ANTIAMOEBIC ACTIVITY OF *ORTHOSIPHON ARISTATUS* (CAT'S WHISKERS) ON ACANTHAMOEBA FROM CONTACT LENS

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ABSTRACT

Acanthamoeba, a free-living protozoan, is the causative agent of granulomatous amebic encephalitis and Acanthamoeba keratitis, which highly associated with contact lens wearers. The present study aimed to investigate the antiamoebic effect of *Orthosiphon aristatus* extracts against *Acanthamoeba* genotype T4 *in vitro*. The isolated Acanthamoeba from contact lens was maintained on 1.5% non-nutrient agar for cystic stage and trophozoite cultured in PYG-medium. The ethanolic extracts of *O.aristatus* leaves were prepared in different concentrations of 125, 250, and 500 mg/ml. The viability of the trophozoites and cysts of *Acanthamoeba* were observed after incubation with the plant extracts at 24, 48 and 72h. Chlorhexidine 0.02% was used as the positive control. The experiments of trophozoites and cysts were test in triplicate for testing the viability by trypan blue vital staining and hemacytometer. The crude leaf extract of *O.aristatus* compounds showed significant growth inhibition effects 100% of trophozoites as well as interrupted the encystation activity at the minimum concentrations of 250 µg/ml after 24 hours. The *O.aristatus* compounds could not kill the cyst after 72 hours. Further investigations are needed to find the effective fractions of *Orthosiphon aristatus* and the mechanisms of effective constituents for development of alternative therapeutics against Acanthamoeba infections. It can also be tested *in vivo* or even against other microorganisms.

Keywords: *Orthosiphon aristatus*. *Acanthamoeba* genotype T4, antiamoebic

INTRODUCTION

Contact lens wearers are increasing in number and highly related to infection of cornea by free-living amoebae and causing *Acanthamoeba* keratitis (Lorenzo-Morales et al., 2013). *Acanthamoeba* is protozoan microorganism, widely distributed in nature with high adaptive capacity to survive in many varieties of environments. (Nacapunchai et al 1999; Salazar-Ardiles et al, 2022). *Acanthamoeba* consists of two stages, an active motile stage called "trophozoite", and converts to non-motile stage called "cyst" when the environment is inconvenience for survival (Marciano-Cabral and Cabral 2003). Some acanthamoebae can exist as both free-living and pathogenic parasites (CDC, 2020).Currently, the genus *Acanthamoeba* have been identified by based on the rRNA sequence into 23 genotypes, ranging from T1 to T23 (Putaporntip et al. 2021) and *Acanthamoeba* genotype T4 has been reported to be the most causative agent of both lethal granulomatous amoebic encephalitis (GAE) and AK (Niyyati et al. 2016). Pathogenic strains of Acanthamoeba produces specific adhesive proteins, such as mannose-binding

proteins and laminin-binding proteins which can bind to those on the corneal epithelium (Huth et al. 2021).

Treatments of *Acanthamoeba* keratitis usually used a combination of topical 0.02% chlorhexidine and polymyxin B neomycin, added to ketoconazole 200 mg orally (Kosrirukvongs et al, 1999; Hargrave et al, 1999). Recently, there is no agent has been described as a single fully effective treatment therefore several herbal medicine have been widely studied and found to have anti-Acanthamoeba properties against trophozoites and cysts. (Derda and Hadas, 2014; Lorenzo-Morales et al. 2013; Sifaoui et al. 2014).

Orthosiphon aristatus (Blume) Miq. (Cat's whiskers) is a traditional folk-herb (Fakkham et al,2018) that has been included in Thai National List of Essential Medicines for using in anti-nephrolithiasis and anti-urolithiasis or stone removal treatment (National drug information. 2016). The reports of therapeutic effects and their biological activities were that of : anticancer (Pauzi et al. 2018; Halim et al. 2017; Abdelwahab et al. 2011), antiepilepsy (Kar et al. 2018), anti-hepatotoxic (Yuniarto et al. 2017), hepatoprotective (Alshawsh,et al. 2011)anti-hypertensive (Adnyana et al. 2013), antiarthritic (Tabana et al. 2016), antidiabetic & anti-inflammatory (Wang et al, 2022), enhancing memory (George et al. 2015), antioxidant (Manivasagan et al. 2022) and antimicrobial (Ho et al. 2010; Ripim et al. 2018). In previous studies, several aspects of the phytochemistry of *O. aristatus* extract were investigated. However, the crude extract of *O. aristatus* leaf have not been examined for their potential anti-*Acanthamoeba* properties.

OBJECTIVE

This study was aimed to determine the anti-Acanthamoeba activity of *Orthosiphon aristatus* (Blume) Miq. (Cat's whiskers), a medicinal plant. Effects of crude leaf extract from plant were investigated against trophozoite and cyst forms.

MATERIALS AND METHODS

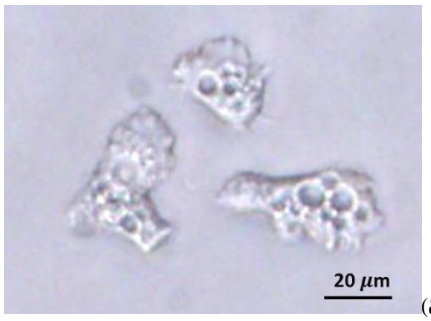
Herbal plant extracts

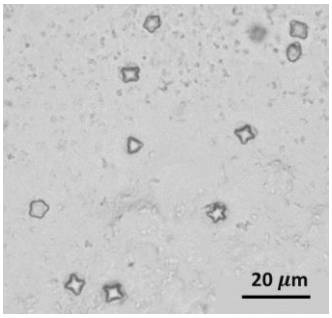
The fresh leaf samples of *Orthosiphon aristatus* (Blume) Miq. were collected from Mahasarakam province, Thailand, and sent for identification at the Royal Forest Department, Ministry of Agriculture and Cooperatives. The collected leaves were washed, chopped into pieces and dried under the hot air condition at 50°C. The dry materials were ground and the extraction procedure was maceration method using 95% ethanol at room temperature with occasional shaking for two days and drying by rotary evaporator. All dried extracts were stored in a freezer at -20°C until use.

Acanthamoeba strain

Acanthamoeba spp. T4 genotype were isolated from contact lenses of our previous study confirmed by morphology and sequencing. (Nacapunchai et al, 1999; Mahittikorn et al., 2015) Briefly, trophozoites (Fig. 1a) were maintained by cultivation in Proteose peptone - yeast extract - glucose medium (PYG) at 30°C (Duarte et al., 2013). Trophozoites in exponential growth (48-72h) and presenting at least a 95% viability by using trypan blue staining and hemacytometer. The cysts (Fig. 1b) were performed by inoculation of trophozoites onto the 1.5%

non-nutrient agar (NNA) plates after 1 week. In order to obtain the parasites for testing by harvesting the amoebae from the media and washing 3 times with sterile Page's saline. The final concentration of both cysts and trophozoites were adjusted to 2×10^5 cell/ml for the activity assays.



(a)


(b)

Figure 1 *Acanthamoeba* trophozoites (a), and cyst (b), on non-nutrient agar (NNA) medium as shown by inverted microscopy. Magnification 400x

Antiamoebic activity assay.

Fifty microliter (50 µl) of the calibrated cyst/ trophozoite suspension (2×10^5 cell /ml) was inoculated and 100µl of each extract concentration (125, 250, 500 µg/ml) was added to each well of the 96-well plate. The plates were incubated at 26 °C for 24, 48, and 72 h. The negative control compound was sterile Page's saline and a positive control one was Chlorhexidine 0.02% eye drop. To evaluate the anti-*Acanthamoeba* effects of extract, different concentrations of the *O. aristatus* (125, 250, 500 µg/ml) were examined at three difference times (24, 48 and 72 h) on trophozoites and cysts of *Acanthamoeba*. Each concentration and experiments were performed in triplicate. At the end of each incubation time, the culture plate were observed under microscope and then 25 µl of 0.4% trypan blue was used for cell viability in each test. Those trophozoites and cysts that absorb the color are dead, but the living ones are colorless. The number of live and dead trophozoites and cysts was counted with a hemocytometer the percentage of viable acanthamoeba cells was calculated.

Statistical Analysis

The number of trophozoites and cysts was expressed as mean ± standard deviation and percent survival. Statistical significance was considered as $P < 0.05$. A comparison of three or more dependent groups was performed using One-way ANOVA and repeated measures tests with Statistical software,

RESULTS

The normal morphological characteristics of the *Acanthamoeba* trophozoites as observed by trypan blue exclusion assay and inverted microscope, such as higher number of acanthopodia like spine on the cell surface membrane (Fig. 2a). In contrast, the amoebae treated with *O. aristatus* extracts have lost their strong acanthopodia (Fig. 2b). Those of the cystic stage such as triangular shape and soft surface of the cysts were observed in the control (Fig. 1b,2a).

It has been found that the cysts treated with of *O. aristatus* extracts demonstrated forms of retraction and shrink cell wall surface (Fig. 2c), compared to the control.

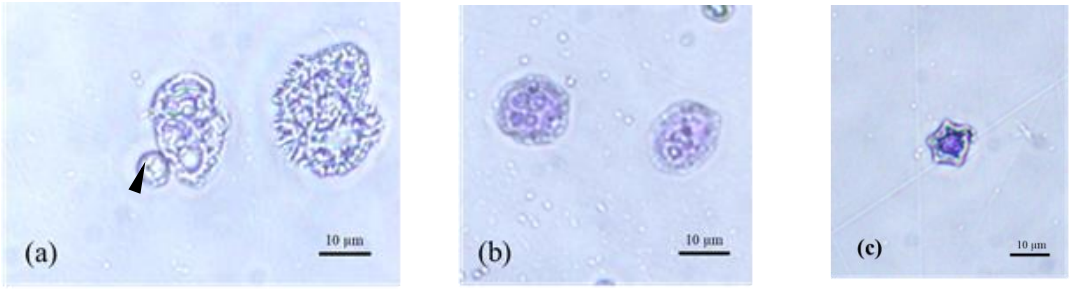


Figure 2. The viability of *Acanthamoeba* by trypan blue exclusion assay: (a) two living trophozoites and one cyst (arrow), (b) two dead trophozoites and (c) dead cyst.

The results show that the leaf extract of *O. aristratus* at the concentration of 250 and 500 µg/ml killed 100% of trophozoites and cysts from 24 h. The O. *aristratus* leaf extract of 125 µg/ml concentration killed 47.1, 54.6 and 69.4% of trophozoites and 43.8, 52.4 and 59.6 % of cysts after 24, 48 and 72h, respectively. The results show that the extract has a dose- and time-dependent activity on trophozoites and cysts. The anti- *Acanthamoeba* effect of the ethanolic extract was statistically significant compared to the negative control group at a concentration of 125 µg/ml after 24, 48, and 72 h ($P < 0.05$) as shown in Table1.

Table 1. Effects of *O. aristratus* leaf extract on viability of *Acanthamoeba* trophozoites and cysts.

***O. aristatus* Extract**	**Stage of *Acanthamoeba***	**Viable of *Acanthamoeba* (%) after incubation time**			**P. value**
		24 h	**48h**	**72h**	
125 µg/ml	Trophozoites	52.83 ±1.39	46.38±1.51	30.65 ±3.99	□P□0.05
	Cysts	56.29±0.82	47.63±5.30	40.48±2.67	□P□0.05
250 µg/ml	Trophozoites	00.00	00.00	00.00	P>0.05
	Cysts	36.21±1.92	49.63±4.20	45.48±3.61	P>0.05
500 µg/ml	Trophozoites	00.00	00.00	00.00	P>0.05
	Cysts	56.29±0.82	47.63±5.30	40.38±2.67	P>0.05
Negative control	Trophozoites	85.96±4.12	92.33±6.20	100.00	□P□0.05
	Cysts	100.00	100.00	100.00	□P□0.05
Positive control	Trophozoites	00.00	00.00	00.00	P>0.05
	Cysts	00.00	00.00	00.00	P>0.05

Data were expressed as mean±SD,
Positive control: Positive control group receiving Chlorhexidine 0.02% drop,
Negative control: Negative control receiving sterile Page's saline

DISCUSSION

Acanthamoeba infections are challenging to treat trophozoites and the difference in sensitivity to drugs have been observed but the transformation of the trophozoites into cysts make it more difficult for treatment. (Niyyati et al, 2016). Moreover, the challenging to treat is mainly due to the strong nature of the two layers of cyst walls. The present study showed that ethanolic extracts of *O. aristratus* are more effective on trophozoites than cysts of *Acanthamoeba* at the tested concentrations. Trophozoites showed strong loss of acanthopodia and thorn-like projection pseudopodia, while few cyst demonstrated retraction appearance. The *O. aristratus* leaf extracts showed some activity against cyst wall but could not inhibit the activity of acanthamoeba cystic stage which is the main factor against treatment of the parasite. This suggests the potential in benefits of the medicinal plants *O. aristratus* as an option for Acanthamoeba infections.

CONCLUSION AND FUTURE WORK.

It can be concluded that upon treatment with the leaf extract of. *O. aristratus* reduction in the number of viable trophozoites and cysts of *Acanthamoeba* was statistically significant with an increase in time and concentration. Finally, further studies are needed to identify the active components of the plants that have the potential toxic effects to be used for the production of drugs towards the treatment of *Acanthamoeba* infections.

Potential conflicts of interest

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

Abdelwahab SI, Mohan S, Elhassan MM, et al. 2011. Antiapoptotic and antioxidant properties of *Orthosiphon stamineus* Benth (Cat's Whiskers): Intervention in the Bcl-2-Mediated apoptotic pathway. Evid Based Complement Alternat Med 2011: 156765. DOI: 10.1155/2011/156765

Adnyana IK, Setiawan F, Insanu M. 2013. From ethnopharmacology to clinical study of *Orthosiphon stamineus* Benth. Intl J Pharm Pharm Sci 5 (3): 66-73.

Alshawsh MA, Abdulla MA, Ismail S, Amin ZA. Hepatoprotective Effects of *Orthosiphon stamineus* Extract on Thioacetamide-Induced Liver Cirrhosis in Rats. Evid Based Complement Alternat Med. 2011: 103039. doi: 10.1155/2011/103039 PMCID: PMC3106356PMID: 21647311

Cdc—Dpdx—Free living amebic infections, "Recuperado 22 de octubre de 2020," 2019, https://www.cdc.gov/dpdx/freelivingamebic/index.html

Chegeni TN, Fakhar M, Ghaffarifar F, Saberi R. Medicinal Plants with anti-Acanthamoeba Activity: A Systematic Review. Infect Disord Drug Targets. 2020; 20(5):620-650. doi: 10.2174/187152651966 6190716095849. PMID: 31322072

Derda M, Hadaś E. The use of phytotherapy in diseases caused by parasitic protozoa. Acta Parasitol. 2014 Mar;60(1):1-8. doi: 10.1515/ap-2015-0001. PMID: 26204014.

Duarte JL, Furst C, Klisiowicz DR, Klassen G, Costa AO. Morphological, genotypic, and physiological characterization of Acanthamoeba isolates from keratitis patients and the domestic environment in Vitoria, Espírito Santo, Brazil. Exp Parasitol. 2013;135(1):9–14. https://doi.org/10.1016/j. exppara.2013.05.013

Fakkham S, Puttanamatakun P. Herbal plants in suansunandha royal garden and Thai traditional medicine. Proceedings of 135th the IRES International conference, Moscow Russia, August,2018

George A, Chinnappan S, Choudhary Y, Choudhary V K, Bommu P, Wong HJ. 2015. Effects of a proprietary standardized Orthosiphon stamineus ethanolic leaf extract on enhancing memory in Sprague Dawley rats possibly via blockade of adenosine A2A receptors. Evid Based Complement Alternat Med 2015: 375837. DOI: 10.1155/2015/375837

Halim NH, Pauzi N, Hamil SHR, Shafaei A, Ismail Z, Mohd KS. 2017. Standardization of *Orthosiphon stamineus* raw material and extracts for anti-uterine fibroid. Intl J Pharmacogn Phytochem Res 9 (4): 512- 515.

Hargrave SL, McCulley JP, Husseini Z. Results of a trial of combined propamidine isethionate and neomycin therapy for Acanthamoeba keratitis. Brolene Study Group. Ophthalmology. 1999 May;106(5):952-7. doi: 10.1016/s0161-6420(99)00515-1. PMID: 10328395.

Ho C, Noryati I, Sulaiman S, Rosma A. 2010. In vitro antibacterial and antioxidant activities of *Orthosiphon stamineus* Benth. extracts against food-borne bacteria. Food Chem 122 (4): 1168-1172. DOI: 10.1016/j.foodchem.2010.03.110

Huth S. Reverey JF. Leippe M. Selhuber-Unkel C. Adhesion forces and mechanics in mannose-mediated acanthamoeba interactions. *PLoS ONE* 2017, *12*, e0176207.

Kar B, Choo M, Kundap UP, Kumari Y, Hue S, Othman I, Shaikh MF. 2018. Orthosiphon stamineus leaf extract affects TNF-α and Seizures in a zebrafish model. Front Pharmacol 9: 139. DOI: 10.3389/fphar.2018.00139

Kosrirukvongs P, Wanachiwanawin, D, and Visvesvara GS. Treatment of Acanthamoeba keratitis with chlorhexidine. Ophthalmology 1999; 106: 798–802.

Lorenzo-Morales J, Martim-Navarro CM, LopézArencibia A, Arnalich-Montiel F, Piñero JE, Valladares B. Acanthamoeba keratitis: an emerging disease gathering importance worldwide? Trends Parasitol. 2013; 29(4):181-187. https://doi.org.10.1016/j.pt.2013.01.006

Mahittikorn A, Kittichathanakul T, Jongdee T, Nacapunchai D. Knowledge, Behavior, and Free-Living Amoebae Contamination of Cosmetic Contact Lens Among University Wearers in Thailand: A Cross-Sectional Study. Eye & Contact Lens: Science & Clinical Practice 43(2): 81-88, March 2017. | DOI: 10.1097/ICL.0000000000000246

Mahittikorn A, Mori H, Popruk S , Roobthaisong A, Sutthikornchai C, Koompapong K, Siri S, Sukthana Y, Nacapunchai D. Development of a rapid, simple method for detecting *Naegleria fowleri* visually in water samples by loop-mediated isothermal amplification (LAMP). PLoS One. 2015 Mar 30; 10(3):e0120997. doi: 10.1371/journal.pone.0120997. eCollection 2015

Manivasagan V , Saranya K , Poojashree S, Ankitha K, Niyathi V. *In vitro* Antioxidant, Antidiabetic and Antibacterial Activities of *Orthosiphon diffusus*. Res J Pharmacog Phytochem. 2022; 14(3):163-0. doi: 10.52711/0975-4385.2022.00030.

Marciano-Cabral F, Cabral G. Acanthamoeba spp. as agents of disease in humans. Clin Microbiol Rev. 2003 Apr ; 16 (2) : 273 - 307. doi: 10.1128/ CMR.16.2.273-307.2003. PMID : 12692099 ; PMCID: PMC153146.

Nacapunchai D, Lamom C, Ruangsittichai C, Sriwichai P. Isolation of free-living amebae from soil and water resources in Thailand. *J Trop Med Parasitol*. 1999;22: 22–26.

National drug information. 2016. Herbal medicine list. Available Source: http://ndi.fda.moph.go.th/ drug _national, April 23, 2020.

Niyyati M, Dodangeh S, Lorenzo-Morales J. A review of the current research trends in the application of medicinal plants as a source for novel therapeutic agents against *Acanthamoeba* infections. Iran. J. Pharm. Res., 2016, *15*(4), 893-900.[PMID: 28243287]

Nuengchamnong N, Krittasilp K, Ingkaninan K. Characterisation of phenolic antioxidants in aqueous extract of *Orthosiphon grandiflorus* tea by LC–ESI-MS/MS coupled to DPPH assay. Food Chem 2011: 127; 1287-93.

Pauzi N, Mohd KS, Abdul Halim NH, Ismail Z. 2018. Orthosiphon stamineus extracts inhibit proliferation and induces apoptosis in uterine fibroid cells. Asian Pac J Cancer Prev 19 (10): 2737-2744. DOI: 10.22034/APJCP.2018.19.10.2737

Prajuabjinda O, Panthong S, Itharat A. Antimicrobial Activity of Thai Medicinal Preparation of Khampramong Temple Used for Cancer Treatment and Its Plant Components. J Med Assoc Thai 2012; 95 (Suppl. 1): S159-64.

Putaporntip C, Kuamsab N, Nuprasert W, et al. Analysis of *Acanthamoeba* genotypes from public freshwater sources in Thailand reveals a new genotype, T23 *Acanthamoeba bangkokensis* sp. nov. Sci Rep. 2021; 11: 17290. doi: 10.1038/s41598-021-96690-0 PMCID: PMC 8397737 PMID: 34453084

Ripim M, Fazil N, Ibrahim K, Bahtiar A, Wai C, Ibrahim N, Nor M. 2018. Antiviral properties of *Orthosiphon stamineus* aqueous extract in Herpes Simplex Virus Type 1 infected cells. Sains Malaysiana 47 (8): 1725-1730.

Salazar-Ardiles C, Asserella-Rebollo L, David C. Andrade, "Free-Living Amoebas in Extreme Environments: The True Survival in our Planet", BioMed Research nternational, vol. 2022, Article ID 2359883, 10 pages, 2022. https://doi.org/10.1155/2022/2359883

Sifaoui I, López-Arencibia A, Ticona JC, Martín-Navarro CM, Reyes-Batle M, Mejri M, et al. Bioassay guided isolation and identification of anti *Acanthamoeba* compounds from Tunisian olive leaf extracts. Exp Parasitol. 2014; 145(Suppl):111-114. https://doi.org/10.1016/j.exppara.2014.02.018.

Tabana YM, Al-Suede FS, Ahamed MB, Dahham SS, Hassan LE, Khalilpour S, Taleb-Agha M, Sandai D, Majid AS, Majid AM. Cat's whiskers (Orthosiphon stamineus) tea

modulates arthritis pathogenesis via the angiogenesis and inflammatory cascade. BMC Complement Altern Med. 2016 Nov 24;16(1):480. doi: 10.1186/s12906-016-1467-4. PMID: 27881135; PMCID: PMC5122152.

Taokaenchan, N, Areesrisom, K, Areesrisom, P, & Sateinperakul, S. (2021). Effect of Harvest Maturity on Potassium Value Secondary Metabolites and Antioxidant Activity of *Orthosiphon aristatus* (Blume) Miq. Rajamangala University of Technology Srivijaya Research Journal, 13(3), 754–763. Retrieved from https://li01.tcithaijo.org/index.php/rmutsvrj/article/view/241942

Wang Q ,Wang JN, Liu J, Zhou J, Zhuang P, Chen H. A Systematic Review of *Orthosiphon stamineus* Benth. in the Treatment of Diabetes and Its Complications. Molecules 2022, *27*, 444.

Wuttithammavead W. Pharmaceutical Science in Thai tradition medicine. Bangkok: Wuttithamma vead Cooporation; 2004.

Yam MF, Lim CP, Fung Ang L, et al. Antioxidant and toxicity studies of 50% methanolic extract of *Orthosiphon stamineus* Benth. Biomed Res Int 2013; 2013:351602.

Yuniarto ARI, Susilawati E, Khairunnisa I, Juanda D, Setiawan F. 2017. Antioxidant and gastric ulcer healing effect of *Orthosiphon Stamineus* (Benth.) leaves extract in aspirin-induced rats. Asian J Pharm Clin Res 10 (2): 2-4.