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Antiviral Properties of Berembang Bukit and Kandis Hutan Against Pseudorabies Virus in Animal Cell Culture

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Abstract

The tropical rainforest in Malaysia represents an untapped potential source of antiviral compounds. Bioactive compounds in plant species from the same genus as Kandis Hutan such as xanthones, benzophenones, biflavonoids and lupeol had been studied. Eugeniin is an anti-herpesvirus compound which had also been found in Berembang Bukit. This preliminary study was carried out to discover the presence of antiviral properties in Berembang Bukit and Kandis Hutan using different antiviral assays. In this study, MTT cell viability assay was used in addition to microscopic evaluation of pseudorabies virus (PrV)–induced cytopathic effect (CPE) on Vero cells. The cellular toxicity of DMSO was also evaluated. DMSO was less than 10% cytotoxic at concentration of 0.1% to Vero cells and its effect can be negligible. Both plants had demonstrated antiviral properties in ethyl acetate and ethanol extracts. From our findings from all three antiviral assays, the ethanol-extracted Kandis Hutan possessed the most promising antiviral properties. Nevertheless, antiviral potential of ethyl acetate and ethanol-extracted Berembang Bukit and ethyl acetate-extracted Kandis Hutan also merit further investigation.

Keywords: Pseudorabies virus, plant extracts, antiviral assays, MTT assay, DMSO, cytotoxicity

Introduction

All herpesviruses are morphologically similar. Pseudorabies virus (PrV) and other closely related homologs such as BHV-1, BHV-5, SHV-1, CHV-1, EHV-1, EHV-3, EHV-4, and FHV-1 are members of the genus *Varicellovirus* and subfamily *Alphaherpesvirinae*. The viruses have a short replication cycle and can establish latency or recrudescence characteristic in infected animal host. Veterinary important pathogenic herpesviruses are contagious or infectious and affected animals have

poor prognosis for recovery or survival. Specific treatment with antiviral drugs have side-effects and its efficacy may be impaired by resistant virus strains.

Driven to identify compounds with antiviral properties for future clinical use as antiviral drugs or antiviral agents, researchers discovered that plants possess various biologically active compounds with potential therapeutic use (Xu *et al.,* 1999). The preserved biodiversity in Malaysian tropical rainforest allows numerous antiviral medicinal plants to be discovered (Ali *et al*., 1996). In addition, anti-PrV and anti-herpesvirus activity had been studied in numerous plant species (Summerfield *et al.,* 1997; Kurokawa *et al*., 1998).

Berembang Bukit is a medium sized to large tropical rainforest tree. The seeds of the tree had been used to treat abdominal pain, food poisoning and peptic ulcer and the leaves applied on the skin by local folks (Tsukiyama *et al.,* 2010). Berembang Bukit had been found to have anti-aging, anti-inflammatory and antimicrobial activities (Tsukiyama *et al.,* 2010; Othman *et al.* 2011).

Kandis Hutan had been used to treat stomachache and fever by local folks (Jabit*et al*., 2009). The leaves of Kandis Hutan contain cytotoxic xantones (Khalid *et. al.,* 2007). Other compounds such as benzophenones, biflavonoids, biphenyls and alkaloids have also been found in plants of this genus (Chiang *et al*., 2003; Jabit *et al*., 2009). The plants of this genus have selective cytotoxic, anti-inflammatory, free radical scavenging, antimicrobial, larvicidal, and anti-HIV activity (Goh, 2011). Although extensive studies had been conducted on plants of this genus, the antiviral potential of Kandis Hutan against herpesvirus had not been elucidated.

The objective of this study was to evaluate the antiviral potential of Berembang Bukit and Kandis Hutan plant extracts against PrV *in vitro.*

Material and Methods

Crude plant extracts

Samples of crude plant extracts from the leaves of Berembang Bukit (UNMC 37) and Kandis Hutan (UNMC 45) were obtained from the Faculty of Bioscience, University of Nottingham, Malaysia. Both plant extracts were crude and extracted with 3 organic solvents namely hexane, ethyl acetate and ethanol.

Pseudorabies virus (PrV) and Vero cells

An established strain of PrV was used in this study. One hundred pfu/mL of PrV was inoculated into each experimental flask-well-seeded with Vero cells. Vero cells (ATCC No. CCL-81) were seeded into sterile 96-well flat bottom plates at 1 X 10^4 cells/well, maintained in RPMI media supplemented with 1% FBS and incubated at 37^{o}C with 5% CO_2 humidified atmosphere

Cytotoxicity Assay

Plant extracts from Berembang Bukit and Kandis Hutan were evaluated for Vero cells cytotoxicity effect *in vitro* at a concentration ranging from 1.56 - 100 µg/mL in 0.1% DMSO. Besides, DMSO cytotoxicity was also evaluated. MTT assay was conducted following cytotoxicity assay to determine the remaining number of viable cells in the experimental wells (Goh, 2011).

Antiviral Assays

Three antiviral assays were carried out in this study namely virucidal assay, attachment assay and prophylaxis study (Goh, 2011). MTT assay was also conducted following antiviral assays.

Statistical Analysis

All samples were tested in triplicate and the results are expressed as Mean ± Std Dev. Percentage of cell cytotoxicity was calculated with the formula: % cell cytotoxicity = OD_{sample} / $OD_{Cell\ ctrl}$ X 100%. The percentage of viral inhibition was calculated using the formula: % viral inhibition =(OD_{sample} – $OD_{Virus\ Ctrl}$) / ($OD_{Cell\ ctrl}$ – $OD_{Virus\ Ctrl}$) X 100%. One-way ANOVA was used to determine the means difference between samples and controls using SPSS 16.0. The significant value is set at $P< 0.05$.

Results and Discussion

Crude extracts from both plants exhibited antiviral properties. They inhibited cytopathic effect (CPE) formation at higher concentrations [100 - 12.5 µg/mL] in virucidal and attachment assay. Anti-PrV properties of Berembang Bukit were likely due to the presence of bioactive compound Eugeniin (Tsukiyama *et al*., 2010). Caged xanthones, benzophenones, biflavonoids or lupanes which were found to be anti-HIV may also be present in Kandis Hutan. However, the hexane extraction of this plant contain cytotoxic compound(s) and hence its antiviral property cannot be evaluated in this study. It is noteworthy to mention that at a concentration of ≤0.1% DMSO, less than 10% cytotoxic effect was observed in control cells and can be regarded as negligible.

Both plants demonstrated better virucidal effects than their attachment and prophylaxis ability, with the overall lowest $IC_{50.}$ They exert their virucidal effect by inactivating the virion through stably binding to it (Carlucci *et al.,* 1999).

To conclude, among all the plant extracts, ethanol-extracted Kandis Hutan was the most promising antiviral sample because it had overall high antiviral potential and it was easily dissolved and hence, will be a more economical and less time consuming antiviral agent to manufacture.

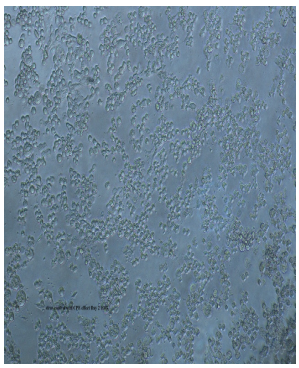

Figure 1. CPE formation in positive control well at day-2 post-attachment assay (100X).

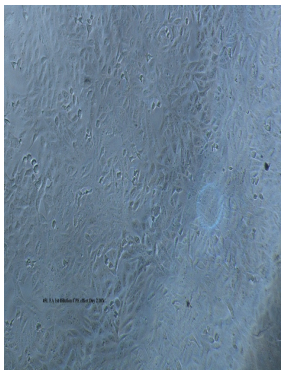

Figure 2. Absence of CPE on day-2 post- attachment assay in wells containing ethyl acetate-extracted KandisHutan at 2^{-1}dilution (100X).

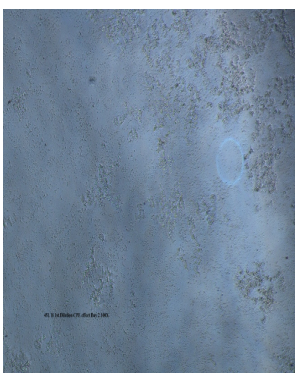

Figure 3. Drastic reduction in the number of viable Vero cells on day-2 post-virucidal assay in wells containing hexane-extracted Kandis Hutan at 2^{-1}dilution due to cytotoxicity (100X).

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