In Vitro Amoebicidal Activity of *Rhinacanthus nasutus* Ethanolic Extracts on *Acanthamoeba* Cysts

ฤทธิ์ในการฆ่าอะมีบาของสารสกัดจากทองพันชั่งด้วยเอทานอลต่อเชื้ออะแคนทามีบาซีสต์ในหลอดทดลอง

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ABSTRACT

Amoebae of the genus *Acanthamoeba* genus are causative agents of amoebic keratitis, granulomatous amoebic encephalitis and other disseminated diseases. Treatment of acanthamoebiasis is difficult because the cyst form is less susceptible to amoebicidal agents than the trophozoite stage. The long duration of treatment and lack of a suitable drug to combat *Acanthamoeba* infections is a hindrance to success. The efficacy and low toxicity of plant extracts medical usage suggest a promising alternative approach. In this study, ethanolic extracts from *Rhinacanthus nasutus*, known as the white crane flower, were used for *in vitro* cysticidal assays against *Acanthamoeba* cysts at various concentrations (0.012, 0.12, 1.2, 12 and 120 mg/ml). A concentration of 120 mg/ml killed 100% of *Acanthamoeba* cysts after exposure for 24 h, while the other lower concentrations could not completely kill. In conclusion, ethanolic extract of *R. nasutus* can be considered a new natural agent against *Acanthamoeba* cysts.

บทคัดย่อ

เชื้ออะแคนทามีบาเป็นเชื้อสาเหตุของโรคกระจกตาอักเสบ โรคสมองอักเสบและโรคติดเชื้อที่อวัยวะอื่นๆ จากโรคกระจายทั่งร่างกาย โรคติดเชื้ออะแคนทามีบารักษาให้หายได้ยากเนื่องจากระยะซีสต์ดื้อต่อยาเคมี ทำให้ผู้ป่วยที่ติดเชื้ออาจเกิดอาการรุนแรงได้ เนื่องจากสารสกัดจากธรรมชาติมักจะมีพิษต่อเซลล์ต่ำและสามารถนำมาพัฒนาเป็นยาฆ่าชื้อได้ดังนั้นในการศึกษาครั้งนี้จึงได้ทำการทดสอบประสิทธิภาพของสารสกัดจากพืชสมุนไพรทองพันชั่งหรือมีชื่อวิทยาศาสตร์คือ *Rhinacanthus nasutus* โดยทำการศึกษาสารสกัดที่ความเข้มข้นต่างๆกัน (0.012, 0.12, 1.2, 12 และ 120 มก./มล.). ผลการทดสอบพบว่าที่ความเข้มข้น 120 มก./มล. สามารถฆ่าเชื้อระยะซีสต์ได้ 100% หลังจากเวลาทดสอบผ่านไป 24 ชั่วโมงในขณะที่การทดสอบที่ความเข้มข้นอื่นๆที่ต่ำกว่านี้ไม่สามารถฆ่าเชื้อได้หมด จากการศึกษาสรุปได้ว่า สารสกัดทองพันชั่งสามารถนำมาพัฒนาเป็นสารที่ต้านเชื้ออะแคนทามีบาระยะซีสต์ได้

Keywords: *Rhinacanthus nasutus*, Cyst, Acanthamoebiasis

คำสำคัญ: ทองพันชั่ง ซีสต์ โรคติดเชื้ออะแคนทามีบา

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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). Cutaneous acanthamoebiasis, most common disseminated disease, could be detected with the presence of *Acanthamoeba* trophoziote or cyst form on the infected site of human skin lesion or wound. Due to this route provided, this amoeba could have circulated in the patient blood stream into central nervous system which lead to co-occur with GAE risk (Khan, 2006). In addition, there are several reports from around the world about other diseases associated or caused by *Acanthamoeba*, including rhinosinusitis (Rivera and Padhya, 2002), peritonitis (Tilak et al., 2008), and peptic ulcer (Thamprasert et al., 1993). In Thailand, these amoebae have been detected from patients and also isolated from the environment (Chusattayanond et al., 2010; Lek-Uthai et al., 2009; Wanachiwanawin et al., 2012). Various genotypes are known to be associated in disease causation but the most predominant in both clinical and environmental isolations is T4 genotype (Di Cave et al., 2014; Thammaratana et al., 2016; Todd et al., 2015). Amongst all genotypes, *Acanthamoeba* T4 genotype have caused both amoebic keratitis and granulomatous amoebic encephalitis (Khan, 2006). *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, herbal extracts, which generally have low toxicity for host cells, have been studied for their amoebicidal effects (Aqeel et al., 2012; El-Sayed et al., 2012). *Rhinacanthus nasutus* (white crane flower) is in the family Acanthaceae. This plant occurs in southeast Asia and China. In traditional treatments, this plant is used in dermatologic diseases such as versicolor, ringworm, pruritic rash, abscess pain, and other skin diseases (Puttarak et al., 2010). *R. nasutus* composes of flavonoids, steroids, terpenoids, anthraquinones, lignans and especially naphthoquinone analogues. Some known bioactive components are naphthoquinones such as rhinacanthins (A-D, G-Q), rhinacanthone and lignin groups (Nirmaladevi et al., 2010). In addition, it is also known to have anti-microbial (Puttarak et al., 2010), anti-tumor (Siripong et al., 2006), anti-oxidant (Siriwatanametanon et al., 2010), anti-inflammatory (Siriwatanametanon et al., 2010), anti-proliferative (Gotoh et al., 2004) and anti-allergic (Tewtrakul et al., 2009) properties. In addition, extracts of white crane flower leaves have been tested against malarial vectors including *Culex quinquefasciatus, Anopheles stephensi* and *Aedes aegypti* and has shown to be adulticidal and to have an effect on fertility of the mosquitoes (Jayapriya and Shoba, 2015; Muthukrishnan and Pushpalatha, 2001). However, there have been no studies on parasites.

Objectives of the study

In this study, we will test the effect of herbal extracts of *Rhinacanthus nasutus* (white crane flower) on the cyst form of *Acanthamoeba* of the T4 genotype isolated from the environment.

Methodology

1. *Acanthamoeba* cysts collection

Acanthamoeba sp. (genotype T4 isolate SU2; Thammaratana et al., 2016) used in this study was previously isolated from natural water in Thailand as shown in figure 1 (Thammaratana et al., 2016). 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 × 10^5 cysts/mL.

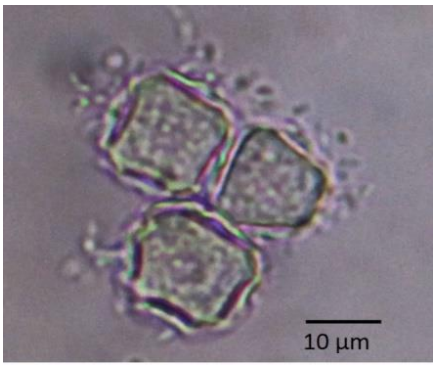

Figure 1 *Acanthamoeba* cysts with double cyst walls used in this study.

Figure 2 *Rhinacanthus nasutus* (white crane flower).

2. *Rhinacanthus nasutus* extract preparation

Fresh leaves of *Rhinacanthus nasutus* were collected from household areas in Khon Kaen province, Thailand as shown in figure 2. These leaves about 500 grams were macerated in 1 litre of absolute ethanol and incubated for 7 days with shaking daily. After, incubation, the solution was filtrated with Whatman® filter paper grade

1 (pore size 11 µm). The filtrate was evaporated in a rotary evaporator for 8 hours at 60 °C (Deepa, N., Ravichandran, 2008). The extract, a sticky material, was obtained after evaporation and kept at -20 °C until used.

3. Cysticidal assay

In the present study, 500 µl of the calibrated amoeba cyst suspension and the same volume of the test *R. nasutus* extract solution (at concentrations of 0.012, 0.12, 1.2, 12 and 120 mg/ml) were mixed thoroughly in 1.5 ml microcentrifuge tubes. Aliquots of amoeba cysts incubated with phosphate buffered saline (PBS) served as the negative control and cysts incubated with 0.06% chlorhexidine in PBS as the positive 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 as shown in figure 3. 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.

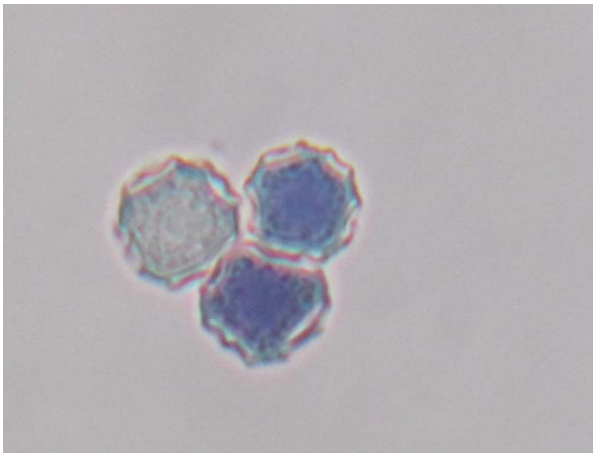

Figure 3 Viability of cysts was determined by 0.4% trypan blue staining. Viable cysts appear unstained while non-viable cysts are stained blue.

4. Evaluation of *R. nasutus* extract 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).

5. Statistical analysis

Data are presented as mean ± SD of triplicate experiments and percent of growth reduction. The data were analyzed using one-way ANOVA followed by Student's t test. A p value < 0.05 was considered significant and a p value < 0.001 was regarded as highly significant.

Results

The results are shown in Table 1. At 120 mg/ml, *R. nasutus* extract killed all *Acanthamoeba* cysts ($p < 0.001$), even after the shortest incubation period (24h). At all lower concentrations of *R. nasutus* extract, the percentage of cysts killed was statistically significantly higher than in the non-treated control ($p < 0.05$). The percentage of cysts killed increased with concentration of extract and also with time, as shown in figure 4.

Table 1 Effect of *R. nasutus* ethanolic extract on the *in vitro* growth *of Acanthamoeba* sp. cyst for different incubation period.

Treatment	Duration of treatment (hours)					
	24h		48h		72h	
	Mean±SD	% cysts killed	Mean±SD	% cysts killed	Mean±SD	% cysts killed
Non-treated control	20±2.1	0	19.8±2	0	19±3.1	0
Chlorhexidine 0.06%	0**	100	0**	100	0**	100
R. nasutus, 120 mg/ml	0**	100	0**	100	0**	100
R. nasutus, 12 mg/ml	6.3±1.5	68.5	4.1±1*	79.3	2.1±1*	89.1
R. nasutus, 1.2 mg/ml	7.2±1.5*	64.0	6.6±1.5*	66.6	5.7*	70
R. nasutus, 0.12 mg/ml	9.3±1*	53.5	8±2.3*	59.6	7±2*	63.2
R. nasutus, 0.012 mg/ml	12±1*	40	9.4±2*	52.5	8.3±1*	56.3

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

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

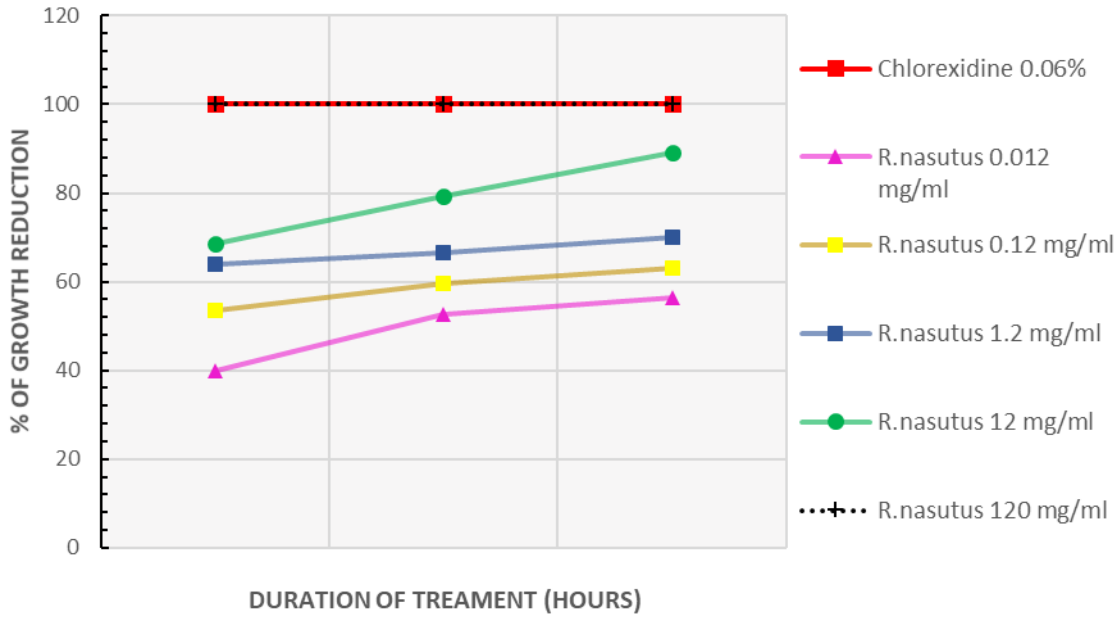

Figure 4 Percentages of *Acanthamoeba* growth reduction (relative to untreated controls) after exposure to different concentrations of *Rhinacanthus nasutus* extract at different incubation times.

Discussion and Conclusion

Acanthamoeba infection is important because severe complications such as blindness and even death in misdiagnosed cases, may occur (Yagi et al., 2007; Zamora et al., 2014). To date, there are no potential agents against this amoeba. The long treatment duration and combination of drugs used are not satisfactory. New therapeutic compounds should be developed to combat this issue. In this study, ethanolic extract of *Rhinacanthus nasutus* leaf have showed a potent effect on cysts of the *Acanthamoeba* T4 genotype. Extracts of *R. nasutus* at 120 mg/ml killed all cysts even after the shortest incubation period, whereas lower concentrations killed percentages of the cysts in a dose- and time-dependent manner as seen in Table 1. Extracts of *R. nasutus* have been used in traditional medicine for various skin diseases with high efficacy. As mention above, such extracts possess antioxidant and anti-inflammatory effects as other herbal plants. Furthermore, this plant also has antibacterial (Antonysamy, 2017; Prabakaran and Pugalvendhan, 2009), antifungal (Shoba, 2015), antiviral (Kernan et al., 1997; Sendl et al., 1996), anti-proliferative properties and causes induction of apoptosis (Kupradinun et al., 2009; Siripong et al., 2009). Apparently, there is now a need to develop a local topical agent from this plant to treat *Acanthamoeba* infections of the eye and to prevent infection through skin lesions or wounds leading to granulomatous amoebic encephalitis. Similarly, formulation for administration via the oral route drug is necessary. Oral administration might offer synergistic effects with other drugs, enhancing amoebicidal effects. However, only one genotype has been used in this study. In order to develop the potent drug, this plant extracts should be tested against other acanthamoebiasis associated genotypes. Furthermore, the active ingredient detection is needed in the future study with using of chromatography, mass spectrometry, or other methods. The optimal conditions of active ingredient such as solubility, pH, humidity and other parameters are crucial for using compound in drug formulation. Such studies could investigate further along with the mechanism of action of these compounds, toxicity, and to proceed to animal-model investigations in the future. This plant is common in Thailand, even around dwellings, making it easy to acquire material for further study of the active compounds. In conclusion, this is the first study of ethanolic extract of *R. nasutus* leaves as a killing agent of *Acanthamoeba* cysts. This plant shows promise as a source of a new drug for treatment of *Acanthamoeba* infection.

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