Several Fractions of *Pouzolzia indica* Methanolic Extract were Lethal to the Acanthamoeba Cyst: *in vitro* study


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ABSTRACT

**Objective:** The purpose of this study was to compare the least concentrations of 5 fractions of *Pouzolzia indica* methanolic extract which can be lethal to the cyst form of the *Acanthamoeba spp*.

**Methods:** *Acanthamoeba spp.* was isolated from a keratitis patient and was cultured using nonnutrient agar plates enriched with heat-killed *E.coli* for seven days at room temperature for the production of mature cysts. The cysts were harvested, washed in normal saline solution and adjusted to the final concentration of 10^5 cysts/ml. They were mixed with several dilutions of each fraction of *Pouzolzia indica* methanolic extract. After incubation for 1 hour, they were washed and centrifuged to remove the herbal extract supernatants. The cysts were recultured in the same medium for 7 days to confirm that they were all dead.

**Results:** *Pouzolzia indica* methanolic extract fraction No.1 which was eluted by water could not kill the cyst, while the crude extract (Fraction C) could at the concentration of 1:2. The fraction No.2 which was eluted by water: methanol had the minimal cysticidal concentration of 1:4, fraction No.3 which was eluted by methanol had the minimal cysticidal concentration of 1:8 and the fraction No.4 which was eluted by ethyl acetone had the minimal cysticidal concentration of 1:4.

**Conclusion:** Our results demonstrated that the *Pouzolzia indica* methanolic extract of several fractions can be cysticidal to an Acanthamoeba cyst, and this can modified to be a better disinfecting solution for contact lens cases.

**Keywords:** *Pouzolzia indica* Benn, *Acanthamoeba spp.*

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Acanthamoeba is a group of single-celled free-living amoeba that are opportunistic pathogens of human. There are two stages of life cycle of this environmental amoeba, an infective trophozoite and a resilient cyst. Trophozoites live on a variety of bacteria, when environmental conditions become unfavourable, the organism encysts. In the cyst form, the amoeba is capable of surviving up to a year and is resistant to temperature and pH. *Acanthamoeba spp.* have been isolated from several habitats, including soil, dust, air, natural and treated water, sea water, drinking water, bottled water, dental treatment units, dialysis units, eyewash stations, and contact lenses and lens cases. Acanthamoeba spp. commonly causes Acanthamoeba keratitis (a painful eye infection). The first signs of Acanthamoeba keratitis are inflammation with redness, epithelial defects and photophobia, edema, pain due to radial neuritis, epithelial loss and stromal abscess formation with vision-threatening consequences. The most characteristic clinical feature of Acanthamoeba keratitis is the presence of a ring-like stromal infiltrate, thought to be correspond to the infiltrating inflammatory cell. Acanthamoeba keratitis is typically associated with the use of contact lenses and seems to be the most important factor. Treatment of Acanthamoeba keratitis regimen includes combination therapy which use 2 or 3 biocides such as biguanides or chlorhexidine and polyhexamethylene biguanides (PHMB) or in combination with diamidines which are effective in the treatment of Acanthamoeba keratitis. Resistance to
chemotherapeutic agents is probably the principal factor contributing to the increase in cases of *Acanthamoeba* keratitis that may result in loss of vision.

**Pouzolzia indica** Benn, with Thai name “Kob-cha-nang-dang”, is a Thai medicinal plant in the family of Urticaceae. It is a shrub of 0.5-3.0 m tall. Its leaves are alternated, stipules lanceolate of 3.5 mm. Its petiole is 2.6-11.0 cm. The leaf shape is blade-like or lanceolate to rhombic-ovale of 3.19 x 5.9 cm. with a papery appearance. Its secondary veins comprise 2 apical pairs. The abaxial surface is strigosed or dense. The adaxial surface is scabrous and sparsely pubescent. The base is round or cuneate. The margin is dentate, the apex is acute or acuminate. It is a monocotyledon with little hair on the stems and leaves. It has a cluster of little flowers between the corners of leaves and its branches.**Pouzolzia indica** can evacuate parasites in children, expel menstruation, discharge urine and treat pus. It has been used in dermatological and urological disease.

*Acanthamoeba* keratitis has been increasingly identified in the contact lens wearers, several hundreds of human cases have been reported worldwide.**Acanthamoeba* were grown on nonnutrient agar plates enriched with heat-killed *E.coli* (NNA-E.coli) for seven days at room temperature. The cysts were harvested, washed in normal saline solution to disperse the parasites and resuspend the cysts while swabbing with a sterile loop. The suspended **Acanthamoeba** cysts were passed into normal saline solution in plastic tubes, and adjusted to a final concentration of 10⁵ cysts/ml.

**Pouzolzia indica** methanolic extract.Dried stems and leaves of **Pouzolzia indica** Benn. were purchased from Chao-Krom-Po, Thai Herb Pharmacy - a traditional drug store and were processed following the method of Trakulsomboon et al., 2006. The dried stems and leaves were cut into small pieces and ground into powder. Nine hundred grams of the powder were macerated with ethylalcohol. The extract was concentrated under reduced pressure to yield the crude ethanolic dry extract of 43.7 g, then dissolved in 120 ml water and the water soluble part was chromatographed on an Diaion® HP-20 column, which was prepared from 111 g adsorbent, which was prepared from 111 g adsorbent. The column was eluted with water yielding the water fraction (No.1). The water-insoluble part was dissolved in water: methanol (1:1), the soluble part was chromatographed on the same column and eluted with water: methanol. The insoluble parts in water-methanol and methanol were dissolved in methanol and ethyl acetate respectively, and the column chromatographic process was repeated. The water (fraction 1), water-methanol (fraction 2), methanol (fraction 3) and ethyl acetate (fraction 4) were obtained.

All fractions in dry extract form were dissolved in dimethyl sulfonide or DMSO (Sigma, USA), except the dry water extract which was dissolved in sterile water. For all experiments, the extracts were diluted with medium to attain the required concentrations varying from 1 to 500 µg/ml.

**The experimental procedures**

Fifty µl of dimethyl sulfonide (DMSO) was added into the 2nd to 6th wells and another 50 µl of DMSO was added into the 7th well which was the control well. **Pouzolzia indica** methanolic extract solution of 5 fractions, fraction C (crude), fraction 1, fraction 2, fraction 3 and fraction 4 were used in this experiment. 100 µl of each fraction was added into the first well, then 50 µl of each fraction of the 1st well was brought into the 2nd well and mixed thoroughly. Another 50 µl of the mixture of the 2nd well were brought to the 3rd well and mixed; and the procedures were repeated until the dilution of the 6th well were 1:32 (by discarding the last 50 µl of the mixture of the 6th well). The six dilutions (crude, 1:2, 1:4, 1:8, 1:16 and 1:32) and the control wells were further tested for their amoebicidal effects of each fraction of **Pouzolzia indica** methanolic extract.

In the cyst assay, 50 µl amount of standardized cysts suspension of 1x10⁵ amoeba/ml was added into each well. The final six dilutions of each fraction were

**Fig 1. Photograph of fresh plant of **Pouzolzia indica** Benn.**

**MATERIALS AND METHODS**

*Acanthamoeba* cyst

The cystic form of *Acanthamoeba spp.*, isolated from a human eye with keratitis, was obtained from the Department of Parasitology, Faculty of Medicine Siriraj Hospital, Mahidol University. *Acanthamoeba* were grown on nonnutrient agar plates enriched with heat-killed *E.coli* (NNA-E.coli) for seven days at room temperature. The cysts were harvested, washed in normal saline solution to disperse the parasites and resuspend the cysts while swabbing with a sterile loop. The suspended *Acanthamoeba* cysts were passed into normal saline solution in plastic tubes, and adjusted to a final concentration of 10⁵ cysts/ml.

**Pouzolzia indica** methanolic extract

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In the cyst assay, 50 µl amount of standardized cysts suspension of 1x10⁵ amoeba/ml was added into each well. The final six dilutions of each fraction were
1:2, 1:4, 1:8, 1:16, 1:32 and 1:64. The wells were incubated in the incubator at 37°C. After 24 h, the wells were checked microscopically with the inverted microscope to detect viable cysts.

Then 200 μl of normal saline was added into each well to stop the reaction. The wells were aspirated and washed twice with 200 μl of normal saline. Then 50 μl of living E.coli suspension (optical density = 0.2) was added to each well. The tests were performed in triplicate per fraction of *Pouzolzia indica* methanolic extract solution. One of the triplicates was transferred from the wells of each dilution to non-nutrient agar with heat killed E.coli (NNA-E.coli) plates and sealed. Both wells and plates were incubated at 37°C in an incubator for 7 days in order to test the viability.

The minimum cysticidal concentration (MCC) was defined as the lowest concentration of *Pouzolzia indica* methanolic extract solution that resulted in no excystment and trophozoite replication after repeated reculture for 7 days of incubation.

**RESULTS**

From this experiment when *Acanthamoeba* was treated with *Pouzolzia indica* methanolic extract solutions for 24 h, and reculture repeated for 7 days, the minimum cysticidal concentrations (MCC) of *Pouzolzia indica* methanolic extract solutions were 1:2 (undiluted of fraction C), 1:4 (fraction 2), 1:8 (fraction 3) and 1:4 (fraction 4) at the final concentrations. At these concentrations of each fraction there was no viable of *Acanthamoeba* while in fraction 1, no dilution could kill the cyst. Table 1 indicates the results positively or negatively after reculture in the wells and non-nutrient agar plates.

The viable and non-viable cysts were viewed under light microscopically as illustrated in Fig 2-Fig 5. The normal cyst appears round or oval (Fig 2) with double cyst walls, the ectocyst and the endocyst. The ectocyst appears wrinkled and clearly separated from the endocyst which is thin and smooth.

Light micrographs of the *Acanthamoeba* cysts treated with the fraction 2 (Fig 3), fraction 3 (Fig 4), fraction 4 (Fig 5) *Pouzolzia indica* methanolic extract which are all non-viable after repeated reculture for 7 days. All showed the double cyst walls with shrunken cells inside. Some showed the cytoplasmic clumping with the destruction of the cytoorganelles.

**DISCUSSION**

*Acanthamoeba* keratitis is a severe disease related to the use of soft contact lenses.6,11-13,19 Over the years various therapeutic regimens have been proposed, but none has shown constant effectiveness in achieving clinical and parasitological cure.7-9,16,20 An important factor that might influence treatment is the identification and culture of the *Acanthamoeba* strains and a subsequent in vitro assay for known antiparasitic agents.20 This experiment would aid the clinician in planning the therapeutic regimen using the medicinal plant *Pouzolzia indica* in order to obtain the best possible outcome. The effectiveness of an antimicrobial agent on *Acanthamoeba* is of clinical value when it can destroy the cystic form completely. In this study, the Thai medicinal plant *Pouzolzia indica* methanolic extract of 4 fractions can destroy the cystic form except fraction 1. Subculture of cysts treated with *Pouzolzia indica* on NNA-E.coli plates was done to determine the cidal or static effects of this agent.

**TABLE 1.** Five fractions of *Pouzolzia indica* which can kill the *Acanthamoeba* cysts - = Negative growth of trophozoite; + = Positive growth of trophozoite in the reculture wells and NNA plates.

<table>
<thead>
<tr>
<th>Final concentrations</th>
<th>1 : 2</th>
<th>1 : 4</th>
<th>1 : 8</th>
<th>1 : 16</th>
<th>1 : 32</th>
<th>1 : 64</th>
<th>Control</th>
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<tbody>
<tr>
<td>Fraction C (crude)</td>
<td>-</td>
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<td>+</td>
<td>+</td>
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<td>+</td>
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<td>Fraction 1</td>
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<td>Fraction 2</td>
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<td>Fraction 3</td>
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<td>Fraction 4</td>
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*Fig 2.* Light micrograph of normal viable cyst.

*Fig 3.* Light micrograph of the cyst treated with fraction 2 of *Pouzolzia indica* methanolic extract.

Evaluation of the efficacy of 4 fractions of Pouzolzia indica methanolic extract indicated that fraction 1 could not kill the cyst while fractions C, 2, 3, 4 could at the concentrations of 1:2, 1:4, 1:8 and 1:4 respectively. In vitro study of the sensitivity assay is helpful for beginning therapy or at the later stage when resistance develops. Pouzolzia indica is an alternative way if the chemicals are too dangerous, as in this study this plant’s extracts have a very good antiamoebic activity on the cystic stage of Acanthamoeba. However, the results need to be confirmed with other amoebic strains and species, in association with other chemicals in vitro. Pouzolzia indica will be another Thai medicinal plant that will prove to be very important in the future.

REFERENCES