PARASITOLOGY

Anti-*Acanthamoeba* Activity of Garden Croton (*Codiaeum variegatum*) Extract on the Cysts of *Acanthamoeba*

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ABSTRACT

Introduction: ***Acanthamoeba*** **is an opportunist protist pathogen that is known to infect the cornea to produce keratitis. Early diagnosis, followed by aggressive treatment using a combination of drugs is a prerequisite in successful treatment but even then, prognosis remains poor due to lack of effective drugs.** ***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) has been identified as a rich source of many medicinal properties. This study was performed to evaluate the anti-*Acanthamoeba* activity of** ***Codiaeum variegatum's*** **leaf extract as an anti-*Acanthamoeba* agent against the cysts of environmentally 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 environmental isolates comprising TTAL 1 and TL 3.**

Results: ***Codiaeum variegatum's*** **leaf extract at concentration of 150 µg/ml has good cysticidal effect on both of the environmental isolates studied. The average MCC values obtained for** ***Codiaeum variegatum's*** **leaf extract of both distilled water and absolute ethanol methods were 56.25 µg/ml and 37.5 µg/ml respectively.**

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

KEY WORDS

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

INTRODUCTION

Acanthamoeba is a free-living protozoan widely distributed in the environment, occurring in vegetative trophozoite, and resistance cyst stages during its life cycle. *Acanthamoeba* can cause two well-recognized diseases: *Acanthamoeba* keratitis and *Acanthamoeba* granulomatous encephalitis. *Acanthamoeba* keratitis has been recognized as a significant ocular microbial infection, being an acute inflammation of the cornea that can result in blindness when not properly treated in the initial stage (Schuster and Visvesvara, 2004). The incidence of the illness is on the rise due to the increasing number of contact lens wearers.

Acanthamoeba eradication from the infection site is very difficult because under unfavourable conditions (i.e. drug treatment), the amoebas trophozoite switch over to cyst stage and medical treatment (drugs) is mostly less effective against amoeba cysts than trophozoites due to the stiff double wall of the cysts which render it extremely resistant to anti-amoebic drugs. This is also problematic as cysts have the capability to resist and survive after initial successful chemotherapeutic administration and cause deterioration of the disease. Encystment is the strategic mechanism amoeba adopts to combat the external threat i.e. harsh environmental conditions and/or drug treatment. The capability of cysts to resist chemotherapeutic agents is perhaps a key factor which contributes the increased cases of protozoan infections. There is an essential need to have a better antimicrobial chemotherapy and alternate strategies to build up therapeutic interventions. The search for new compounds originating from natural resources is an important research area. Many new natural product groups have been identified for antiparasitic properties of with their astonishing efficacy and selectivity such as plant-derived terpenes, alkaloids and phenolics (Kayser *et al*. 2003).

According to Liska (2000), natural plants have become an option as a source of medication in treating various diseases. The use of natural ingredients is an alternative that is often considered in various types of studies. In traditional medicine, medicinal plants has been selected as a source of new drugs. Zubeyde *et al*. 2008 demonstrates the in vitro effectiveness of garlic against the A. castellanii growth curve. Evaluations revealed that garlic inhibits trophozoite growth in dose and time-dependent ways. The garden croton, *Codiaeum variegatum"s* leaf, has been used in traditional medicine a long time ago. For example, decoction of the freeze-dried leaves of *Codiaeum variegatum's* was taken as a tea by the Filipinos and its crushed leaves were believed to treat diarrhea (Gertrudes 2006; Saffoon *et al*. 2010). Besides that, *Codiaeum variegatum's* leaves are said to contain antibacterial and antiamebic properties (Moundipa *et al*. 2005). Therefore, this study was done to evaluate the anti-*Acanthamoeba* activity of *Codiaeum variega-*

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Table 1: Weight and percentage *(w/w) of Codiaeum variegatum's leaf 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 immersion	500 g	1.210 g	0.242 %
Codiaeum variegatum	Leaves	Absolute ethanol immersion	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 ?g/ml	*C.v* L. extract of absolute ethanol immersion 150 ?g/ml	DMSO 2 %	PBS 0.1M (pH 7.3)
TTAL 1	+	-	+	+	X	X	X	X	X
TL 3	+	-	+	+	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

tum's leaf extract against the cysts of environmentally isolated *Acanthamoeba*.

MATERIALS AND METHODS

Samples of *Acanthamoeba* sp.:

Acanthamoeba sp. isolates were obtained from the *Acanthamoeba* sp. Culture Laboratory, Universiti Kebangsaan Malaysia. The isolates were previously derived from environmental specimens; sea water (TTAL 1) and soil (TL 3). *Acanthamoeba* sp. were subcultured on non-nutrient agar (NNA) and left for about 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℃ 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* leaves of absolute ethanol immersion was weighed and recorded.

b) Preparation of treatment agents' solution:

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

Codiaeum variegatum's leaves extract solution were prepared at 2.4 mg/ml as a stock solution. The stock solution was then diluted to a concentration of 0.15 mg/ml which is equivalent to 150 µg/ml before it was tested. Diluent used for distilled water extraction of *Codiaeum variegatum's* leaves was 0.1 M PBS solution (pH 7.3). Diluent used for the absolute ethanol extraction was 2 % DMSO.

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 ?g/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 environmental isolates; TTAL 1 and TL 3 based on modifications of the filtration-culture method by Gradus *et al*. (1989) and the dilution method by Narasimhan *et al*. (2002). To ensure the cysts were well distributed in the PAS solution, the *Acanthamoeba's* cyst suspensions were vortexed for approximately one minute. Then, a total 10 µl of cyst suspension was pipetted into the microtiter plate containing 100 µl of treatment agents that has been diluted. The cysts suspension were pipetted in all wells except for the negative control. Then all of the 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 no cysts were

Table 3: Efficacy of Treatment Agents

Isolates	Effectiveness of treatment agents	
	Codiaeum variegatum's leaf extract of distilled water immersion 150 µg/ml	*Codiaeum variegatum's* leaf extract of absolute ethanol immersion 150 µg/ml
TTAL 1	√	√
TL 3	√	√

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

Table 4: Minimum cysticidal concentration (MCC) Values

Isolates	MCC values	
	Codiaeum variegatum's leaf extract of distilled water (µg/ml)	*Codiaeum variegatum's* leaf extract of absolute ethanol (µg/ml)
TTAL 1	75 µg/ml	37.5 µg/ml
TL 3	37.5 µg/ml	37.5 µg/ml
Average	56.25 µg/ml	37.5 µg/ml

left in the microtiter plate and to eliminate 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 leaf extraction:

Table 1. shows the weight and percentage of *Codiaeum variegatum's* leaf 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 test:

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* leaf extracts (distilled water and ethanol immersion) at a concentration of 150 µg/ml, exhibit anti-*Acanthamoeba* activity against both environmental isolates tested (Table 3).

Results of minimum cysticidal concentration (MCC) test:

Table 4. shows the results of minimum cysticidal concentration (MCC) test obtained for both TTAL 1 and TL 3. The average MCC values for *Codiaeum variegatum's* leaves extract of distilled water was at 56.25 µg/ml while for the absolute ethanol method was at 37.5 µg/ml.

DISCUSSION

Acanthamoeba infections are always difficult to treat and resistance to most antimicrobial agents. Treatment and eradication of *Acanthamoeba* from the infected tissues are exceedingly difficult. Therefore, more effective drugs against *Acanthamoeba* must be developed and medicinal plants can be useful in this search. Natural compounds have been studied extensively for their therapeutic role in the protection against cardiovascular diseases, cancer, diabetes, neurological disorders, infectious diseases, etc. Presently, more than two-third of the antibiotics that are used against common bacterial pathogens are derived from natural compounds or their semi-synthetic derivatives (Fischbach and Walsh, 2009).

Investigations of plants used by traditional folk medicine is one strategy for finding alternative compounds although, it does not ensure a lack of toxicity or side effects but increases the pool of compounds to use against diseases. This would also represent a linkage between modern technology and the knowledge accumulated by different cultures through history. Several dozens of plant species are used for treating infections and diarrhoea, but most of them have not been tested for antimicrobial activity (Zaragoza et al.1999). Hafiz et al. 2016, demonstrated for the first time that methanolic seed extract of *Peganum harmala* exhibits remarkable inhibition of *Acanthamoeba* cysts and encystment in vitro which could serve a potential new natural agent against *Acanthamoeba*. Ismael et al. 2011, analyzed *Pterocaulon polystachyum* essential oil and assessed its amoebicidal activity against *Acanthamoeba polyphaga*. Plants of the genus *Pterocaulon* (Asteraceae) are used in folk medicine as an antiseptic and antifungal agent. The extract exhibited amoebicidal activity at concentrations of 20, 10, 5, 2.5, and 1.25 mg/mL of essential oil tested. Yousuf et al. 2012, discovered that pre-exposure of *Acanthamoeba castellanii* to 100 lg resveratrol and demethoxy curcumin exhibited significant amoebicidal effects (23% and 25%, respectively) at 100 lg concentrations ($P < 0.01$).

Based on the results of our current study, the extraction weight obtained from the distilled water immersion method was 1.210 g which is 0.242% of the total weight while the extraction weight obtained from the absolute ethanol immersion method was 12.650 g which is 2.530% of the total weight of the sample. These results show that the extract's weight obtained from the absolute ethanol immersion method is higher than the extract's weight obtained from distilled water immersion method. This condition may indicates that the *Codiaeum variegatum's* leaves are more likely to have more bioactive substances that are soluble in organic solvents compared to water.

Both ethanolic and aqueous extracts of *Codiaeum variegatum's* leaves at concentration of 150 µg/ml were effective at inhibiting the excystation of both isolates of Acanthamoeba cysts studied (TTAL 1 and TL3). This proves that *Codiaeum variegatum's* leaves extract possesses anti-*Acanthamoeba* activity. Previous studies have also demonstrated that *Codiaeum variegatum's* leaf has anti-amebic 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 both *Acanthamoeba* isolates is probably due to the bioactive substances found in the leaves of the plant. This is consistent with our recent findings (Mohamed Kamel et al. 2021) whereby the leaves extract also showed cysticidal activity against clinical isolates of *Acanthamoeba*. 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. Martín-Escolano *et al*. 2021, evaluated the trophozoital and cysticidal activity of 13 flavonoid glycosides isolated from *Delphinium gracile*, *D. staphisagria*, *Consolida oliveriana* and from *Aconitum napellus* subsp. They discovered that flavonoids 1, 2, 3 and 4 showed higher trophozoital activity and selectivity indexes than the reference drug chlorhexidine digluconate. These results suggest that the flavonoids might be used for the development of novel therapeutic approaches against *Acanthamoeba* infections after satisfactory *in vivo* evaluations.

The MCC value of *Codiaeum variegatum's* leaves extract of absolute ethanol immersion method tested on both isolates; TTAL 1 and TL

3 was 37.5 µg/ml. This is even lower than the MCC of flavonoid 2 that showed 100% cysticidal activity at a concentration of 50 µM by Martín-Escolano *et al*. 2021. However, the MCC values for the aqueous extract on TTAL 1 was 75 µg/ml and 37.5 µg/ml for TL 3. Our previous study on the clinical isolates of Acanthamoeba, gave an average MCC value of 75 µg/ml for both distilled water and absolute ethanol extraction methods (Mohamed Kamel *et al*. 2021).

This might indicate that the bioactive compounds in *Codiaeum variegatum's* leaves that are soluble in absolute ethanol are better at giving lower MCC value to inhibit the growth of *Acanthamoeba* from environmental isolates used in this study. A study conducted by Moundipa *et al*. (2005) found that *Codiaeum variegatum's* leaf 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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