PARASITOLOGY

The Effect of Garden Croton (*Codiaeum variegatum*) Extract on the Cysts of Clinically Isolated *Acanthamoeba* spp.

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ABSTRACT

***Introduction: Acanthamoeba's* cyst resistance towards a variety of existing treatment agents has led to numerous studies in discovering new alternative drugs to be used as anti-Acanthamoeba agents. Leaf extract of *Codiaeum variegatum* (garden croton) or locally known as "Pokok Puding" has been identified as a rich source of many medicinal properties. This study was performed to evaluate the efficacy of *Codiaeum variegatum's* leaf extract as an anti-*Acanthamoeba* agent against the cysts of clinically isolated *Acanthamoeba*.**

***Materials and Methods:* Samples of treatment agents ie; *Codiaeum variegatum's* leaves were extracted using distilled water and absolute ethanol immersion methods. The efficacy test of the treatment agents as well as the minimum cysticidal concentration (MCC) test were performed to study its effect on the cysts of *Acanthamoeba* from two clinical isolates comprising HS 72 and HUKM 74.**

***Results: Codiaeum variegatum's* leaf extract at concentration of 150 µg/ml has good cysticidal effect on both of the clinical isolates studied. The average MCC value obtained for *Codiaeum variegatum's* leaf extract of both distilled water and absolute ethanol methods was at 75 µg/ml.**

***Conclusion: Codiaeum variegatum's* leaf extract exhibits good anti-*Acanthamoeba* activity towards *Acanthamoeba* cysts from clinical isolates and has the potential to be further developed for therapeutic purposes in future.**

KEY WORDS

***Acanthamoeba*, Garden Croton (*Codiaeum variegatum*), Malaysia**

INTRODUCTION

Acanthamoeba sp. is a free-living, single celled microscopic organism that is widely distributed in the natural environment. It has also been found in the nasal cavity, throat and intestine (De Jonckheere, 1991). *Acanthamoeba* sp. can cause a variety of diseases such as *Acanthamoeba* keratitis, granulomatous amebic encephalitis (GAE) and cutaneous lesions. *Acanthamoeba* keratitis is one of the most severe and potentially sight-threatening ocular parasitic infectious diseases and is recognized as the most challenging among ocular infections because of the protracted painful clinical course and frequently encountered treatment failures (Auran, 1987). It mostly affects contact lens wearers that results in corneal ulcer, partial vision loss as well as blindness. Substandard contact lens hygiene is a major risk factor for this condition where contact lens storage cases contaminated with micro-organisms are optimal for *Acanthamoeba* sp. growth. The trophozoites and cysts can transfer onto the eye leading to keratitis

Globally, *Acanthamoeba* keratitis is a cause for concern. In Malaysia, the first case of *Acanthamoeba* keratitis was reported in 1995 involving a woman who was a long-term contact lens wearer. Since then, this condition is no longer a rarity and is seen with increasing frequency especially among contact lens wearers. In 2001, 10 cases of *Acanthamoeba* keratitis were diagnosed in Hospital Universiti Kebangsaan Malaysia (HUKM) alone (Mohamed Kamel *et al*. 2005). Most cases in Malaysia were contact lens related, particularly involving the use of soft lenses (Mohamed Kamel *et al*. 2018).

Acanthamoeba keratitis is one of the most difficult ocular infections to manage successfully due to the resistance of the organism's cyst stage to most antimicrobial agents at concentrations tolerated by the cornea (Kilvington & White, 1994; Larkin *et al*. 1992). Hence, the drugs used for the treatment of this ocular infection must be cysticidal to prevent the recurrences of infection from the dormant cysts that have survived from antimicrobial treatment (Schuster, 2004).

Because of the serious nature of *Acanthamoeba* infections in humans and because of an almost complete lack of therapeutic agents available against *Acanthamoeba*, any new agent, compound or drug treatment would be welcomed. Our previous attempt by using the epidermal mucus of catfish (*Clarias batrachus*) as anti-*Acanthamoeba* against clinical isolates of *Acanthamoeba* was not successful (Mohamed Kamel *et al*. 2017). The use of natural ingredients in the production of treatment agents is a major factor that is often being considered in many studies. According to Di (1995), the selection of medicinal plants as a source of new drugs is based on herbal medicines that have been used in traditional medicine. *Codiaeum variegatum*'s leaves have been used in traditional medicine a long time ago. For example, the freeze-dried *Codiaeum variegatum*'s leaves decoction was taken as a tea by the Philipinos and its crushed leaves were used to treat diarrhea (Saffoon *et al*. 2010). *Codiaeum variegatum*'s leaves are also said to have many medicinal properties including purgative activity, sedative activity, antifungal, antiamebic and anticancer (Deshmukh & Borle 1975; Kupchan *et al*. 1976). Therefore, this research was conducted to study the efficacy of *Codiaeum variegatum's* leaf extract as an anti-*Acanthamoeba* agent.

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Table 1: Weight and percentage *(w/w) of *Codiaeum variegatum's* leaves extract of distilled water and absolute ethanol extraction.

Plant species	Type of samples	Extraction methods	Weight of samples (g)	Weight of extracts (g)	Percentage of extracts (%)
Codiaeum variegatum	Leaves	Distilled water	500 g	1.210 g	0.242 %
Codiaeum variegatum	Leaves	Absolute ethanol	500 g	12.650 g	2.530 %

Notes: *(w/w) weight per weight

Table 2: Results of the Control tests

Isolates	Positive control				Negative Control				
	PAS solution + cyst	H_2O_2 3 % + cyst	DMSO 2 % + cyst	PBS 0.1M (pH 7.3) + cyst	PAS solution	C.*v* L. extract of distilled water immersion 150 µg/ml	C.*v* L. extract of absolute ethanol immersion 150 µg/ml	DMSO 2 %	PBS 0.1M (pH 7.3)
HS 72	+	-	+	+	X	X	X	X	X
HUKM 74	+	-	+	+	X	X	X	X	X

Notes: + Presence of cyst and trophozoite of *Acanthamoeba* sp.
- Absence of trophozoite of *Acanthamoeba* sp.
X Absence of cyst and trophozoite of *Acanthamoeba* sp.
PAS Page amoebic saline
H2O2 Hydrogen peroxide
DMSO Dimethyl sulfoxide
PBS Phosphate buffered saline
C.v L. *Codiaeum variegatum's* leaves

Table 3: Efficacy of Treatment agents

Isolates	Effectiveness of treatment agents	
	Codiaeum variegatum's leaves extract of distilled water immersion 150 µg/ml	*Codiaeum variegatum's* leaves extract of absolute ethanol immersion 150 µg/ml
HS 72	√	√
HUKM 74	√	√

Notes: √ Effective (absence of trophozoites)
X Not effective (presence of trophozoites)

Table 4: Minimum Cysticidal Concentration (MCC) values

Isolates	MCC value	
	Codiaeum variegatum's leaves extract of distilled water 150 µg/ml	*Codiaeum variegatum's* leaves extract of absolute ethanol 150 µg/ml
HS 72	75 µg/ml	75 µg/ml
HUKM 74	75 µg/ml	75 µg/ml
Average	75 µg/ml	75 µg/ml

MATERIALS AND METHODS

Samples of *Acanthamoeba* sp.

Acanthamoeba spp. isolates were obtained from the *Acanthamoeba* Culture Laboratory, Dept of Medical Parasitology, Universiti Kebangsaan Malaysia. The two clinical isolates were previously derived from keratitis cases and were coded as HS 72 and HUKM 74. *Acanthamoeba* were subcultured on non-nutrient agar (NNA) and left for 14 days to allow the transformation of trophozoites into cysts as well as standardizing the age of the cysts.

Samples of treatment agents

Codiaeum variegatum's leaves were obtained from a residential area in Kulim, in the state of Kedah. The obtained samples were cleaned using sterile distilled water to eliminate all dirt attached on the surfaces of the leaves.

Sample Processing

a) Preparation of Codiaeum variegatum's leaf extract:

The extraction technique used in this study was modified from the extraction methods by Irjayanti *et al*. 2015 and Olukoya *et al*. 1993. *Codiaeum variegatum*'s leaves were left to freeze overnight to break down the leaf's cell wall. The leaves were finely cut, dried and grounded. The 500 g of the grounded leaves were then immersed in 2 L of sterile distilled water and left for 24 hours at room temperature. Then, the extract of the distilled water immersion was filtered using a gauze filter and Whatman no. 1 filter paper into a new sterile beaker and was transferred to sterile cryovial to be frozen at -80°C for at least 18 hours. The frozen extract was then freeze dried and the powder obtained was weighed and recorded.

The filtered *Codiaeum variegatum*'s leaves obtained before were then immersed in 1 L of ethanol in a new sterile beaker and left for 24 hours at room temperature. Then, the leaves extract of the absolute ethanol immersion was filtered using a gauze filter and Whatman no. 1 filter paper into a new sterile beaker and was concentrated in the rotavapor. The extract obtained was then left in a sterile petri dish under the fume hood to eliminate all excess ethanol. The powder of the *Codiaeum variegatum*'s leave of absolute ethanol immersion was weighed and recorded.

b) Preparation of treatment agent solution

Codiaeum variegatum's leave extract of distilled water and absolute ethanol immersion:

Codiaeum variegatum's leave extract solution was prepared at 2.4 mg/ml as a stock solution. The stock solution was then diluted to a concentration of 0.15 mg/ml, equivalent to 150 µg/ml before being tested. This method was applied to both extracts of *Codiaeum variegatum's* from distilled water and absolute ethanol immersion.

c) Testing methods

There were four positive controls and five negative controls used in this study. Positive controls consisted of Page Amebic Saline (PAS) solution with cyst, 3% hydrogen peroxide (H2O2) solution with cyst, 2% DMSO solution with cyst and 0.1 M PBS solution (pH 7.3) with cyst.

Negative controls consisted of PAS solution, *Codiaeum variegatum's* leave extract solution of both distilled water and absolute ethanol immersion at 150 µg/ml.concentration, 0.1 M PBS solution (pH 7.3) as well as 2% DMSO solution without cyst mixture or other ingredients.

This test was conducted on the isolates studied based on modifications of the filtration-culture method by Gradus *et al*. (1989) and the dilution method by Narasimhan *et al*. (2002). The *Acanthamoeba's* cyst suspensions were vortexed for one minute to ensure the cysts were well distributed in the PAS solution. A total 10 µl of cyst suspension was

pipetted into the microtiter plate containing 100 µl of antimicrobial agents that has been diluted. The cyst suspensions were included in all wells except for the negative control wells. Then, the all mixtures were incubated for 24 hours at 30□.

After incubation was completed, the mixture in the well was rinsed with 100 µl of PAS solution into a 1.5 ml vial to ensure that there were no cysts were left in the microtiter plate. This method was also to get rid of the antimicrobial agents around the cysts. This step was repeated three times for the same purposes. The vials were then centrifuged at a speed of 2570 rpm (740 xg) for 5 minutes (Niszl *et al*. 1995). Sediments (cysts) in the vial were transferred onto the NNA agar plate containing heat-killed *Escherichia coli* followed by incubation for 48 hours at 30□. The plates were observed under inverted microscope for the presence of trophozoites. Observations were made for 14 consecutive days before declaring it as negative. The results of this test were compared with the controls.

The minimum cysticidal concentration (MCC) of the treatment agent is defined as minimum concentration in which there is no excystation of cyst to trophozoite stage after 14 days of incubation.

RESULTS

Codiaeum variegatum's leaves extraction

Table 1. shows the weight and percentage of *Codiaeum variegatum's* leaves extract obtained using distilled water as well as absolute ethanol immersion methods. The extraction weight obtained from the distilled water immersion method was 1.210 g representing 0.242% of the total weight while the extraction weight obtained from the absolute ethanol immersion method was 12.650 g representing 2.530% of the total weight of the sample.

Results of the control tests

Both positive and negative controls used in the study have provided the expected and appropriate results as shown in table 2.

Results of efficacy test of treatment agents

Both *Codiaeum variegatum's* leaves extracts (distilled water and ethanol immersion) at a concentration of 150 µg/ml, exhibit anti-*Acanthamoeba* activity against both clinical isolates studied (Table 3).

Results of minimum cysticidal concentration (MCC) test:

The average MCC value for *Codiaeum variegatum*'s leaves extract of both distilled water and absolute ethanol methods was at 75 µg/ml (Table 4).

DISCUSSION

Based on the results of this study, extraction of *Codiaeum variegatum*'s leaves by absolute ethanol immersion yielded higher amount of extract as compared to the distilled water immersion method. This may indicate that the *Codiaeum variegatum*'s leaves are likely to have more bioactive substances that are soluble in organic solvents compared to water.

Both *Codiaeum variegatum*'s leaves extracts (distilled water and absolute ethanol immersion) at 150 µg/ml were capable of inhibiting the excystation of the both *Acanthamoeba* isolates studied. This proves that *Codiaeum variegatum*'s leaves extract possesses anti-*Acanthamoeba sp*. activity. Previous studies have also showed that *Codiaeum variegatum*'s leaf has antiamebic activity (Deshmukh & Borle 1975; Kupchan *et al*. 1976; Njoya *et al*. 2014; Moundipa *et al*. 2005; Ogunwenmo *et al*. 2007).

Effectiveness of *Codiaeum variegatum*'s leaves extract solution in inhibiting the growth of all strains of *Acanthamoeba sp*. studied is probably due to the bioactive substances found in the leaves of the plant. According to Bijekar and Gayatri (2014), the medicinal value of plants is due to the presence of several phytochemical compounds contained in the plants. This phytochemical substance is divided into two types, namely primary and secondary metabolites. Primary metabolites are needed for plant growth while secondary metabolites are by-products of metabolic pathways that play an important role in plant defense systems. *Codiaeum variegatum*'s have been found to be rich in secondary metabolites such as flavonoids, phenols and terpenoids. Flavonoids are substances that are able to inhibit the growth of microorganisms by depleting the membrane, inhibiting synthesis of DNA, RNA and even proteins (Dzoyem *et al*. 2013). Therefore, the high content of flavonoids in *Codiaeum variegatum*'s leaves may have successfully disturbed the wall stability of *Acanthamoeba sp*. studied and inhibited its growth.

The MCC value of *Codiaeum variegatum*'s leaves extract of each immersion method tested on both for HS 72 and HUKM 74 was obtained at a concentration of 75 µg/ml. A study conducted by Moundipa *et al*. (2005) found that *Codiaeum variegatum*'s leave extract solution at a concentration of 10 µg/ml and 100 µg/ml exhibited anti-*amoeba* activity. In fact, its in vitro activity was higher than that of metronidazole, the reference drug used for the treatment of amoebiasis (Moundipa *et al*. 2005). This plant is a good potential candidate for future studies, mainly to confirm the true amoebicidal activity in axenical culture, and biochemical mechanism of anti-Acanthamoebic inhibition.

CONCLUSION

Codiaeum variegatum's leaf extract does possess anti-*Acanthamoeba* activity and has the potential to be further developed for therapeutic purposes in future.

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