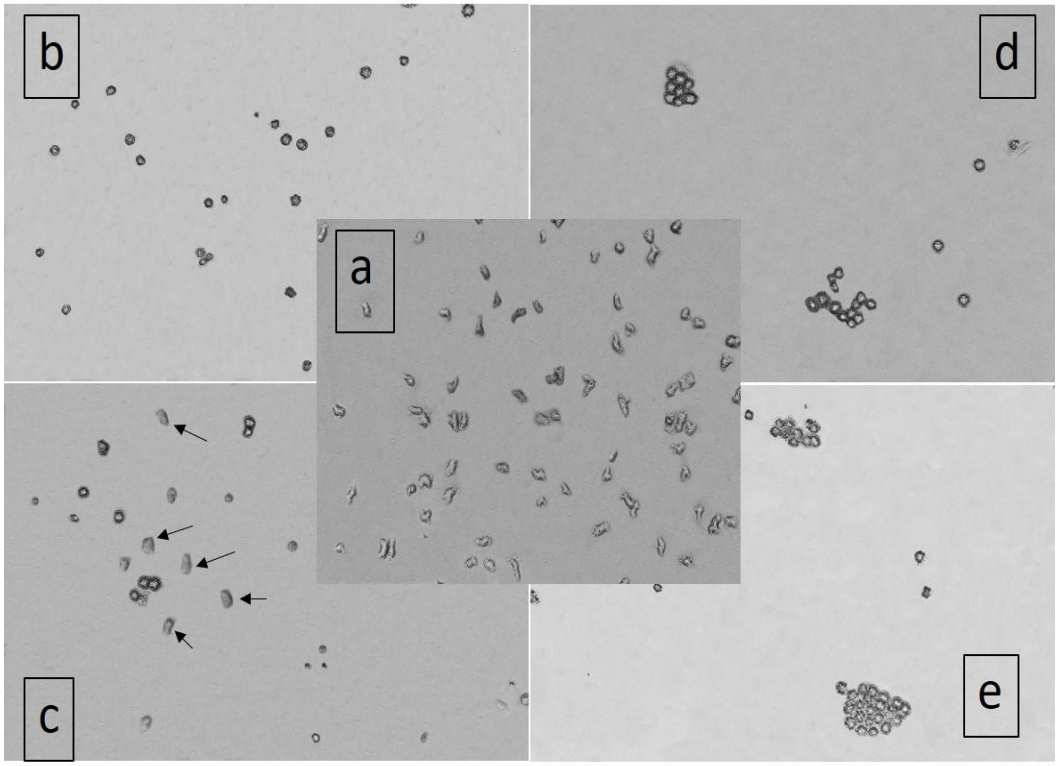

Fig. 2 *Acanthamoeba* cysts of T4 genotype treated with each eye drop for 72 h and the viability were tested by PYG culture for 72 h and observed by inverted microscopy (×20). a. non-treated control; b. 0.02% chlorhexidine c. 0.5% levofloxacin; d. 0.3% gentamicin; e. 0.3% gatifloxacin

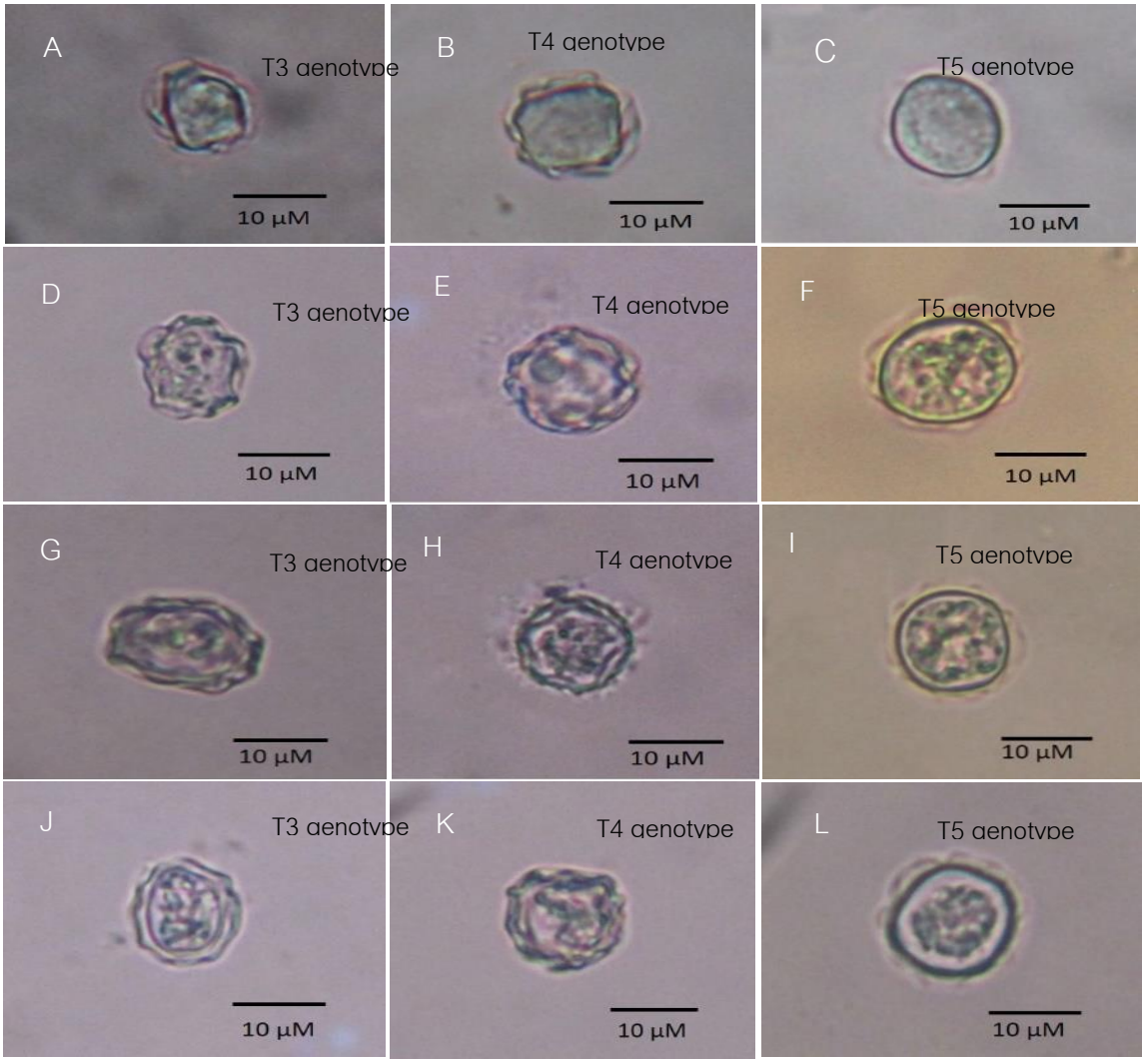

Fig. 3 Effect of each eye drop against *Acanthamoeba* cysts of three genotypes after exposure for 24 h, then observed by light microscopy (×40). A-C. untreated cyst; D-F. treated with 0.02% chlorhexidine; G-I. treated with 0.3% gentamicin; J-L. treated with 0.3% gatifloxacin

Discussion

Our study has presented the susceptibility of cysts of three *Acanthamoeba* strains, to three commercially available as eye drops, 0.3% gentamicin, 0.3% gatifloxacin and 0.5% levofloxacin. To determine the cysticidal activity of the drugs, we examined the treated cysts by culturing in PYG medium for 72 h and on NNA medium for 1 month to make sure there were completely killed for qualification assay, and staining with trypan blue for quantification assay. Gentamicin and gatifloxacin could completely kill cysts of all three genotypes after 1 h and 24 h of incubation, respectively. Whereas levofloxacin could not even after 72 h of exposure. These drugs are primarily known for killing bacteria. In this study, gentamicin had the highest cysticidal activity because it could kill using the shorter time. Another study has found that a lower concentration of gentamicin was also effective against clinical and environmental strains of *Acanthamoeba* [16]. They reported the mean minimum cysticidal concentration (MCC) at 30 °C in all tested stains was 0.193 mg/ml and 0.029 mg/mL at 37 °C (range 0.031-1 mg/ml) after incubation for 48 h. Gentamicin is a member of the aminoglycoside group act by inhibiting protein synthesis [17]. Another member of the aminoglycoside group that have been reported, to have high efficacy against *Acanthamoeba* cysts such as neomycin sulfate and tobramycin. [18]. There have been no previous studies using gatifloxacin and levofloxacin alone *in vitro* for amoebicidal activity. Gatifloxacin, a fourth-generation fluoroquinolone antibiotic, affected morphology of cysts belonging to genotypes T3, T4 and T5 after 24 h. Its activity is the inhibition of DNA replication. In this study, levofloxacin was the least effective of the three drugs tested against on cysts stage. Levofloxacin is in the same group as gatifloxacin, members of the fluoroquinolone group, but produced different results. *Acanthamoeba* cysts belonging to genotype T3, T4 and T5 were resistant to this solution at 5 mg/ml despite 72 h of incubation.

However, it was successful at 5 mg/ml as part of combined drug treatment of bacterial coinfection in an *Acanthamoeba* keratitis case [19]. As used by us *in vitro*, drugs were diluted to 50% of their supplied concentration. In clinical use, drugs applied to the eye will also be diluted, and eventually flushed out, by the production of lachrymal fluid [20].

We have presented data showing that commercially available ophthalmic drugs differ in their ability to kill *Acanthamoeba* cysts. Three different genotypes of *Acanthamoeba* were selected based on their potential to be pathogenic strains that can cause keratitis [1]. Gentamicin and gatifloxacin have high ability for cyst killing. Although our results need to confirm *in vivo* study, these two drugs may be the optimum choices for treating the early stages of eye infection caused by *Acanthamoeba* spp. However, not only the efficacy of drugs should be considered in AK treatment, but also issues relating to the duration of treatment and the effects of dilution by the lachrimal fluid.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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การศึกษาที่ 3

Evaluating the *in vitro* cysticidal activity of miltefosine against *Acanthamoeba* genotype T3, T4 and T5

บทคัดย่อ:

เชื้ออะแคนทามีบา ก่อโรคที่สำคัญโรคกระจกตาอักเสบ โรคเยื่อสมองอักเสบและ โรคในระบบอื่นๆที่เกิดจากการแพร่กระจายของเชื้อไปตามกระแสเลือด การกำจัดเชื้อนะยะซีสต์จะช่วยป้องกันการเสียชีวติที่เกิดจากการการติดเชื้อได้ การศึกษาวิจัยนี้เป็นการศึกษาประสิทธิภาพของยา miltefosine ในการฆ่าเชื้ออะแคนทามีบาระยะซีสต์ เพื่อหาความเข้มข้นที่น้อยที่สุดของยาที่สามารถฆ่าเชื้อระยะซีสต์ได้ 100% ได้เตรียมความเข้มข้นตั้งแต่ 9.81, 19.62, 24.53 และ 49.06 mM ตามลำดับ และหลังการบ่มเชื้อกับยาที่ความเข้มข้นต่างๆ คือ 1, 3, 5, 7 วันและตรวจสอบการตายของเชื้อ โดยเลี้ยงเชื้อในอาหารเลี้ยงเชื้อ NNA และ ย้อมสีด้วย 0.4% trypan blue ผลการศึกษาพบว่า ความเข้มข้นน้อยที่สุดที่จะฆ่า T4 T5 ได้คือ 24.53 mM สำหรับ T3 ต้องใช้ความเข้มข้นน้อยที่สุดคือ 49.06 mM ผลการศึกษาสามารถใช้เป็นแนวทางการโดยใช้ยา miltefosine หรือใช้ยาดังกล่าวร่วมกับยาอื่นเพื่อผลการรักษาที่มีประสิทธิภาพ

คำหลัก: *Acanthamoeba* cyst, Miltefosine, Genotypes, Cysticidal

Abstract

Acanthamoeba species are known as the causative agents of amoebic keratitis, GAE, and disseminated infection. Miltefosine, anticancer drug, had been developed and currently recommended for treatment of *Acanthamoeba* infection. However, there is no full treatment regimnt are not established, particularly for killing of cyst stage. In the present study, we present the cysticidal concentration against three *Acanthamoeba* strains belonging to T3, T4, and T5, Thailand isolates. The study demonstrated that miltefosine exhibited the minimal cysticidal activity against T4 and T5 at the concentration of 9.81, 19.62, 24.53 and 49.06 mM, while against T3 needs the concentration of 9.81, 19.62, 24.53 and 49.06 after 7, 5, 3 and 1 days, respectively. Although there are variable in regiment treatment of *Acanthamoeba* in each species. This study will be the guideline for optimizing therapy or consider to use the combination with other amoebicidal drugs.

Key words: *Acanthamoeba* cyst, Miltefosine, Genotypes, Cysticidal

Introduction

Acanthamoeba is a genus of free-living amoebae distributed worldwide in the environment (fresh water, brackish water, soil and even in the air) and known to be a causative agent of acanthamoebiasis (amoebic keratitis, granulomatous amoebic encephalitis and other disseminated diseases).[1,2] As its abundance, various genotypes of *Acanthamoeba* are accounted for diseases. In Thailand, these amoebae have been detected from patients and also isolated from the environment.[3–6] The treatment of *Acanthamoeba* keratitis is challenging and chances of recurrent infection are high.[7] With the delay of treatment could lead to blindness .[8] On the other hand, there are no

recommended treatments for disseminated amoebic diseases and amebic encephalitis. The mixture of antimicrobial agents is mostly used but the outcome still remains poor. Although the trophozoite is infective stage, the eradication of cyst form is problematic as medical therapy is often less effective against cysts than trophozoites due to the rigid double-layered wall of the cysts which makes it highly resistant to biocide and anti-amoebic drugs.[7,9,10] In addition, the risk of drug resistance and frequent development of undesirable side effects are major limitations.[11] Miltefosine is FDA approval drug for cutaneous and visceral lesihmaniasis.[12] Nowadays, the drug of choice for free-living amoeba treatment recommended by CDC is miltefosine as well with several studies against free-living amoeba including *Acanthamoeba* genus and other protozoa.[13–18] The aim of this study is to detect the effect of miltefosine on *Acanthamoeba* spp cyst, isolate in Thailand.

Materials and Methods

In the present study, Three Thailand strains of *Acanthamoeba* spp. (KT897271, KT897274, KT897268) belonging to T3, T4, and T5 genotypes were cultured with non-nutrient agar (NNA) seeded with 5 µl of heat-killed *Escherichia coli* for three week to obtained cyst form.[3] A standardized cyst of 20×10^5 cysts/mL used in all experiments. Miltefosine (Sigma, MO, USA) were dissolved in solvent base on company recommendation and used. Briefly 500 µl of each calibrated amoeba cyst strains were incubated with Miltefosine (9.81, 19.62, 24.53 and 49.06 mM,) and incubated at 30 °c for 1, 3, 5, and 7 days. Aliquots of amoeba cysts incubated with phosphate buffered saline (PBS) served as the negative control. Following incubation, viability of cysts was determined by staining them with 0.4% trypan blue and counting cysts in a hemocytometer. Viable cysts appear unstained while non-viable cysts will stain blue. For confirmation, all strained treated were cultured on 1.5% NNA medium, incubated at 30 °C for 10

days and observed every day using stereomicroscopy. At least three independent experiments were done, each in triplicate.

Results and Discussion

The different strains of *Acanthamoeba* have varying response toward chemotherapy agents. Amoeba cysts belong to T3 and T4 genotype showed complete eradication at concentration 4.84 mM after 5 days of exposure, whereas 100% growth reduction of T5 genotype amoeba cysts were achieved at 9.68 mM at 7 days of exposure as showed in figure 1. The growth reduction of amoeba cyst was increased with dose and time dependent manner. The minimal cysticidal concentration (MCC) for all three strains were obtained at 19.36 mM at first day of incubation as showed in Table 1.

As cyst form of *Acanthamoeba* is more resistance to biocide, environmental conditions and many chemical substances than trophozoite form, that leads into the problem in treatment and relapse cases.[19] Schuster et al. (2006) have seek that MIC and MAC of miltefosine on *A. polyphaga* trophozoite was in between 40 µM and 80 µM, as the recovery of amoeba in culture medium at 40 µM have been observed but not in 80 µM at 7 days.[17] Here, we found the 100% cyst killed of all three strains tested, even though the concentration might be higher from the previous study by Walochnik et al. (2002). In the previous study, the eradication of the cyst was not completely obtained using APC1 with just showed killing effect varied from 60% to 80% depend on amoeba strains tested, but sensitive to trophozoite form.[15] Trophozoite was more sensitive to chemical agents than the cystic form but response variation with drug have occurred between strains of *Acanthamoeba* species. Three clinical isolations of amoeba trophozoite illustrated different MTC with miltefosine. The minimal trophicidal concentration of miltefosine against *A. castellanii* was 125 µM and 500 µM for *Acanthamoeba* sp. and *A. hugdunensis* at 1 hr

incubation time.[18] Miltefosine is belonged to alkylphosphocholine drug which involved in cell survival signaling pathway.[20] It is effective for cutaneous and visceral leishmaniasis treatment and showed potential effect in treating free-living amoeba as well. In our present study, we here to report the sensitivity and MCC of miltefosine against *Acanthamoeba* cysts belong to three genotype of Thailand isolations. Our results illustrate the optimal dosage in treatment using this chemotherapy agent should be adapt in clinical setting as its variation between strains and resistance prevention using low dose to some strains.

Table 1 The minimal cysticidal concentration (MCC) of mitelfosine in three strains of *Acanthamoeba* spp.

	MCC (mM)		
Incubation time (d)	T3 genotype (KT897271)	T4 genotype (KT897274)	T5 genotype (KT897268)
1	49.06	24.53	24.53
3	24.53	19.62	19.62
5	19.62	9.81	9.81
7	9.81	4.90	4.90

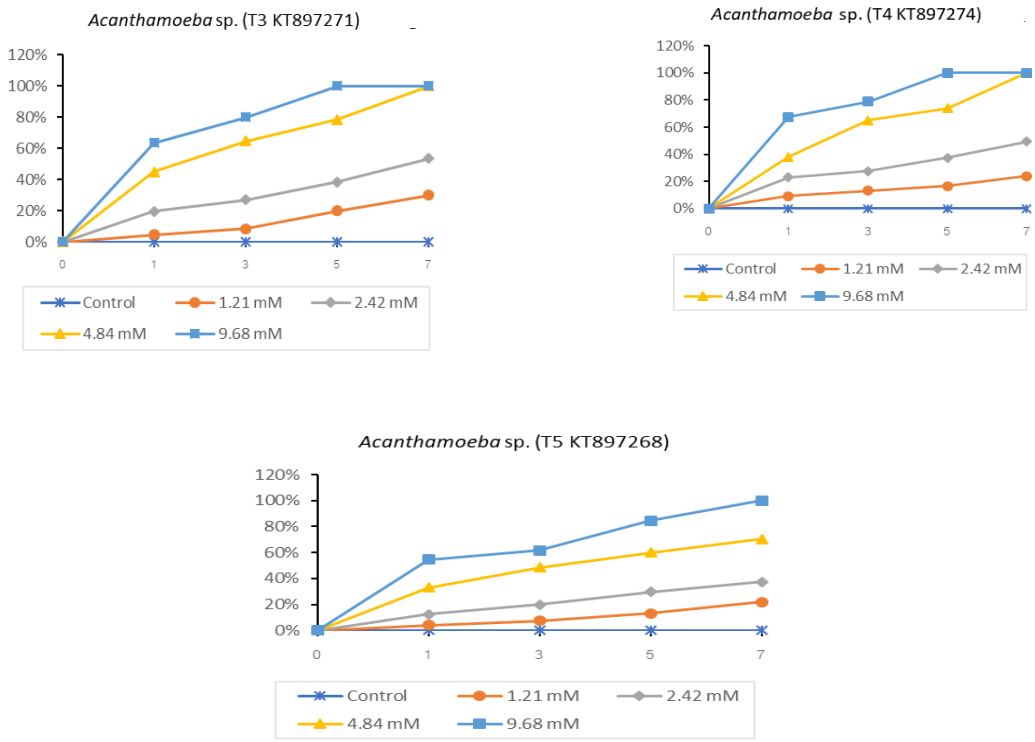

Figure 1. effect of different concentrations of Miltefosine against three strains of *Acanthamoeba* spp. (a) *Acanthamoeba* sp. (T3 KT897271), (b) *Acanthamoeba* sp. (T4 KT897274), (c) *Acanthamoeba* sp. (T5 KT897268) at various incubation times represent in growth reduction percentages.

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การศึกษาที่ 4

In vitro evaluation of antiseptic agents and disinfectant solutions against *Acanthamoeba* spp.

Abstract

This study investigated the efficacy of skin antiseptic agents and disinfectant solutions, commonly used in Thailand, against three genotypes of *Acanthamoeba* spp. The survival cysts after treatments were determined by NNA culture. The results demonstrated that the antiseptic agents containing 0.1% mercury, 10% povidone-iodine, 2.5% iodine, 2% merbromin killed all genotypes after 10 min, while 10 mg/mL ciclopirox olamine had killed after 24 h. The agent containing 0.1% acriflavine was the least effective because only the T4 genotype had been killed after 24 h, while the T3 and T5 genotypes were not. The disinfectant, Dettol®, containing 4% 4-Chloro-3 methylphenol had efficiently killed after 10 min. The disinfectant named Virkon® and Clorox® showed different degrees of effectiveness, Virkon® had killed after 24 h, while Clorox® was ineffective. This is the first report demonstrating the efficacy of mercury, merbromin, iodine, ciclopirox olamine, and 4-Chloro-3-methylphenol. This demonstration could be the guideline for choosing the appropriate skin antiseptic agents and disinfecting biocides in order to reduce the possibility of *Acanthamoeba* infection and decontaminate medical equipment that can prevent the transmission of *Acanthamoeba* and reduce the microbial pathogens that have been found associated with *Acanthamoeba* spp.

Key words: *Acanthamoeba* cysts, Genotypes, Disinfectant solution, Skin antiseptic agent, Household and Hospital

Introduction

Free-living *Acanthamoeba* have been isolated from natural and artificial environments such as soil, water, and even in the air [1]. Based on rRNA gene sequencing, at least 20 genotypes (T1–T20) have been reported [2]. Some genotypes are known to be the causative agents of granulomatous amoebic encephalitis (GAE) and have disseminated infection in immunocompromised individuals and *Acanthamoeba* keratitis (AK) in immunocompetent individuals. In addition, *Acanthamoeba* can also act as a host for pathogenic microorganisms, such as *Cryptosporidium parvum* oocysts [3], adenoviruses [4], *Helicobacter pylori* [5], *Legionella* [6], and *Pseudomonas* [7]. In 2009, *Acanthamoeba* spp. was positively diagnosed in patients' urine samples in the intensive care units of hospitals, which suggests that *Acanthamoeba* might be associated with infection by facilitating transmission of bacteria to the patient [8] In its life cycle, there are two forms, a trophozoite and cyst stage. Under adverse conditions, the amoebae will encyst and while medical therapy is often more effective against trophozoites, it is considerably less effective against cysts due to the cysts' rigid double-layered wall, which makes them highly resistant to various biocides. At present, successful AK treatment depends upon an early diagnosis and prompt treatment using various drug combinations. Yet, GAE and disseminated infection treatments are limited and are rarely successful [9]. The portals of entry for GAE are the nasal passages, the lower respiratory tract, and skin lesions [10].There have been at least 4 cases of GAE reported in Thailand and all cases were diagnosed post-mortem [11]. Of these four cases, one case was demonstrated the possible of GAE primarily infected by skin infection. Therefore, using effective antiseptic agents might reduce the possibility of *Acanthamoeba* infection through skin lesion.

The aim of this study was to demonstrate the efficacy of first aid antiseptic agents, which are commonly used in households in Thailand to treat skin lesions. In addition, our goal was to test disinfectant solutions, which are used to decontaminate medical equipment in hospitals because we have realized that the effective biocides can prevent the transmission of *Acanthamoeba* itself. Furthermore, these biocides can reduce the risks of infection from other microbial pathogens that have been found to be intracellularly associated with *Acanthamoeba*.

The commercial antiseptic agents and disinfectant solutions were purchased from the market in Khon Kaen Province, Thailand. These agents are commonly used in Thailand. The details for each of the biocides are shown in Table 1. In this study, three different genotypes with the following accession numbers were examined: T3 (KT897271), T4 (KT897267), and T5 (KT897268). These environmental strains had been previously isolated from natural water sources in Thailand [12] and maintained in 4 °C in our lab. Cysts of these three genotypes were prepared by sub-culturing them on non-nutrient agar (NNA) plates and by transferring them onto new 1.5% NNA plates seeded with 5 μL of heat-killed *Escherichia coli*. The edges of the culture plates were then sealed with parafilm and incubated at 30 °C for 3 weeks to collect the cyst forms. The cysts were harvested, were then washed in phosphate-buffered saline (PBS) (pH 7.5), and were centrifuged at 12,000 × *g* for 5 min. After centrifugation, the supernatant was discarded and the residue was suspended in PBS (pH=7.5) plus 0.5% sodium dodecyl sulfate (SDS) to lyse the immature cysts. Finally, the cysts were standardized to 25×10^4 cysts/mL by counting them with a hemocytometer.

Next, 100μL of the cyst suspension was mixed with 100 μL of each of the biocides in sterile microtubes except untreated control tubes which had only pure phosphate buffer, and

afterward, they were incubated at room temperature. The incubation times for all of the experiments are shown in Table 2. After treatment, all experimental samples were inoculated onto NNA–*E. coli* plates and were incubated at 30 °C for an additional 10 days and examined daily under stereo microscope. All treatments were performed in triplicate and were repeated in at least three independent experiments.

The results, indicating the efficacy of all of the biocides used, are shown in Table 2. It was found that there had been no significant differences in the three experiments. There were six types of therapeutic agents used in this study: five types were antibacterial agents and the sixth type was an antimycotic agent. The antiseptic agents, containing 0.1% mercury, 10% povidone-iodine, 2.5% iodine, and 2% merbromin, had effectively killed all genotypes at 10 min of exposure, while the agent containing 0.1% acriflavin had been less effective because it had only killed T4 after 1 h of exposure, but had been unable to kill T3 and T5 even after 24 h of exposure. Regarding the antimycotic agent containing 10 mg/mL Ciclopirox olamine, it had effectively killed all genotypes at 24 h of exposure. The solution containing 4% 4-Chloro-3 methylphenol had killed the cysts at 10 min of exposure. Virkon®, which contains 1% potassium peroxymonosulfate, demonstrated that its efficacy had varied based on the strains tested. T4 had been killed at 1 h of exposure, while T3 and T5 had been killed after 24 h of exposure. In contrast, Clorox®, containing 6% sodium hypochlorite, had shown an inability to kill all genotypes even after 24 h of exposure. For untreated control, all tubes were viable determined by NNA culture.

Granulomatous amoebic encephalitis caused by *Acanthamoeba* is difficult to treat and mostly results in death. This life-threatening disease is thought to be initiated by entering into the nasal passages and into skin lesions, followed by a hematogenous spread to CNS [13]. Using

efficient therapeutic agents for skin infection might be the first line of defense to deter CNS infection. This study investigated the efficacy of six therapeutic agents used in first aid antiseptics in households in Thailand and a disinfectant multipurpose cleansing solution, Dettol®, that are used against *Acanthamoeba* cysts. The environmental *Acanthamoeba* isolates used in this study belonged to T3, T4, and T5. Previous studies have reported that T3, T4, and T5 genotypes are all highly pathogenic [10]. After treatment, the treated cysts were examined by culture on NNA for 10 days and monitored daily to make sure that there were no excystments of trophozoites. Our results showed that four therapeutic agents containing providone-iodine, mercury, mercurochrome, and iodine had efficiently killed the cysts of the tested *Acanthamoeba* isolates at 10 min of exposure. The other chemical solution, ciclopirox olamine, had displayed a lesser degree of effectiveness, whereas the acriflavine solution had proven to be the least effective because T4 and T5 had not been killed even when the exposure time reached 24 h. Of these chemical solutions, only providine-iodine has been reported to effectively kill *Acanthamoeba in vitro* as shown in a study carried out on ophthalmic agents [14], while the others have not been previously studied. Acriflavin was introduced as an antiseptic in 1912 by Paul Ehrlich, German medical research worker, used in the first world war to kill the parasite against sleeping sickness and then tested as an antimalarial drug to treat quinine resistant strains since 2014 [15]. Merbromin is an organomercuric disodium salt compound and fluorescence used to treat minor wounds.

Therefore, this is the first study to be conducted using mercury, mercurochrome, ciclopirox olamine, and unformulated iodine solution. The side effects of long term using of these antiseptic agents is skin irritation that might be leading to scar information. Therefore, short time of *Acanthamoeba* killing can reduce the risk of irritation. The disinfectant multi-use solution

containing 4-Chloro-3 methylphenol were shown to be strongly efficient at killing at 10 min after exposure. This chemical compound is used as a disinfectant and as a pharmaceutical preservative to prevent microorganisms from degrading organic materials, such as cosmetics and medications, etc. Moreover, this study also investigated the efficacy of disinfectant solutions, Virkon® and Clorox®, which are commonly used for decontamination of the medical equipments in Thailand's hospitals. The final concentrations of both were prepared following the manufacturer's instruction. in The results from testing these two solutions indicated different degrees of effectiveness. In order to kill all three genotypes, the exposure time should be at least 24 h for Virkon® treatment. The results with Clorox®, the formulated form of sodium hypochlorite, proved to be ineffective at killing all genotypes even when incubated for 24 h. Previous studies reported the efficacy of using unformulated sodium hypochlorite to kill *Acanthamoeba* [16]. This might be due to the fact that the biocidal activity against cysts of the active molecule could have been reduced by the additional compounds in the formulation. It is noteworthy to mention that when compared with T4, both T3 and T5 showed greater resistance. This finding is similar to the results from a previous study, which reported that T3 and T5 had been more resistant to contact lens cleaning solution than T4 [17]. The differences in the resistance of each genotype or strain may partly be due to the thickness of the ectocyst and the amount of cellulose content [16]. An increase in the cellulose content in the wall of the cyst has been demonstrated to increase its resistance to biocides [18].

Our studies are the first to be carried out on the *in vitro* effects on the population of cysts of *Acanthamoeba* strains using mercury, mercurochrome, iodine, acriflavine, 4-chloro-3 methylphenol, ciclopirox olamine, and the formulated form of sodium hypochlorite. Not only do the results of the study serve as an important guideline for choosing the appropriate therapeutic

agents to prevent of *Acanthamoeba* infections, but it has also demonstrated the efficacy of medical equipment decontamination. The results could also be helpful in preventing the transmission of the organism itself given that there are other risks, which have also been identified and which have been intracellularly associated with a range of microbial pathogens associated with these microorganisms.

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การศึกษาส่วนที่ 5

เป็นการศึกษาสมุนไพร 3 ชนิด คือ Greater galangal (*Alpinia galanga*), Lemongrass (*Cymbopogon citratus*)**,** *Zingiber officinale* (ginger)

1. Introduction

Acanthamoeba is a genus of free-living amoebae distributed worldwide in the environment (fresh water, brackish water, soil and even in the air) and known to be a causative agent of acanthamoebiasis (amoebic keratitis, granulomatous amoebic encephalitis and other disseminated diseases) (Khan, 2006; Siddiqui and Khan, 2012). Healthy individuals who wear contact lenses are at risk of amoebic keratitis (AK), whereas granulomatous amoebic encephalitis (GAE) and disseminated diseases occur particularly in immunocompromised hosts (Lorenzo-Morales et al., 2015). Various genotypes of *Acanthamoeba* have caused diseases in individuals but the most predominant in both clinical and environmental isolation is T4 genotype following by T3 and T5 genotypes are also found to be risk agents induce diseases (Aghajani et al., 2016; Behera et al., 2016; Lass et al., 2017; Todd et al., 2015). In Thailand, these amoebae have been detected from patients and also isolated from the environment (Chusattayanond et al., 2010; Lek-Uthai et al., 2009; Thammaratana et al., 2016; Wanachiwanawin et al., 2012). Moreover, Thamprasert and his colleague have reported *Acanthamoeba* association with peptic ulcer in Thailand (Thamprasert et al., 1993). The current regimen treatment of amoebic keratitis consists of the combination of topical antimicrobial agents (Lorenzo-Morales et al., 2015). The duration of treatment can last several months to a year and recurrence rate also exiting more than 10% (Baig et al., 2013). The treatment of *Acanthamoeba* keratitis is challenging and chances of recurrent infection are high (Pérez-Samonja et al., 2003). With the delay of treatment could lead to blindness (Mahgoub and Abdelmageed,

2008). On the other hand, there are no recommended treatments for disseminated amoebic diseases and amebic encephalitis. Current therapeutic agents includes of a mixture of ketoconazole, fluconazole, sulfadiazine, pentamidine, isethionate, amphotericin B, azithromycin B, itraconazole or rifampicin, but the outcome still remain poor (Khan, 2006; Siddiqui and Khan, 2012). This maybe most cases due to GAE are identified post-mortem and the low penetration of the compounds that can cross blood brain barrier. Most of the GAE patients were death as the suffered from drug side effects and non-potential compound for amoeba elimination (Webster et al., 2012; Yagi et al., 2007; Zamora et al., 2014). *Acanthamoeba* has two stages in its life cycle; cyst and trophozoite. The eradication of *Acanthamoeba* from the infection site is difficult because under adverse conditions, the amoebae encyst and medical therapy is often less effective against cysts than trophozoites due to the rigid double-layered wall of the cysts which makes it highly resistant to anti-amoebic drugs. This is a problem as cysts can survive after initial successful chemotherapeutic treatment and cause relapse of the disease (Leitsch et al., 2010). In addition, the risk of drug resistance and frequent development of undesirable side effects are major limitations (Wilson, 1991).

Over the years, the research on the natural biomolecule from plants have increasing dramatically as its usefulness in medical usage. Plant metabolites such as flavonoid, terpenoids, steroids, tannins, alkaloids, and phenolic compounds have provided wide range of pharmacological properties and health beneficial (Aziman et al., 2014). Three medicinal plants have employed in ethnomedicine long ago have used in this study.

Ginger (*Zingiber officinale*), zingiberaceae family, is the perennial plant which distributed in India and southeast Asia used as culinary spice and folk medicine. This plant is about 1.2-meter-high which has thick rhizome and root spreading underground (Heber, 2004). Ginger rhizome and its essential oil has been employed in traditional medicine as potential

compound with their biological activities for treatment many diseases (Sharifi-Rad et al., 2017). There were many studies in this plant compound in medical field. The pharmacological properties of ginger that have been known so far are anti-oxidant (Stoilova et al., 2007), anti-inflammatory (Nile and Park, 2015), anti-obesity (Tabibi et al., 2016), anti-diabetic (Daily et al., 2015; Mozaffari-Khosravi et al., 2014), antimicrobial (Aghazadeh et al., 2016; Wang and Ng, 2005), anti-tumor (Akimoto et al., 2015), and other health benefits (gastro-protective, hepato-protective, and neuroprotective) (Rahmani et al., 2014). Ginger is also served anti-protozoal in several studies (Abdel-hafeez et al., 2015; Arbabi et al., 2016; Dyab et al., 2016; Sohni et al., 1995).

Greater galangal (*Alpinia galanga*) is the perennial plant which has hot spicy taste and aromatic ginger like odor in the family Zingiberaceae. It is widely cultivated in Asia such as Sri lanka, India, Indonesia, Malaysia, Thailand and many more as this plant served as culinary spice (Kaushik, D., Yadav, Y., Kaushik, P., Sacher, D., Rani, R., 2011). Biological activity of this plnat have been reported as antioxidant (Juntachote and Berghofer, 2005), anti-inflammatory (Bhattacharyya et al., 2011), antimicrobial (Hsu et al., 2010; Khodavandi et al., 2013; Oonmetta-aree et al., 2006; Rao et al., 2010), anti-allergic (Matsuda et al., 2003), antiplatelet (Jantan et al., 2005), anticancer (Hadjzadeh et al., 2014; Samarghandian et al., 2014), gastro-protective (Hadjzadeh et al., 2014), hypoglycemia and hypolipidemia activity (Achuthan and Padikkala, 1997; Akhtar et al., 2002). Research on greater galangal activity on protozoa has tested (Kaur et al., 2010; Sawangjaroen et al., 2006).

Lemongrass (*Cymbopogon citratus*), is perennial grass, which is known for lemon scented essential oil in Poaceae family. This grass is distributed worldwide particularly in tropical regions (Federal and Postal, 2007). As it unique characrteristic, lemongrass have been

used in food flavoring, perfume, and other cosmetics application. Lemongrass has employed in traditional medicine in various country such as India, China, Nigeria, Africa, Cuba, brazil, Thailand and other regions in the world (Federal and Postal, 2007; Shah et al., 2011). This herb have provided various pharmacological properties known so far as antioxidant (Cheel et al., 2005), anti-inflammatory (Francisco et al., 2013), anti-diarrhea (Tangpu and Yadav, 2006), antimicrobial (Ewansiha, J.U., Garba, S.A. and Mawak, J.D., Oyewole, 2013; Naik et al., 2010; Singh et al., 2011; Soares et al., 2013), anti-proliferative (Halabi and Sheikh, 2014), anti-nociceptive (Viana et al., 2000), hypoglycemia and hypolipidemia effects (Adeneye and Agbaje, 2007). In addition, lemongrass have possess antimalarial and anti-leishmanial activity (Okere et al., 2014; Santin et al., 2009).

Therefore, the seeking in the potential compounds on acanthamoebiasis elimination is needed. The aim of this study, we would like to investigate the effect of herbal plants on *Acanthamoeba* spp. from various environmental isolations in Thailand.

2. Materials and Methods

2.1 *Acanthamoeba* cysts collection

Five strains of *Acanthamoeba* sp. used in this study were previously isolated from natural water in Thailand as shown in table 1. Cysts were prepared by culturing trophozoites on 1.5% non-nutrient agar (NNA) plates seeded with 5 µl of heat-killed *Escherichia coli* (Thammaratana et al., 2016). The plates were wrapped with parafilm and incubated at 30 °C for 3 weeks to collect the cyst forms. After three weeks all trophozoites have transformed into cysts. The cysts were harvested in phosphate-buffered saline (PBS) (pH 7.5) plus 0.5% sodium dodecyl sulfate (SDS) to lyse non-mature cysts, and then centrifuged at 12,000 × g for 5 min and standardized using a hemocytometer to a concentration of 20×10^4 cysts/mL.

Table1. *Acanthamoeba* strains used in cysticidal assay.

Acanthamoeba strains	Genotype	Reference
KT897271	T3	(Thammaratana et al., 2016)
KT897265	T4	
KT897268	T5	

2.2 Drug and Plant extracts preparation

All plants (ginger, greater galangal, lemongrass) were purchased in local market in Khon Kaen province, Thailand. Fresh rhizomes of ginger and greater galangal were washed, sliced into thin pieces and dried in the incubator while lemongrass stalks were also done in same processes. The dried plants have blend into powder. About 500 grams of each plant powder were macerated in 1 liter of absolute ethanol in separated containers and incubated for 7 days with shaking daily. After, incubation, the solutions were filtrated with Whatman filter paper 1. The filtrates were concentrated in a rotary evaporator at 60 °C. The extracts, a sticky material, were obtained after evaporation and kept at -20 °C until used. Miltefosine (Sigma, MO, USA) were dissolved in distilled water and used in the experiment.

2.3 Cysticidal assay

In the present study, 500 µl of the calibrated amoeba cyst suspension and the same volume of the test extracts of ginger (at concentrations of 50 to 600 mg/ml), greater galangal and lemongrass (at concentrations of 50, 100, 200 mg/ml) were mixed thoroughly in 1.5 ml microcentrifuge tubes. Aliquots of amoeba cysts incubated with phosphate buffered saline (PBS) served as the control. All samples were then incubated for 24, 48 or 72 h. Following incubation, viability of cysts was determined by staining them with 0.4% trypan blue and counting cysts in a

hemocytometer. Viable cysts appear unstained while non-viable cysts will stain blue. For confirmation, all tested cysts were cultured on 1.5% NNA medium, incubated at 30 °C for 10 days and observed every day using stereomicroscopy (El-Sayed et al., 2012). At least three independent experiments were done, each in triplicate.

2.4 Evaluation of herbal extracts efficacy

Counting the number of cysts using the hemocytometer after each period of incubation and calculation of the percent of growth reduction according to the equation (Palmas et al., 1984). Percent of growth reduction = a -b/a ×100 (a is the mean number of cysts in control sample and b is the mean number of cysts in treated samples).

2.5 Statistical analysis

Data are represented as mean and standard deviation, minimal cystidal concentration (MCC) and percent of growth reduction. The data were analysed using one way ANOVA following by student's t test using SPSS software. P value < 0.05 defined as statistically significant while P value < 0.001 is highly significant.

3. Results

Crude extracts of ginger exhibit 100% cyst killed at 800 mg/mL for T3 strain of *Acanthamoeba* where other two strains were achieved at 400 mg/mL and 200 mg/mL for T4 and T5 strains after 24 h exposure as showed in Table 2 with highly significant P value < 0.001. For other concentrations, the effect was observed based on dose and time dependent manner as in Table 2 and Figure 1. On the other hand, the complete eradication of crude extracts of greater galangal and lemongrass on amoebae were reached at 200 mg/mL after 24 h exposure (P value < 0.001) in all strains tested. Lower concentrations of both herbal extracts showed inhibitory effect

according to time incubations and dose dependent manner (Table 3, Table 4, Figure 2, and Figure 3).

Table 2 Effect of *Zingiber officinale* (ginger) ethanol extract on the *in vitro* growth of *Acanthamoeba* spp. cysts for different incubation periods.

Strains	Concentrations (mg/ml)	24h		48h		72h	
		Mean± SD	% of growth reduction	Mean± SD	% of growth reduction	Mean± SD	% of growth reduction
T3	Non-treated control	19.9±1.1	0	19.8±2.3	0	19.7±1.6	0
T4	Non-treated control	19.8±1.6	0	19.6±1.4	0	19.7±2.1	0
T5	Non-treated control	19.8±1.3	0	19.7±2.3	0	19.6±1.5	0
T3 (KT897271)	50	16.0±1.0*	20	14.3±1.1*	28.5	12.5±2.1*	37.5
	100	14.4±1.2*	28	11.3±2.0*	43.5	9.3±1.5*	53.5
	200	10.1±0.6*	49.5	6.3±2.6*	68.5	0**	100)
	400	5.2±2.3*	74	0**	100	0**	100
	800	0**	100	0**	100	0**	100
T4 (KT897265)	50	15.0±2.4*	25	12.0±1.3*	40	11.3±2.4*	43.5
	100	11.0±1.6*	45	9.0±1.6*	55	7.0±1.7*	65
	200	6.3±2.6*	68.5	0**	100	0**	100
	400	0**	100	0**	100	0**	100
T5 (KT897268)	50	12.0±2.4*	40	10.2±2.1*	49	8.3±2.3*	58.5

	100	10.3±2.1*	48.5	8.5±3.4*	57.5	6.3±3.1*	68.5
	200	0**	100	0**	100	0**	100

*p<0.05, statistically significant difference in comparison to non-treated control in the same time interval; **p<0.001, statistically highly significant difference in comparison to non-treated control in the same time interval.

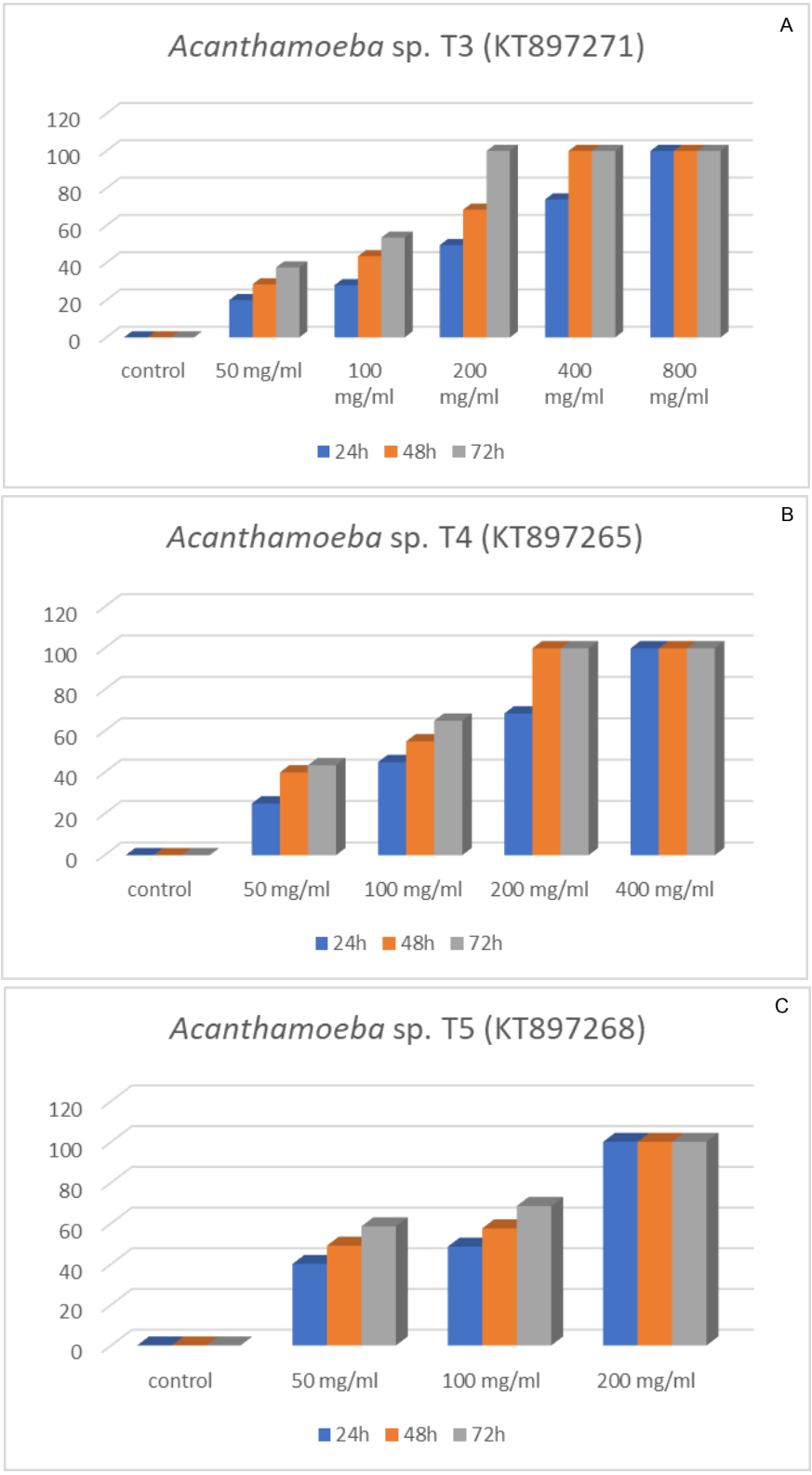

Figure 1 Percentages of *Acanthamoeba* growth reduction after exposure to different concentrations of *Zingiber officinale* (ginger) ethanol extract at different incubation times. (A) T3(KT897271); (B) T4(KT897265); (C) T5(KT897268).

Table 3 Effect of *Alpinia galanga* (greater galangal) ethanol extract on the *in vitro* growth of *Acanthamoeba* spp. cysts for different incubation periods.

Strains	Concentrations (mg/ml)	24h		48h		72h	
		Mean±SD	% of growth reduction	Mean±SD	% of growth reduction	Mean±SD	% of growth reduction
T3	Non-treated control	19.8±1.6	0	19.7±2.3	0	19.5±2.6	0
T4	Non-treated control	19.8±1.1	0	19.6±1.5	0	19.4±2.4	0
T5	Non-treated control	19.8±1.4	0	19.7±2.4	0	19.5±1.6	0
T3 (KT897271)	50	12.7±1.3*	36.5	10.3±1.2*	48.5	7.3±1.0*	63.5
	100	10.1±1.1*	49.5	8.9±2.1*	55.5	5.7±2.1*	71.5
	200	0**	100	0**	100	0**	100
T4 (KT897265)	50	12.1±2.6*	39.5	11.7±1.1*	41.5	9.9±1.2*	50.5
	100	10.2±3.1*	49	8.2±2.1*	59	7.1±2.3*	64.5
	200	0**	100	0**	100	0**	100
T5 (KT897268)	50	11.0±2.3*	45	9.3±2.1*	53.5	7.3±2.2*	63.5
	100	10.0±1.7*	55	8.3±2.4*	58.5	6.2±1.1*	69
	200	0**	100	0**	100	0**	100

*$p<0.05$, statistically significant difference in comparison to non-treated control in the same time interval; **$p<0.001$, statistically highly significant difference in comparison to non-treated control in the same time interval.

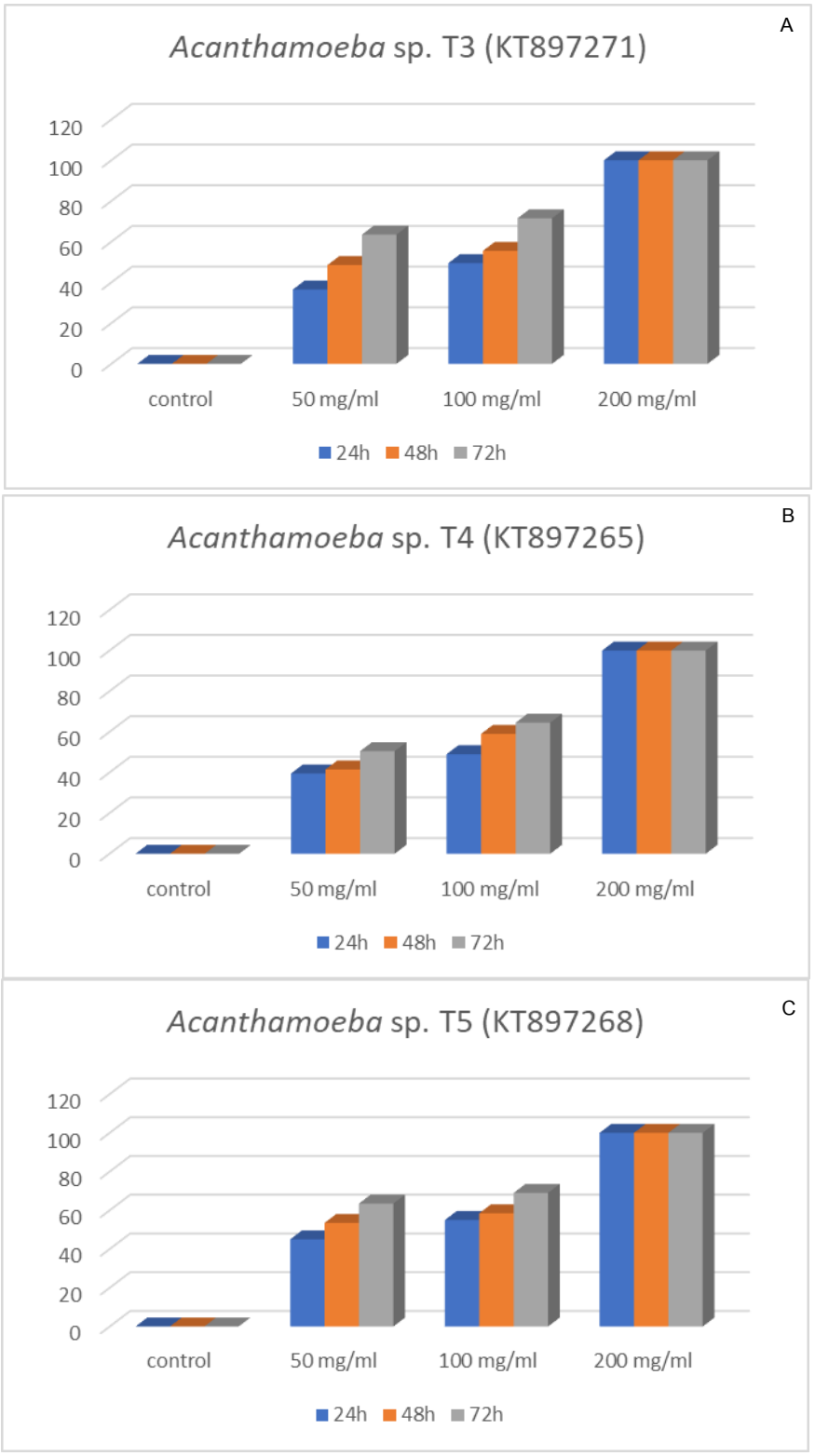

Figure 2 Percentages of *Acanthamoeba* growth reduction after exposure to different concentrations of *Alpinia galanga* (greater galangal) ethanol extract at different incubation times. (A) T3(KT897271); (B) T4(KT897265); (C) T5(KT897268)

Table 4 Effect of *Cymbopogon citratus* (lemongrass) ethanol extract on the *in vitro* growth of *Acanthamoeba* spp. cysts for different incubation period.

Strains	Concentrations (mg/ml)	24h		48h		72h	
		Mean±SD	% of growth reduction	Mean±SD	% of growth reduction	Mean±SD	% of growth reduction
T3	Non-treated control	19.8±1.5	0	19.6±2.1	0	19.5±2.6	0
T4	Non-treated control	19.8±1.4	0	19.7±1.6	0	19.6±2.2	0
T5	Non-treated control	19.8±1.1	0	19.6±2.3	0	19.4±3.1	0
T3 (KT897271)	50	12.0±1.3*	40	10.3±1.1*	48.5	8.3±2.1*	58.5
	100	10.4±2.2*	48	8.7±2.1*	56.5	6.4±3.2*	68
	200	0**	100	0**	100	0**	100
T4 (KT897265)	50	12.1±2.6*	39.5	10.7±1.5*	46.5	8.9±1.2*	55.5
	100	9.7±3.1*	51.5	8.5±2.1*	57.5	6.1±2.3*	69.5
	200	0**	100	0**	100	0**	100
T5 (KT897268)	50	12.0±2.3*	40	10.0±2.1*	50	8.3±2.6*	58.5
	100	10.0±2.7*	50	8.3±3.1*	58.5	6.3±2.1*	68.5
	200	0**	100	0**	100	0**	100

*$p<0.05$, statistically significant difference in comparison to non-treated control in the same time interval; **$p<0.001$, statistically highly significant difference in comparison to non-treated control in the same time interval.

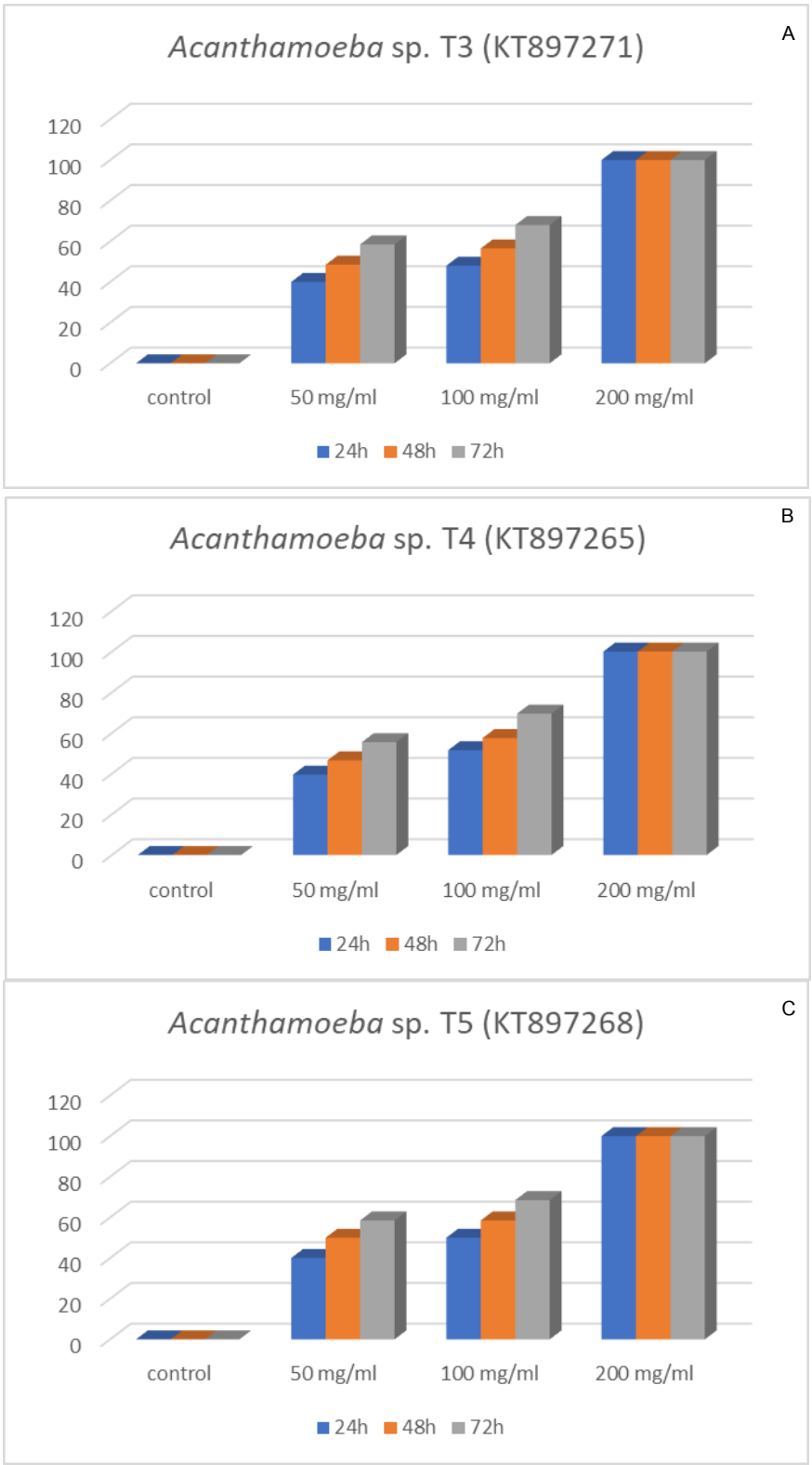

Figure 3 Percentages of *Acanthamoeba* growth reduction after exposure to different concentrations of *Cymbopogon citratus* (lemongrass) ethanol extract at different incubation times. (A) T3(KT897271); (B) T4(KT897265); (C) T5(KT897268).

4. Discussion

To date, there are no potential agents against this amoeba. The long treatment duration and combination of drugs used are not satisfactory. Severe complications due to *Acanthamoeba* infection such as blindness and even death in misdiagnosed cases, existed (Yagi et al., 2007; Zamora et al., 2014). So alternative therapeutic compounds should be developed to combat this issue. Some active compounds isolate from ginger including gingerol, shaogoal, zingerone, terpemioid, flavonoid possess various biological activities (Rahmani et al., 2014). As antioxidant, ginger extract could inhibit 2,2-Diphenyl-1-picril hydrazyl radical (DDPH) up to 90.1%, lipid peroxidation, and powerful hydroxide ion scavenger (Stoilova et al., 2007). Ginger extracts compound, 6-gingerol at 1mM, showed greater inhibition of all enzyme activity tested (β-glucuronidase, diene-conjugate, hyaluronidase, and lipoxidase) which involved in inflammatory pathway (Nile and Park, 2015). The ethanol extract of ginger showed anti-proliferative and induced autotic cell death in pancreatic cancer cell line by suppressed cell cycle arrest, increased reactive oxygen species production, and inhibited negative autophagic regulator (mTOR) (Akimoto et al., 2015). On protozoa, ginger extracts have killed 97% of *Giardia lamblia* cyst in vitro, while inhibited NO productions as its anti-inflammatory activity in *Blastocystic* spp (Abdel-hafeez et al., 2015; Dyab et al., 2016). infected mice. In addition, the components in ginger may disturbed in vital biological activities by inhibiting proteolytic activity of *Trichomonas vaginalis* and antiamoebic effect on *Entamoeba histolytica* at MIC up to1000 µg/ml (Arbabi et al., 2016; Sohni et al., 1995). As in the same family of Zingiberaceae, *Alpinia galanga* have possessed similar pharmacological properties as ginger mention above. Its extract showed antibacterial activity against various gram positive and gram negative bacteria strains (Rao et al., 2010). Moreover, it showed antifungal effect against many *Candida* species which resistant to azoles (Khodavandi et al., 2013). As antiprotozoal, alpinia galanga had greatest effect on *Entamoeba histolytica* and *Giardia lamblia* among medicinal plants tested in Southern

Thailand with IC50 value of 55.2 µg/ml and 37.73 µg/ml (Sawangjaroen et al., 2006, 2005). Phenylpropanoids from this plant extracts were found to be effective against *Leishmania donovani* (Kaur et al., 2010). Similarly, to other medicinal plants, *Cymbopogon citratus*, a perennial grass in Poaceae family, provided anti-oxidant, anti-inflammatory, antimicrobial and anticancer activities which could be applied against other diseases (Cheel et al., 2005; Ewansiha, J.U., Garba, S.A. and Mawak, J.D., Oyewole, 2013; Francisco et al., 2013; Halabi and Sheikh, 2014; Naik et al., 2010; Shah et al., 2011; Singh et al., 2011; Soares et al., 2013). Beside this, the essential oil of lemongrass was caused morphological alteration such as mitochondrical damage, autophagic structures formation on promastigote and amastigote of *Leishmania amazonesis* observed by TEM and SEM (Santin et al., 2009). Aqueous extract of lemongrass, 120 mg/kg in vivo, indicated highly significant parasitemia level reductions of *Plasmodium falciparum* infected mice (Okere et al., 2014).

Ethanol extract of *Zingiber officinale* (ginger) showed 100% cyst killed at concentration 400 mg/ml all T4 genotype strains after 24h of exposure except T5 and T3 as showed in Table 1. Whereas 200 mg/ml of *Alpinia galanga* and *Cymbopogon citratus* extracts produced killing effect at 24 hr after exposure on all strains tested in Figure 2 and Figure 3. The growth reduction of amoebae was reduced base on dose and time-dependent manner as showed in each tables and figures. Yet the underlying mechanism on *Acanthamoeba* species is still unclear, the activity of all this plants mentioned above might be involved in killing of the amoeba treated. Natural products are good candidates for developing as potential agent to eliminate infection as low-cytotoxity property as all this plants are used as daily food spice. A study of Ginger powder consumption at dose 0.5 to 1 gram from 3 months to 2.5 years did not show any adverse effects (Langner et al., 1998). Moreover, ginger extract with various doses up to 1000 mg/kg administered to pregnant rats for 10 days didn’t cause any teratogenic toxicity (Weidner and Sigwart,

2001). The ethanol extract of *Alpinia galanga* is non-toxic up to 3g/kg body weight in mice has been reported (Qureshi et al., 1992). All the plants tested exhibited antiprotozoal activity which is highlight the alternative therapeutic approach using natural products. Development of resistant strains against the recommended drugs and adverse effect of the chemotherapy evoke the importance of using an alternative medicine. Further study is needed to dissect the active molecule in these plants, mechanism of action on amoeba, cell-line toxicity (corneal cell) as topical agent development or others cell line involving amoeba infection site, and proceed to animal model investigation. Ultrastructural of amoeba is also crucial to detect morphological change in treated amoeba.

In conclusion, this is the first study of herbal extracts against environmental isolations of Thailand *Acanthmoeba*. The ethanol extract of herbal plants showed compromised results and illustrate the potential as novel antiparasitic agents. This plant is common in Thailand, even around dwellings, making it easy to acquire material for future study.

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