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Full Length Research Paper

Cytotoxicity activity of root extract/fractions of *Eurycoma longifolia* Jack root against vero and Hs27cells

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One extract (TACME) and four fractions (TAF 273, TAF 355, TAF 191 and TAF 401) from *Eurycoma longifolia* root reported in literature as antimalarial agent were evaluated for their *in vitro* cytotoxicity activity against two mammalian cell lines (Vero/HS27) which will be use as host for our future antiparasitic activity study. The *in vitro* cytotoxicity activity was measured by methyl thiazolyl tetrazolium (MTT) assay. The CC50 values of TACME, TAF 273, TAF 355, TAF 191 and TAF 401 against Hs27 cell were 2.75, 1.50, 3.75, 2.50 and 2.00 µg/ml, respectively. The extract and all fractions of *E. longifolia* root tested have high toxicity (CC50 < 20 µg/ml) against Hs27 cells. The Vero cells toxicity levels (CC50) for TAF 273, TAF 355, TAF 191, TAF 401 and TACME were 9.5, 23.5, 5.0, 15.0 and 6.75 µg/ml, respectively. Hence, four of these extract/fractions demonstrated significant antiproliferative activity against the cell lines. Moreover, TAF 401 showed lower activity and the TAF 355 fraction did not caused any toxicity against Vero cell lines tested in this study. Based on these results TAF 355 and TAF 401 were suggested for further *in vitro* toxoplasmicidal evaluation by using Vero cells as host for *Toxoplasma gondii.*

Key words: *Eurycoma longifolia*, MTT assay, vero cells, Hs27 cells, cytotoxicity.

INTRODUCTION

Parasitic diseases continue to take an enormous toll on human health, particularly in tropical regions (Luder et al., 2001). New antiparasitic drugs are urgently needed to treat and control diseases such as toxoplasmosis, which affect thousands of people each year. Drug discovery is an iterative process which, depending on the strategy used, typically comprises several discrete stages: target identification and validation; assay development; screening (whole cell or molecular target-based) to identify hits; procurement/synthesis and assessment of analogues to develop structure–activity relationships (SAR) and identify leads; iterative medicinal chemistry to optimize leads; and preclinical development prior to clinical evaluation (Pink et al., 2005). A major challenge in this arena of antiparasitic drug discovery was to identify an appropriate host to support the growth of parasite, which will be used for antiparasitic activity evaluation.

Scientific evidence supporting the antiparasitic properties of medicinal plant extract derives mainly from *in vitro* studies. The main advantage of using *in vitro* assays in this context is the extract or compounds can be tested. In order to test the antiparasitic activity and to assess their role in parasite control, it is necessary to ensure that the plant extract/ compound are not toxic to the host and will not affect host performance. Plant extract/ compound can have a role in antiparasitic activity assessment only if parasitized host can obtain a net benefit on their performance from extract/compound exposure. This net benefit can only be achieved if the antiparasitic effects of extract/compound overshadow the adverse consequences on host performance. Hence, the first step in an antiparasitic activity study is to test the

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cytotoxicity activity of the extract/compound against the host cells. The present study was to investigate the *in vitro* cytotoxicity activity of root extract/ fractions of *Eurycoma longifolia* Jack against two mammalian cell lines (Vero/HS27) which will be used as host for our future antiparasitic activity study. Vero/ HS27 cells were selected for this study because in our laboratory theses cells were used to culture *Toxoplasma gondii.*

E. longifolia Jack, from the Simaroubaceae family and identified locally as 'Tongkat Ali and Pasakbumi' has been commonly prescribed in traditional medicine as a febrifuge and a remedy for dysentery, glandular swelling, intermittent fever (malaria) (Gimlette and Thomson, 1977; Perry, 1980). *E. longifolia* is found in primary and secondary, evergreen and mixed deciduous forest in Burma, Indochina, Thailand, Malaysia, Sumatra, Borneo and the Philippines. It is popularly sought after as a singly or an essential component for the treatment of fevers, aches, sexual insufficiency and also as health supplements, but has not been indicated strongly for any specific illness. However, traditional medicinal users usually take a decoction of the roots in water as a health tonic and antistress remedy. Extracts derived from the roots of this plant were also found to demonstrate activity when evaluated with the sarcoma 180 model (Itokawa et al., 1990). Other documented medicinal uses of these plants are based on its antimalarial (Chan et al., 1986; Chan et al., 1989; Chan et al., 2004; Kardono et al., 199; Ang et al., 1995a; Ang et al., 1995b) and cytotoxic (Kardono et al., 1991; Morita et al., 1990; Itokawa et al., 1992; Itokawa et al., 1993) activities.

MATERIALS AND METHODS

Plant material

The roots of *E. longifolia* Jack were purchased from a pharmaceutical company, Hovid Berhad in Ipoh. A voucher specimen (No. 785-117) was deposited in Penang Botanical Garden, Penang, Malaysia.

Extraction and isolation

The air-dried powdered roots of *E. longifolia* was extracted with 6 × 4 L of 95% MeOH for 6 days at 60˚C. The combined MeOH extract then evaporated to dryness to yield a dark brown residue. Subsequently, this dark brown residue was chromatographed on a resin column with several alcohol/water mixtures to yield 4 fractions (Fr 1 - 4) such as alcohols layers, water layer and residue layers. The 4 fraction were concentrated under vacuum and were resuspended in water and then partitioned successively with saturated *n*-butanol to yield several subfractions. Successive column chromatography using silica gel and centrifugal TLC of the subfractions with various $CHCl_3$-MeOH mixtures were yielding the desired active subfractions (namely TAF 273, TAF 355, TAF 191 and TAF 401). The fractions that contained TAF 273, TAF 355, TAF 191 and TAF 401 were identified by TLC comparison. The RPMI-1640 medium was used as the solvent for preparation of different dilutions of plant extracts.

Cytotoxicity screening

Cell lines

All cell lines used during the present study were obtained from Tissue Culture Laboratory of Institute for Research in Molecular Medicine, Universiti Sains Malaysia, Pulau Pinang, Malaysia.

Vero cell line

The Vero cell line was initiated from kidney of a normal adult African green monkey on March 27th, 1962, by Yasummura and Kawakita at the Chiba University, Japan American Public Health Association, 1992). Vero cells was maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 raM), penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were cultured at 37˚C in a humidified 5% CO_2 incubator. Vero/ HS27 cells were cultured and maintained in RPMI 1640 medium supplemented with 10% FBS. The cells were cultured at 37˚C in a humidified 5% CO_2 incubator.

Human foreskin fibroblasts (hFF) Hs27

Human foreskin fibroblast cells are derived from neonatal human foreskins and were obtained from American Type Culture Collection (ATCC: Manassas, VA). Hs27 cells were maintained in RPMI-1640 medium supplemented with 10% FBS, glutamine (2 raM), penicillin (100 units/ml) and streptomycin (100 µg/ml). The cells were cultured at 37˚C in a humidified 5% CO_2 incubator.

Cytotoxicity assay

The extract and subfractions of *E. longifolia* root were tested for *in vitro* cytotoxicity, using vero and hFF cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, 100 µl of media (RMPI 1640) was added into each of the 96-well plates from row B to row G (triplicate). Than, 100 µl of diluted plant extract or fractions were added in row A and row B. Starting from row B the 200 µl of solution (100 µl drug + 100 µl media) were mixed and 100 µl from row B were added into next row (row C) by using micropipetter and a serial dilution was done up to row G. Finally, excessive 100 µl from row G were discarded. The final volume for each well was 100 µl.

The cultured Vero/ HS27 cells were harvested by trypsinization, pooled in a 50 ml vial. Then, the cells were plated at a density of 1 × 10^6 cells/ml cells/well (100 µl) into 96-well micro-titer plates from row B to row G. Finally, 200 µl of cells (Vero/HS27) were added in row H as a control. Each sample was replicated 3 times and the cells incubated at 37˚C in a humidified 5% CO_2 incubator for 24 h. After the incubation period, MTT (20 µl of 5 mg/ml) was added into each well and the cells incubated for 2 to 4 h, until a purple precipitate was clearly visible under a microscope, the medium together with MTT (190 µl) was aspirated off from the wells, DMSO (100 µl) added and the plates shaken for 5 min. The absorbance for each well was measured at 540 nm in a micro-titre plate reader (Mosmann, 1983) and percentage cell viability (CV) calculated manually using the formula:

$$CV = \frac{\text{Average abs of duplicate drug wells}}{\text{Average abs of control wells}} \times 100\%$$

A dose-response curve were plotted to enable the calculation of the concentrations that kill 50% of the Vero/HS27 cells (CC50).

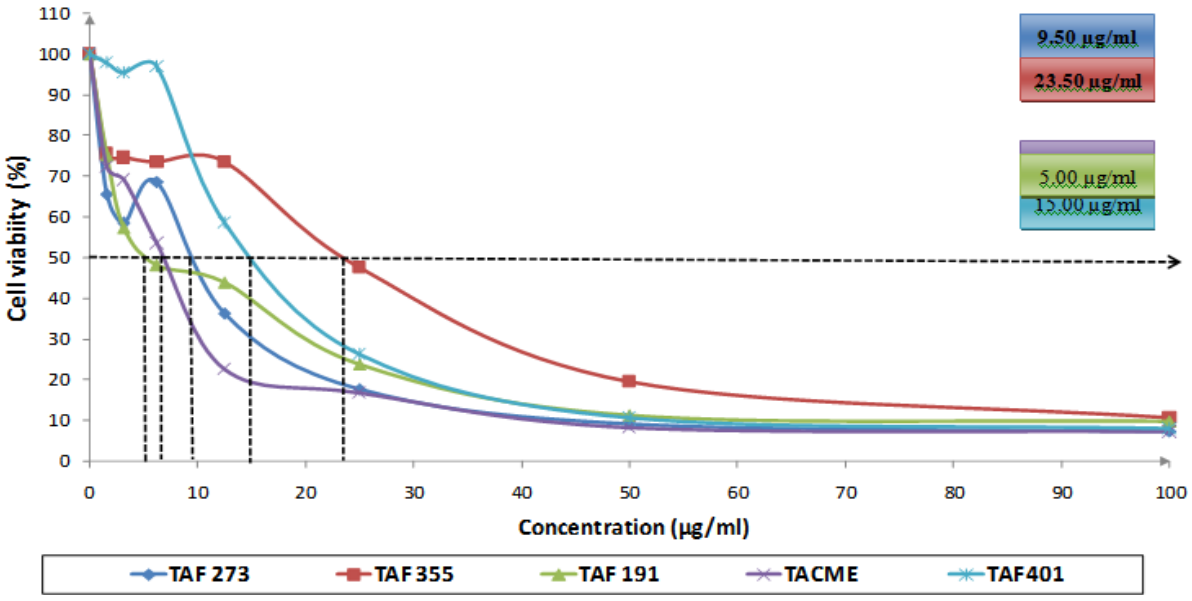

Figure 1. Effect of concentration on cytotoxicity of *Eurycoma longifolia* root extract and fractions on Vero cells.

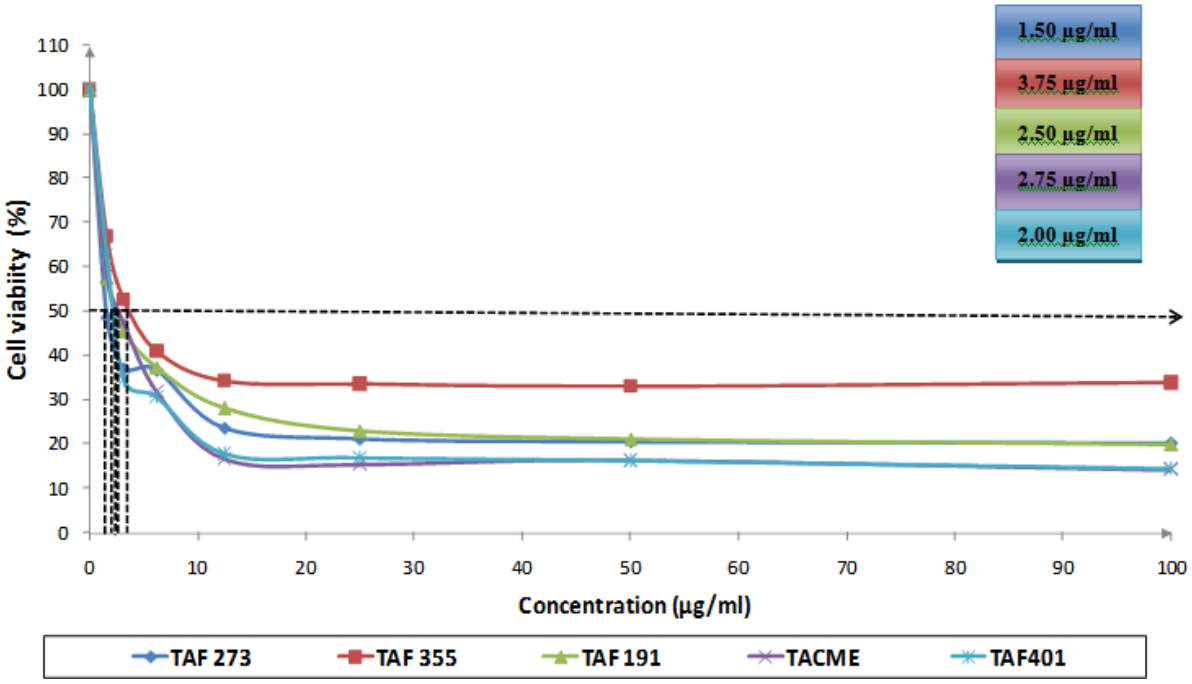

Figure 2. Effect of concentration on cytotoxicity of *Eurycoma longifolia* root extract and fractions on Hs27 cells.

RESULTS AND DISCUSSION

One extract (TACME) and four fractions (TAF 273, TAF 355, TAF 191 and TAF 401) from *E. longifolia* root reported in literature as an antimalarial agent were evaluated *in vitro* for their cytotoxicity activity against Vero and Hs27 cells by using MTT assay. The results of cytotoxicity evaluation of extract and all fractions as CC50 (µg/ml) were shown in Figure 1 and 2. Figure 3 showed the light micrograph of Vero and Hs27 cells used in this study. The extracts were considered safe when CC50 is > 20 µg/ml (Zirihi et al., 2005). This data showed

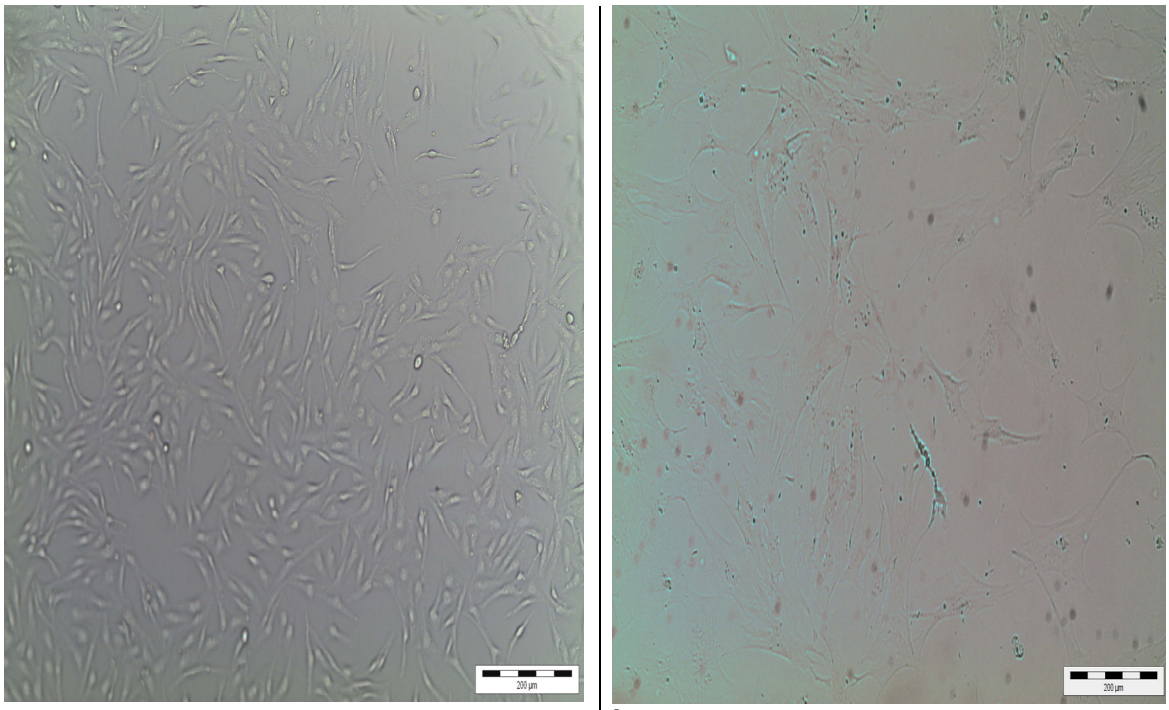

Figure 3. Light micrograph of Vero cells (a) and Hs27 (b).

that the extract and all fraction of *E. longifolia* root possessed the highest cytotoxicity activity against the Hs27 cell line. The CC50 values of TACME, TAF 273, TAF 355, TAF 191 and TAF 401 against Hs27 cells were 2.75, 1.50, 3.75, 2.50 and 2.00 µg/ml, respectively. The extract and all fractions of *E. longifolia* root tested had high toxicity (CC50 < 20 µg/ml) against Hs27 cells, suggesting that they may affects host performance in the antiparasitic activity study. Hence, the Hs27 cell line was not a best candidate as a host for the antiparasitic activity study by using *E. longifolia* root extract and fractions.

On the contrary, the extract and all fractions of *E. longifolia* root possessed a moderate cytotoxicity activity against the Vero cells compared to the Hs27 cells. The CC50 values of TACME, TAF 273, TAF 355, TAF 191 and TAF 401 against Vero cell were 6.75, 9.50, 23.50, 5.00 and 15.00 µg/ml, respectively. The extract TACME and TAF 273, TAF 191 fraction of *E. longifolia* root tested had high toxicity (CC50 < 20 µg/ml) against Vero cells, suggesting that they may affects host performance in the antiparasitic activity study. On the other hand, TAF 401 fraction showed a moderate toxicity (15.00 µg/ml) against Vero cells. The only fraction does not showed any cytotoxicity activity (CC50 > 20 µg/ml) against the Vero cells was TAF 355 with CC50 value of 23.50 µg/ml, suggesting that this fractions may not affect host performance in the antiparasitic activity study. Hence, the Vero cell line was the best candidate as a host for the antiparasitic activity study by using *E. longifolia* root fractions. Previous study also reported that the propagation of *T. gondii* in Vero cells which produced high yield and viability of tachyzoites, with minimal host cell contamination by Saadatnia et al. (2010). Hence, we proposed Vero cell line as a host for antiparasitic activity evaluation for the TAF 355 and TAF 401 fractions, based on the results of cytotoxicity activity against the cells. Similar result was also reported by Chen et al. (2008). They used ginkgolic acids (GAs) isolated from the *Ginkgo biloba* sarcotesta on human foreskin fibroblast (HFF) cells. In order to analyze the toxicity of ginkgolic acids (Gas) and azithromycin on HFF cells, they have used the *in vitro* MTT cell proliferation assay. The results they obtained indicated that at concentrations less than 100 µ g/ml, both GAs and azithromycin did not have a significant effect on HFF cell growth, and this concentration can be used safely for the anti-*Toxoplasma* assay.

Conclusions

In summary, one extracts (TACME) and four fractions (TAF 273, TAF 355, TAF 191 and TAF 401) of *E. longifolia* root showed high cytotoxicity against Hs27 cells compared to Vero cells. TAF 355 fraction of *E. longifolia* root showed no cytotoxicity activity against Vero cells tested in this study. Moreover, TAF 401 fraction showed a moderate toxicity (15.00 µg/ml) against Vero cells.

Therefore, the Vero cell line was the best candidate as a host for the antiparasitic activity evaluation for the

fraction TAF 355 and TAF 401. Further studies involved the investigation about anti-*Toxoplasma* assay of TAF 355 and TAF 401.

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