ANTI-AMEBIC ACTIVITY OF DIOSGENIN ON *NAEGLERIA FOWLERI* TROPHOZOITES

Jundee Rabablert[1], Supathra Tiewcharoen[2], Prasert Auewarakul[3], Thassanant Atithep[4], Natchagorn Lumlerdkij[5] , Renu Vejaratpimol[1] and Virach Junnu[2]

[1]Department of Biology, Faculty of Science, Silpakorn University, Sanamchandra Palace, Amphoe Mueang, Nakhon Pathom; [2]Department of Parasiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok; [3]Institute of Molecular Bioscience, Mahidol University, Salaya, Nakhon Pathom; [4]Center of Nanoimaging, Faculty of Science, Mahidol University, Bangkok; [5]Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

Abstract. The aim of this study was to investigate the activity of diosgenin against *Naegleria fowleri* trophozoites at the cellular and molecular levels. Diosgenin (100 μg/ml; 241.2 μM) had a 100% inhibitory effect on *N. fowleri* trophozoites ($5x10^5$ cell/ml). Scanning electron micrograph revealed diosgenin decreased the number of sucker-like apparatuses and food cup formation among *N. fowleri* trophozoites at 3 and 6 hours post-exposure, respectively. Diosgenin down-regulated the *nf cysteine protease* gene expression of *N. fowleri* trophozoites at 6 and 12 hours post-exposure. The toxicity to mammalian cells caused by diosgenin at therapeutic dose was less than amphotericin B, the current drug used to treat *N. fowleri* infections. Our findings suggest diosgenin has activity against the surface membrane and the *nf cysteine protease* of *N. fowleri* trophozoites. However, the other mechanisms of action of diosgenin against *N. fowleri* trophozoites require further exploration.

Keywords: *Naegleria fowleri*, diosgenin, *nf cysteine protease*, scanning electron micrograph

INTRODUCTION

Naegleria fowleri is a thermophilic free-living ameba found in freshwater environments worldwide. It can cause a rare, potentially fatal disease in humans known as primary amebic meningo-encephalitis (PAM**)** (Tiewcharoen *et al*, 2009). The few surviving cases often have permanent neurological sequale (Schuster and Visvesvara, 2004). The current drug of choice to treat PAM is amphotericin B (AMB). AMB has significant toxicity; is a hydrophobic molecule with negligible solubility in aqueous solutions (Kim *et al*, 2008; Brunton *et al*, 2011). There is an urgent need to develop other agents to treat PAM with fewer side-effects and more rapid onset of action. The World Health Organization (WHO) has encouraged countries to investigate traditional medicines to identify safe and effective remedies (WHO, 2000). *Momordica charantia* and its purified compound, diosgenin, has been found to have anti-viral (Wang

Correspondence: Supathra Tiewcharoen, Department of Parasiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok Noi, Bangkok 10700, Thailand.
E-mail: supathra.tie@mahidol.ac.th

et al, 2011), anti-fungal (Cho *et al*, 2013), anti-helminthic (Wang *et al*, 2010), and anti-malarial activity (Pabon *et al*, 2013). In a previous study, we found the crude methanolic extract of *M. charantia* (5 mg/ml) to have 100% activity against *N. fowleri* trophozoites and to have low cytotoxicity in SK-N-MC or LLC-MK2 cells (Luangboribun *et al*, 2014). In this study, we evaluated the anti-amebic activity of diosgenin against *N. fowleri* trophozoites at the cellular and molecular levels. The cytotoxicity of diosgenin against mammalian cells was also investigated.

MATERIALS AND METHODS

Naegleria fowleri culture

N. fowleri (Siriraj-strain) was first isolated from a PAM patient at Siriraj Hospital, Bangkok, Thailand in 1986. Trophozoites were cultured in Nelson's medium supplemented with 5% fetal bovine serum (FBS) without antibiotics at 37°C. The trophozoites were harvested during the logarithmic phase of growth and determined using the Trypan blue exclusion method (Tiewcharoen *et al*, 2008).

Mammalian cell culture

A rhesus monkey kidney LLC-MK2 cell line donated by the Late Professor Natth Bhamarapravati, Center for Vaccine Development, Institute of Molecular Bioscience, has been maintained in Dulbecco's Modified Eagle medium (DMEM, Invitrogen, Carlsbad, CA). A human neuroblastoma SK-N-MC cell line was purchased from Cell Line Service, Germany, in 2006. The cell has been maintained in DMEM; Ham's F-12 (Cell-Line, Heidelberg, Germany). Ten percent of fetal bovine serum (FCS), 4 mM L-glutamine, 100 μ/ml penicillin, 100 μg/ml streptomycin were added to both cell cultures and incubated at 37°C with 5% CO_2 (Tiewchareon *et al*, 2014).

Reagents

Fifty grams of amphotericin B (AMB, C47H73NO17 MW: 924.07902) was purchased from Bharat Serums and Vaccines (Ambermath, India), and dissolved in 5 ml distilled water, giving a concentration of 10 mg/ml to be a stock solution, and then stored at -20ºC until used. Diosgenin, a purified extract product of *M. charantia*, was donated by the Center for Applied Thai Traditional Medicine, Faculty of Medicine, Siriraj Hospital, Mahidol University. Fifty milligrams of diosgenin was dissolved in 2 ml dimethyl sulfoxide (DMSO), (Sigma-Aldrich, St. Louis, MO), resulting in a concentration of 25 mg/ml to be a stock solution, and then stored at -20ºC until used.

Anti-amebic activity assay

The *N. fowleri* trophozoites were seeded in 1.5 ml microcentrifuge tubes at a density of 5×10^5 cells/ml. Two-fold serial dilutions of diosgenin (0, 25, 50, and 100 μg/ml) were then added to the trophozoites and incubated at 37°C for 6, 24, and 48 hours. AMB at 10 μg/ml was used as positive control, as previously described by Luangboribun *et al* (2014). Nelson's medium and 4% DMSO were used as negative control. At specified times, the untreated and treated trophozoites were then harvested and centrifuged at 5,000*g* for 2 minutes. The cell pellets were then dissolved with 0.1 M phosphate-buffered saline (PBS), at a pH of 7.4. Trophozoite viability was determined using the Trypan blue exclusion method.

Ultrastructure study

N. fowleri trophozoites were placed in a 35 mm dish at a density of 5×10^5 cells/ml. One hundred microliters of diosgenin

was added to the trophozoites and incubated at 37°C for 0, 3, and 6 hours. At the specified times, the untreated and treated trophozoites were then harvested and prepared for study as described previously (Tiewcharoen *et al*, 2014). Finally, the trophozoites were photographed under a scanning electron microscope (SEM, Hitachi S-510, Tokyo, Japan) at an accelerating voltage of 25 kV.

Total RNA extraction and cDNA synthesis

The RNA was extracted from the untreated and treated trophozoite pellets using a NucleoSpin RNA II Kit (Macherey-Nagel, Duren, Germany), following manufacturer's instructions. Twenty nanograms of RNA were used as a template to synthesize the first strand of the cDNA used in the Maxime RT PreMix Kit (iNtRON Biotechnology, Kyungki-Do, Korea). After cDNA synthesis, it was stored at -20°C until used for PCR amplification.

Polymerase chain reaction

PCR amplification was conducted using 2 μl of the synthesized single-stranded cDNA along with 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 0.2 μM *nf cysteine protease* (F; 5′-TTGCTGCTACCATCTCCAGC-3′ R; 5′-GCTCTTACCAACACCATA ACCAAC-3′) and other specific primers; *nfa1*, *Mp2CL5, pB2.5, Naegleria pore B,* Nf314, *nf phospholipase* and *nf actin* of *N. fowleri* trophozoites (Tiewcharoen *et al*, 2014), and 2.5 μmol of *Taq* polymerases (iNtRON Biotechnology) to give a total volume of 20 μl. The cDNA templates were amplified as described previously (Rabablert *et al*, 2011). The PCR products were then placed on 1.8% agarose Tris-borate-EDTA gel and electrophoresed at 100 V for 30 minutes. The gel was then stained with ethidium bromide and visualized under ultraviolet light.

Statistical analysis

The results are expressed as means ± one standard deviation for each of the three independent experiments and were carried out in triplicate. A Student's *t*-test was used for analysis. A p-value <0.05 was considered significant.

RESULTS

Effect of diosgenin on *N. fowleri* trophozoite

The cell viability of the tested trophozoites treated with diosgenin at 0, 50, and 100 μg/ml was determined using the Trypan blue exclusion method. Diosgenin at 100 μg/ml gave 98% inhibition 6 hours post-exposure, but diosgenin at 50 μg/ml did not cause any inhibition 6 hours post-exposure (Fig 1). Diosgenin at 100 μg/ml gave 100% inhibition 24 hours post-exposure but diosgenin at 50 μg/ml gave not significant inhibition 24 hours post-exposure. AMB (10 μg/ml) gave 100% inhibition at 6, 24, and 48 hours post-exposure (Fig 1). Four percent DMSO caused no inhibition (Fig 1), suggesting the diosgenin, not DMSO, caused inhibition. The effect of diosgenin on *N. fowleri* trophozoites was both dose- and time-dependent ($p<0.05$).

Effect of diosgenin on the morphology of *N. fowleri* trophozoites

On scanning electron micrographs of the *N. fowleri* trophozoites 3 and 6 hours post-diosgenin, the morphological characteristics of the *N. fowleri* trophozoites were studied. Untreated trophozoites had a sucker-like apparatus and a wrinkled membrane (Fig 2a). Diosgenin-treated amebae 3 hours post-exposure were smaller in size and had fewer sucker-like apparatuses (Fig 2b). The morphological characteristics of the treated trophozoites

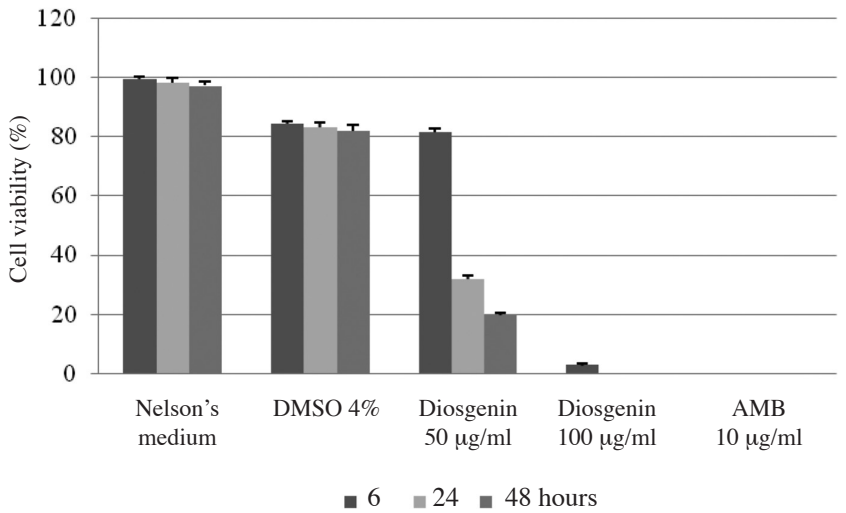

Fig 1–Activity of diosgenin (50, and 100 μg/ml) and amphotericin B (AMB, 10 μg/ml) against *N. fowleri* trophozoites using the Trypan blue exclusion method. Nelson's medium and DMSO (4%) were used as a negative control.

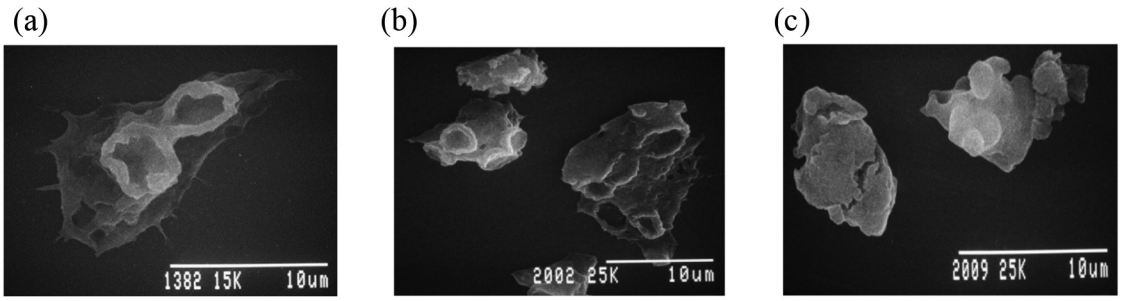

Fig 2–Scanning electron micrographs of *Naegleria* trophozoites: (a) untreated trophozoites with a sucker-like apparatus and wrinkled membrane; (b) diosgenin-treated (50 μg/ml) trophozoites 3 hours post-treatment, small in size; (c) diosgenin-treated (50 μg/ml) trophozoites 6 hours post-treatment, rounded small in size without pseudopodia or sucker like apparati.

6 hours post-exposure: they were small in size, rounded up with cracking of the membrane, free of pseudopodia, and lack of sucker-like apparatus (Fig 2c).

Effects of diosgenin on trophozoite at the genetic level

To study the activity of diosgenin on *N. fowleri* trophozoite genes, we used diosgenin at 50 μg/ml and evaluated 8 genes: *nfa1*, *Mp2CL5*, *pB2.5*, *Naegleria pore B*, *nf cysteine protease*, Nf314, and *nf phospholipase* of *N. fowleri* trophozoites at 1, 3, 6, and 12 hours post-exposure using reverse-transcriptase (RT)-PCR. The *nf actin* was used as house-keeping gene. Diosgenin down-regulated the *nf cysteine protease* gene expression of *N. fowl*eri trophozoites at 6 and 12 hours, post-incubation (Fig 3). Diosgenin at the dose tested, did not affect the other genes of *N. fowleri* trophozoites

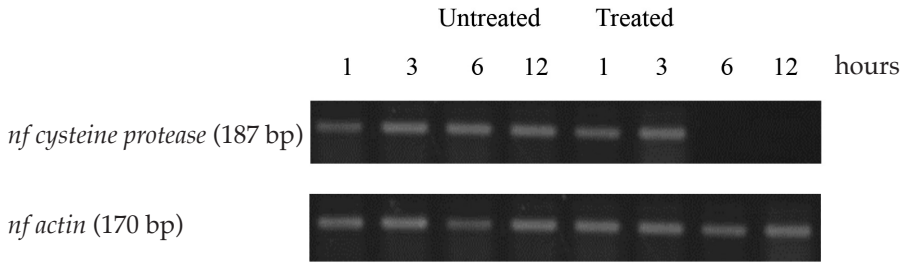

Fig 3–Expression of *nf cysteine protease* in untreated and diosgenin-treated *N. fowleri* trophozoites at 1, 3, 6, and 12 hours post-exposure using RT-PCR. The *nf actin* was used as a house-keeping gene.

at 1, 3, 6, and 12 hours, post-incubation (data not shown).

Effect of diosgenin on mammalian cell viability

Diosgenin at 200 μg/ml, similar to AMB at 10 μg/ml resulted in 63%, 39%, and 24% of SK-N-MC cells being viable at days 1, 2, and 3 post-exposure, respectively (Fig 4a). Diosgenin at concentrations of 50, and 100 μg/ml caused less damage to SK-N-MC cells 3 days post-exposure. Diosgenin (200 μg/ml), similar to AMB (10 μg/ml), resulted in 56%, 45%, and 46% of LLC-MK2 cells being viable 1, 2, and 3 days post-exposure, respectively (Fig 4b). Diosgenin at concentrations of 50, and 100 μg/ml caused less damage to LLC-MK2 cells 3 days post-exposure. Diosgenin had a dose- and time-dependent affect on the tested cells.

DISCUSSION

The free-living ameba *Naegleria fowleri* can cause an acute, fulminent, necrotizing, hemorrhagic meningoencephalitis (PAM) that can cause serious morbility and mortality (Budge *et al*, 2013). AMB is the current drug of choice to treat PAM, but it has significant renal toxicity (Kim *et al*, 2008). Diosgenin [(25*R*)-spirost-5-en-3b-ol] is a steroidal saponin found in *M. charantia* (Danial *et al*, 2014). It can cause changes in membrane permeability and pore formation among chloroquine-resistant *Plasmodium falciparum* (Pabon *et al*, 2013). Diosgenin also causes increased permeability of the cell membrane, disrupts the membrane potential and changes the osmolarity leading to cell shrinkage in *Candida albicans*, resulting in cell death (Nelson, 2009). In our current study, diosgenin at 100 μg/ml (241.2 μM) reduced *N. fowleri* trophozoite viability (Fig 1), and was 50 times more effective than the methanol extract of *M. charantia* (5 mg/ml) (Luangboribun *et al*, 2014). In our current study, diosgenin caused *N. fowleri* trophozoites to develop abnormal membranes and damaged food cups (Fig 2). AMB damages *N. fowleri* trophozoites, causing bleb formation and the disappearance of suckers and pseudopodia (Tiewcharoen *et al*, 2009). Diosgenin, like AMB, appears to be amebocidal.

In our current study, diosgenin at 50 μg/ml (120.6 μM) inhibits *nf cysteine protease* (Fig 3). Aldape *et al* (1994) purified a 30 kDa secreted cysteine protease of *N. fowleri* that had a cytopathic effect on BHK cells. Amin (2004) isolated and characterized the 128 and 170 kDa cysteine protease in cell lysates and purposed these proteases were involved in tissue destruction

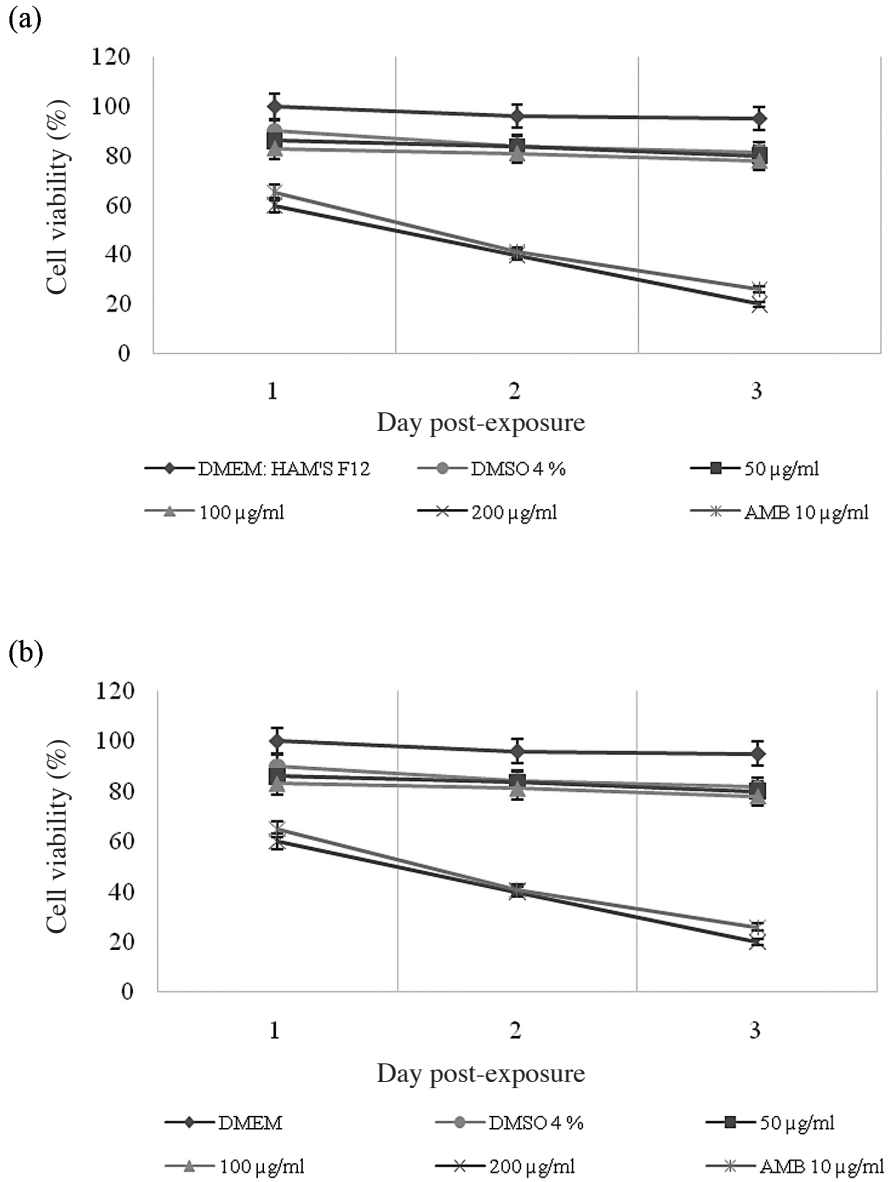

Fig 4–SK-N-MC (a) and LLC-MK2 (b) cell viability 1, 2, and 3 days post-exposure to diosgenin at various concentrations, amphotericin B (10 µg/ml), DMEM and DMSO (4%). DMEM, Dulbecco's Modified Eagle medium; DMSO, dimethyl sulfoxide; AMB, amphotericin B.

and pathogenesis. Rojas-Hernandez *et al* (2004) found *N. fowleri* trophozoites do not damage epithelial nasopharyngeal cells or their extracellular matrix during the initial stages of meningoencephalitis in mice. Tissue damage apparently occurs only when a host inflammatory reaction appears at the site of infection. In addition to amebic proteases, other factors may be contributing to the pathogenesis of PAM.

N. fowleri lysate and excretory-secretory proteins include various pathogenic proteins, such as secreting effect proteins, cysteine proteases (including cathepsin B and cathepsin B-like proteases), secretory lipase, peroxiredoxins, and thrombin receptors, which play a role in the entry of the ameba into the host cell and function as various dominant antigenic proteins; which are involved in pathology (Kim *et al*, 2009). In our study, the diosgenin inhibited the *nf cysteine protease* gene, which prevents the ameba from entering host cells, leading to an aberration in amebic pathogenesis. Therefore, diosgenin had an effect on *N. fowleri* trophozoites at both the cellular and molecular levels (Figs 1-3). Diosgenin at 100 μg/ml caused less damage to SK-N-MC cells and LLC-MK2 cells than AMB. These findings suggest diosgenin may be a good candidate for development as drug against *N. fowleri* trophozoites.

In conclusion, diosgenin had activity against *N. fowleri* at the cellular and molecular levels. The anti-amebic effect of diosgenin needs to be further investigated to better understand the mechanism of action.

ACKNOWLEDGEMENTS

This study was supported by the Siriraj Grant for Research Development and Medical Education No. R015532042, Faculty of Medicine Siriraj Hospital, Mahidol University. This study was also supported by Grant No. SRF-JRG-2557-3, Department of Biology, Faculty of Science, Silpakorn University.

REFERENCES

Aldape K, Huizinga H, Bouvier J, McKerrow J. *Naegleria fowleri*: characterization of a secreted histolytic cysteine protease. *Exp Parasitol* 1994; 78: 230-41.

Amin MN. Proteinases in *Naegleria fowleri* (strain NF3), a pathogenic amoeba: a preliminary study. *Trop Biomed* 2004; 21: 57-60.

Brunton LL, Chaber BA, Knollmann BC, eds. Goodmans, Gilman's pharmacological basis of therapeutics. 12th ed. New York: McGraw-Hill, 2011.

Budge PJ, Lazensky B, Van Zile KW, *et al*. Primary amebic meningoencephalitis in Florida: a case report and epidemiological review of Florida cases. *J Environ Health* 2013; 75: 26-31.

Cho J, Choi H, Lee J, *et al*. The antifungal activity and membrane-disruptive action of dioscin extracted from *Dioscorea nipponica*. *Biochim Biophys Acta* 2013; 1828: 1153-58.

Daniel P, Supe U, Roymon MG. A review on phytochemical analysis of *Momordica chartina*. *IJAPBC* 2014; 3: 214-20.

Kim JH, Jung SY, Lee YJ, *et al*. Effect of therapeutic chemical agents *in vitro* and on experimental meningoencephalitis due to *Naegleria fowleri*. *Antimicrob Agents Chemother* 2008; 52: 4010-6.

Kim JH, Yang AH, Sohn JH, *et al*. Immunodominant antigens in *Naegleria fowleri* excretory-secretory proteins were potential pathogenic factors. *Parasitol Res* 2009; 105: 1675-81.

Luangboribun T, Tiewcharoen S, Rabablert J, *et al*. Screening of anti-amoebic activity on *Naegleria fowleri* in extracts of Thai medicinal plants. *In*: Khusmith S, ed. *JITMM Proceedings* 2014; 3: 16-22.

Nelson N. Plant photosystem I- the most efficient nano-photochemical machine. *J Nanosci Nanotechnol* 2009; 9: 1709-13.

Pabon A, Escobar G, Vargas E, *et al*. Diosgenone synthesis, anti-malarial activity and QSAR of analogues of this natural product. *Molecules* 2013; 18: 3356-78.

Rabablert J, Tiewcharoen S, Junnu V. ITS and pB2.5 gene expression of *Naegleria fowleri* in drug resistance. *Health* 2011; 3: 529-33.

Rojas-Hernandez S, Jarilllo-Luna A, Rodri-

guez-Monroy M, *et al*. Immunohistochemical characterization of the initial stages of *Naegleria fowleri* meningoencephalitis in mice. *Parasitol Res* 2004; 94: 31-6.

Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *Int J Parasitol* 2004; 34: 1001-27.

Tiewcharoen S, Malainual N, Junnu V, *et al*. Cytopathogenesis of *Naegleria fowleri* Thai strains for cultured human neuroblastoma cells. *Parasitol Res* 2008; 102: 997-1000.

Tiewcharoen S, Phurttikul W, Rabablert J, *et al*. Effect of synthetic antimicrobial peptides on *Naegleria fowleri* trophozoites. *Southeast Asian J Trop Med Public Health* 2014; 45: 537-45.

Tiewcharoen S, Rabablert J, Junnu V. In vitro susceptibility of *Naegleria fowleri* trophozoites to amphotericin B-combined chlorpromazine. *Res J Microbiol* 2009; 4: 320-33.

Wang GX, Han J, Zhao LW, *et al*. Antihelmintic activity of steroidal saponins from *Paris polyphylla*. *Phytomedicine* 2010; 17: 1102-5.

Wang YJ, Pan KL, Hsieh TC, *et al*. Diosgenin, a plant-derived, exhibits antiviral activity *in vitro* against hepatitis C virus. *J Nat Prod* 2011; 74: 580-4.

World Health Organization (WHO) General guidelines for methodologies on research and evaluation of traditional medicine. Geneva: WHO, 2000; 1.