SCREENING OF ANTI-AMOEBIC ACTIVITY ON NAEGLERIA FOWLERI IN EXTRACTS OF THAI MEDICINAL PLANTS

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ABSTRACT

Naegleria fowleri, a free-living amoeba-flagellate, is the causative agent of primary amoebic meningoencephalitis (PAM) in humans. Andrographis paniculata, Curcuma longa, Momordica charantia, and Zingiber officinale are plants widely used in the clinical treatment of viral infections, tumors, and inflammations, yet their anti-amoebic properties remain unclear. This study investigates the anti-amoebic activity of A. paniculata, C. longa, M. charantia, or Z. officinale against N. fowleri trophozoites. MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] assay was carried out to determine the maximum non-toxic dose (MNTD) of the crude extracts, followed by the anti-amoebic activity of the four medicinal plants against N. fowleri trophozoites by means of Trypan blue exclusion method. The MNTD values of M. charantia, Z. officinale, A. paniculata, and C. longa on human neuroblastoma (SK-N-MC) cells were 5, 1, 0.5, and 0.5 mg/ml respectively. No significant difference between the four plant extracts at MNTD was observed on rhesus monkey kidney (LLC-MK2) cells. The results reveal that M. charantia and A. paniculata at MNTD values had 100% anti-amoebic efficacy at 24h post-incubation. In contrast, Z. officinale and C. longa did not show amoebic efficacy at 6, 24, and 48h post-incubation. In summary, methanolic crude extracts of M. charantia and A. paniculata inhibits the activity of N. fowleri trophozoites in in-vitro assays. Both of these plants should be further investigated and may have potential as alternatives for PAM treatment.

Keywords: PAM, Naegleria fowleri, plant extracts anti-amoebic activity

INTRODUCTION

The free-living amoeba-flagellate, Naegleria fowleri causes acute fulminant primary amoebic meningoencephalitis (PAM), a disease that most commonly occurs in healthy children and young adults with recent freshwater exposure. PAM caused by N. fowleri is distributed worldwide but is most frequent in tropical areas during the hot summer months (Yoder JS et al., 2010). Infection results from water containing N. fowleri entering the nostrils, followed by migration of the amoeba to the brain via the olfactory nerve. N. fowleri’s clinical manifestations include neurological deficit, ataxia, haemorrhage, necrosis, meningitis and encephalitis in the brain leading to death in 3 to 7 days (Schuster and Visvesvara, 2004, Tiewcharoen et al., 2008). An optimum treatment for PAM has not been well defined: only 10 patients with PAM have been treated successfully with amphotericin B or with a combination of other drugs (Vargas et al., 2005).

Amphotericin B remains a cornerstone in PAM treatment, but it requires a high dosage. The usage of this drug is limited by a narrow therapeutic level and its association with high
toxicity on other organs, particularly the renal organs. In addition, amphotericin B may also cause anaemia, chills, fever, nausea, vomiting, and headache (Goodman et al., 2006). Drugs and chemotherapeutic agents to treat PAM have been investigated in vitro and in vivo studies, and adverse drug effects and toxicity were the main problems. Therefore, there is an urgent need to develop new therapeutic agents for PAM treatment that are quick acting and have low side-effects.

The World Health Organization (WHO) has encouraged countries to interact with traditional medicine to identify and exploit aspects that provide safe and effective remedies (WHO, 2000). As such, our study aimed to evaluate the maximum non-toxic dose (MNTD) of four medicinal plants; Andrographis paniculata, Curcuma longa, Zingiber officinale, and Momordica charantia, against SK-N-MC and LLC MK2 cells in in-vitro assays. We also investigated the anti-amoebic effects of four medicinal plants on N. fowleri trophozoites.

MATERIALS AND METHODS

Cell culture

A human neuroblastoma SK-N-MC cell line isolated from a Caucasian female patient with Askin’s tumour was purchased from Cell Line Service, Germany, in 2006. The cell has been maintained in Dulbecco’s Modified Eagle Medium and HAM’s F-12 (DMEM: HAM’S F-12) medium with 10% foetal bovine serum, 4mM L-glutamine, 100u/ml penicillin and 100µg/ml streptomycin and incubated at 37°C and 5% CO₂ (Tiewchareon et al., 2008). A rhesus monkey kidney LLC-MK2 cell line donated by the Late Professor Natth Bhamarapravati, Center for Vaccine Development, Institute of Molecular Bioscience, was also used. The cell was maintained in DMEM medium with 10% foetal bovine serum, 4mM L-glutamine, 100u/ml penicillin and 100µg/ml streptomycin and incubated at 37°C and 5% CO₂ (Yoksan et al., 2013).

Free living amoeba Naegleria fowleri culture

N. fowleri (Siriraj-strain) was isolated in 1986 from a PAM patient at Siriraj hospital. The trophozoites were cultured in T75-cm² flasks (Corning, USA) containing Nelson’s medium supplemented with 5% fetal calf serum (FCS) without antibiotics at 37°C. The trophozoites (~ 90%) were harvested by incubation at 4°C for 10min, scraping, and centrifugation at 5,000rpm for 2min. The pellet was dissolved with 1ml of medium. The number of total cells was determined using Trypan blue exclusion method (Tiewchareon et al., 2008).

Medicinal plant materials

Methanolic crude extracts of Andrographis paniculata were donated by Assistant Professor Dr. Jundee Rabablert, Department of Biology, Faculty of Science, Silpakorn University. Curcuma longa, Momordica charantia and Zingiber officinale were donated by Miss Natchagorn Lumlerdkij, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. Dimethyl sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) was used as a proper solvent to prepare the compounds’ stock solution (50mg/ml).

MTT colorimetric assay

The MTT colorimetric assay was performed to evaluate the maximum non-toxic dose (MNTD) of the four methanolic crude extracts according to the method described and validated by Zandi et al (2012). The SK-N-MC or LLC-MK2 cells were seeded in 96-well plates at a density of 2×10⁵ cells/well. Following 24h incubation and attachment, the cells were treated with different concentrations of plant extract (0-10 mg/ml) for 24 and 48 hours. At the indicated time, the cells were washed twice with PBS 7.4. The MTT solution was incubated for 2h before cells were lysed with dimethyl sulphoxide (DMSO). The absorbance was measured after 30 min using
a microplate reader (Wallac 1420 Multilabel counter, Perkin Elmer) at a wavelength of 590nm. Medium and DMSO (4%) served as the negative control. The results are generated from two independent experiments; each experiment was performed in triplicate. The MNTD values were calculated with Probit analysis software (LdP Line software, USA).

**Anti-amoebic activity**

*N. fowleri* trophozoites ($5 \times 10^5$ cells/ml) were added to each 1.5ml centrifuged tube. The concentration of each compound at MNTD values was added into each tube and incubated at 6, 24, and 48 h. Nelson’s medium was used as negative control. At indicated times, the amoeba culture tube was placed in an ice box for 5 min and centrifuged for 2min at 5,000 rpm to form a pellet. The supernatant was discarded and 200µl of PBS 7.4 was added to detect anti-amoebic activity by Trypan blue exclusion methods (Tiewcharoen *et al.*, 2008).

**RESULTS**

**Effect of methanolic plant extracts on cell viability.**

The maximum non-toxic dose (MNTD) of the four medicinal plants was determined by testing the methanolic extracts against SK-N-MC and LLC-MK2 cells *in vitro*. The studies were initiated by using various concentrations of methanolic extracts of each plant followed by further optimization in order to achieve a specific cytotoxic concentration. The MNTD of each plant obtained through these optimization steps are presented in Figure 1.

MTT assay revealed that the MNTD of *M. charantia* was 5mg/ml in both cells, the highest value among all the four plants examined (Figure 1A). In contrast, MNTD of *A. paniculata* and *C. longa* were the lowest, at a concentration of 0.5mg/ml in both cells (Figure 1B and 1D). *Z. officinale* recorded the second lowest MNTD (1mg/ml) in both cells (Figure 1C). It was found that the MNTD of methanolic extract was in the decreasing order of *M. charantia* > *Z. officinale* > *A. paniculata* > *C. longa*. Our studies also found that the MNTD values of the four plants in SK-N-MC were similar to those in LLC-MK2 cells.

**Effects of methanolic plant extracts on *N. fowleri* morphology**

The morphology of trophozoites treated with four medicinal-plant extracts at MNTD was observed under Olympus IX70 inverted microscope equipped with a digital camera. Observation after 6 hours revealed that *Naegleria* trophozoites were active and showed progressive movement in Nelson’s medium (Figure 2 A). *A. paniculata* and *M. charantia* halved the amoeba’s viability. The morphology of the living amoeba was affected, and cells were rounder or lifting off the plate (Figure 2 B-C). *C. longa* and *Z. officinale* slightly decreased, but still maintained normal cell morphology (data not shown).

**Effects of methanolic plant extracts on *N. fowleri* trophozoite viability**

The cell viability of trophozoites treated with four methanolic plant extracts at MNTD was measured by Trypan blue exclusion method. Among the four plants studied, our observation at 6h, *A. paniculata* showed the highest anti-amoebic inhibitory activity (98%) whereas *Z. officinale* showed the lowest anti-amoebic inhibitory activity (20%). *M. charantia* and *C. longa* also exhibited 75% and 30% anti-amoebic inhibitory activity, respectively. Test results at 24h and 48h revealed that *M. charantia* and *A. paniculata* exhibited 100% anti-amoebic inhibitory activity. This suggests that the effects of *M. charantia* and *A. paniculata* on *N. fowleri* trophozoites were time-dependent (Figure 3A, B). *Z. officinale* and *C. longa* showed approximately 20% and 30% inhibition at 24h and 48h, suggesting that both of these did not significantly affect anti-amoebic activity (Figure 3C, D).
Fig. 1- Percentage of cell viability treated with methanolic crude extracts of four plants; (A) *M. charantia*, (B) *A. paniculata*, (C) *Z. officinale* and (D) *C. longa* on SK-N-MC and LLC-MK2 at 48 h post-incubation. The data shown are means ± S.D. of two independent experiments performed in triplicate.

Fig. 2- Morphological characteristics of *Naegleria* trophozoites in the absence or presence of crude extracts at 6 h post-incubation were demonstrated; (A) control amoebae trophozoites in medium alone, (B) Amoebae treated with *M. charantia* and (C) Amoebae treated with *A. paniculata* were rounded, smaller in size, and loose from pseudopodia. The cells were viewed under inverted microscope at 400X magnification.
DISCUSSION

Free living amoeba *Naegleria fowleri* causes an acute, fulminant, necrotizing, and hemorrhagic meningoencephalitis called PAM leading to death in healthy children and young adults with a history of recent contact with fresh water (Budge et al., 2013). Amphotericin B is the current drug of choice in treating PAM, however, it has significant toxicity and is a hydrophobic molecule with negligible solubility in aqueous solutions (Soltow and Brenner, 2007).

The present study evaluates the anti-amoebic activity of four medicinal plants; *A. paniculata*, *C. longa*, *M. charantia*, and *Z. officinale*, against *N. fowleri* trophozoites. This study demonstrates that *M. charantia* extracts had low cytotoxicity in both SK-N-MC and LLC-MK2 cells (Figure 1A).

*Fig. 3-* Anti-amoebic activity of four plant extracts; (A) *M. charantia*, (B) *A. paniculata*, (C) *Z. officinale*, (D) *C. longa* against *N. fowleri* trophozoites. The data shown are means ± S.D. of two independent experiments performed in triplicate.

*M. charantia* extracts induced morphological changes, such as rounding and lifting out of the plate (Figure 2C). Additionally, *M. charantia* extract had time-dependent anti-amoebic inhibitory effects on *N. fowleri* trophozoites, thereby reducing the *N. fowleri* trophozoite yield (Figure 3A). *M. charantia* extracts display antiviral activity against dengue virus (Tang et al., 2012a) and anti-parasitic activity against *Trypanosoma*
brucei (Phillips et al., 2013). A. paniculata extracts also induced similar morphological changes such as rounding and lifting out of the plate (Figure 2B), reflecting the results of M. charantia. A. paniculata extract also had time-dependent anti-amoebic inhibitory effects on N. fowleri trophozoites, reducing N. fowleri trophozoite yield (Figure 3B). A. paniculata extracts displays antiviral activity against dengue virus (Tang et al., 2012a) and HIV (Tang et al., 2012b), anti-bacterial activity against Pseudomonas aeruginosa (Ma et al., 2012) and anti-parasitic activity against ticks and fluke (Elango et al., 2011). Our results indicate the anti-amoebic potential of M. charantia and A. paniculata against N. fowleri trophozoites.

CONCLUSION

Further extensive studies of M. charantia and A. paniculata, which show anti-amoebic properties, should be conducted. Isolation, purification, and characterization of active compounds, to discover potential anti-amoebic compounds, should be carried out. Furthermore, investigations into the mode anti-amoebic activity by the active compounds can be conducted, to provide increased insight into the inhibition of N. fowleri trophozoites.

ACKNOWLEDGEMENTS

We are very grateful to Associate Professor Darawan Wanachiwanawin, head of Department of Parasitology, Faculty of Medicine, Siriraj hospital, Mahidol University, for allowing us to carry out the above study.

REFERENCES


