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Antiviral and virucidal activities of *Duabanga grandiflora* leaf extract against Pseudorabies virus in vitro

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Abstract

Background: *Duabanga grandiflora* or known in Malaysia as *Berembang Bukit*, *Megawasih*, or *Pedada Bukit*, is a native plant of the Southeast Asian countries. In this study, the anti-viral properties of *D. grandiflora* were investigated.

Methods: The *D. grandiflora* leaf extracts were obtained with ethyl acetate, hexane, and ethanol as solvents and labelled 37 leaf ethyl acetate (37 L EA), 37 leaf hexane (37 L H), 37 leaf ethanol (37 L ET), respectively. The cytotoxicity of the extracts on Vero cells were determined by the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay.

Results: Among extracts, 37 L EA was most cytotoxic to Vero cells, followed by 37 L H and 37 L ET, with CC_{50} of 218, 833, and >1000 $\mu\text{g/mL}$, respectively. The cytopathic effect (CPE) and plaque reduction, inhibition, and virucidal assays and the selective index (SI) were employed to determine the effect of the extracts on infectivity and replication of pseudorabies virus (PrV) in Vero cells. The *D. grandiflora* leaf extracts showed dose-dependent antiviral activities, with higher activities at high doses. The 37 L ET and 37 L EA showed anti-viral effects through plaque formation and viral replication inhibitions, and virucidal property. The SI of the 37 L ET and 37 L EA by the viral replication inhibition assay was 8.3 and 1.9, respectively, and by the CPE reduction assay, 6.7 and 2.9, respectively.

Conclusion: Ethanol is the best solvent for the preparation of *D. grandiflora* leaf extract as an antiviral agent.

Keywords: Antiviral assay, *Duabanga grandiflora*, Plaque reduction assay, Inhibition assay, Virucidal assay

Background

Duabanga, a genus originally included in the *Sonneratiaceae*, is now classified under the subfamily Duabangoideae of the family *Lythraceae*. *Duabanga*, native to Southeast Asia, consists of three species, *Duabanga grandiflora*, *D. moluccana*, and *D. taylorii* Jayaweera [1]. In Malaysia, *Duabanga* is known as *Berembang Bukit*, *Megawasih* and *Pedada Bukit*. *Duabanga grandiflora* contains eugenin, comprising of gallic acid, ellagic acid, and sugar [2].

Traditionally, the *Duabanga grandiflora* tree has several medicinal properties. The leaf extract is used topically to whiten skin, retard aging, and heal inflammation

through the stimulation of type II collagen production [2]. The *D. grandiflora* extracts and partial purified fractions exhibited bacterial growth inhibition and re-sensitized methicillin-resistant *Staphylococcus aureus* (MRSA) towards ampicillin treatment by inhibiting MRSA biofilm formation and the penicillin-binding protein, PBP2a [3–6]. The plant extracts also possess insecticidal activity [7].

The antimicrobial and phytochemical properties of *D. grandiflora* was first described in 2011 [3] in a report that described the use of higher plant species for combating newly emerging and drug resistant pathogens. The ethanol *D. grandiflora* leaf extract appears to contain a high concentration of phytochemicals including tannins, phenolic compounds, flavonoids and steroids. On the other hand, the ethyl acetate *D. grandiflora* extract contain high level of tannins and moderate levels of alkaloids and phenols [3]. Previously, phytochemicals

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especially tannins, phenolic compounds, flavonoids were reported to possess antiviral activities against human and animal viruses [8–12].

Herpesviruses are highly prevalent in humans and most animals [13]. Members of Herpesviridae are host-specific agents that share properties, which allow them to cross species barriers [14, 15]. This group of viruses has relatively short reproduction cycle, can efficiently destroy infected cells, spread rapidly, and establish latent infection in the host.

In this study, the antiviral properties of *D. grandiflora* plant extracts against PrV was determined. The cellular toxicity and the most effective solvent to be used in the preparation of *D. grandiflora* leaf extracts were also determined.

Methods

Crude plant extract

D. grandiflora was collected from the Semenyih Dam area (3.079066°N 101.886091°E), Selangor, Malaysia, identified by Dr. Christopher Wiart of the University of Nottingham, Malaysia Campus, and the herbarium voucher specimens (UNMC 37) deposited at the Herbarium of Faculty of Science of the University. Crude plant extracts, harvested from the leaves, extracted with hexane, ethanol, and ethyl acetate, and labelled 37 L H, 37 L ET, and 37 L EA, respectively were prepared by Dr. Kang Nee Ting and Prof. Dr. Hwei San Loh of the University of Nottingham, Malaysia.

The leaves of the *D. grandiflora* were cleaned with sterile deionized water, cut into small fragments, and dried in a closed room at 25 to 28 °C for 2 weeks. The dried leaf fragments were grounded, weighed and soaked in 95 % ethanol at a proportion of 1:8 (fragment: ethanol) at room temperature for 24 h. The plant materials were then subjected to sequential fractionation using hexane, ethanol, and ethyl acetate. Each plant material was soaked and fractionated twice before subjecting to rotary evaporation at 40 °C under reduced pressure. The concentrated crude extracts were stored at -20 °C.

Pseudorabies Virus (PrV) propagation

The PrV strain AIP used in this study is from the working stock virus (1×10^8 pfu/mL) prepared and stored at -80 °C in the Virology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia. Quantitation of stock virus was by plaque titration assay using Vero cells.

Vero cell culture

Vero cells (ATCC No. CCL-81) were grown in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS) and antibiotic-antimycotic combination [1 % Penicillin (100 U/mL), 1 % Streptomycin (100 mg/mL) and 1 % Fungi zone (2.5 mg/mL)]. The cells were maintained in

RPMI maintenance media supplemented with 1 % FBS, seeded into sterile 96-well flat bottom and 24-well plates at 1×10^4 cells/well, and incubated under 5 % CO₂ humidified atmosphere at 37 °C.

Plant extract

All plant extracts were dissolved, with frequent shaking for 48 h in dimethyl sulfoxide (DMSO) and then diluted with sterile deionized water to obtain stock concentrations of 10 mg/mL in 10 % DMSO and 50 mg/mL in 50 % DMSO. The 37 L ET extract was the most soluble followed in order by 37 L EA, and 37 L H. The solutions were stored as stock at -20 °C.

Cytotoxicity assay

The stock solutions were diluted serially with RPMI media without FBS to obtain extract concentrations of 62.5, 125, 250, 500, and 1000 µg/mL. In general, the final concentration of DMSO in the working concentration was below 0.3 %. 100 µL of each concentration was used to treat confluent monolayer Vero cells in a 96-well flat-bottom plate. For reference the Vero cells were treated with 100 µL of either 0.05, 1.0, 10, or 12 % DMSO in PBS. Non-treated Vero cells served as the negative control. Positive controls were treated with DMSO at the same concentrations as the plant extracts. All experiments were performed in triplicates. The Vero cells were then incubated for 48 h under 5 % CO₂, humidified atmosphere at 37 °C and visualized under light microscopy. The cytotoxic effect of treatments was determined by MTT assay. Cytotoxicity scoring was according a 5-point scale: 5 = confluent well-defined monolayer with cell-to-cell contact and the cell morphology and density intact; 4 = occasional cell lysis with ≤ 20 % of the cells round in shape, loosely attached, devoid of intracytoplasmic granule; 3 = prevalent cell lysis with < 50 % of cells round in shape and devoid of intracytoplasmic granule; 2 = the majority of the cells are affected but < 70 % of cells are round in shape or lysed; 1 = almost total lysis and destruction of cells and presence of spaces between cells [16]. The cytotoxic concentrations (CC₅₀) of crude plant extracts, which are the concentrations causing 50 % cell cytotoxicity of Vero cells were determined (Additional file 1).

Antiviral assays

Cytopathic Effect (CPE) reduction assay

The CPE reduction assay was conducted according to method described previously [17]. The Vero cells were seeded in a 96-well flat-bottom plate for 24 h to obtain a monolayer. 50 µL of 50 TCID₅₀ PrV with 50 µL of either 18.75, 37.5, 75, 150, or 300 µg/mL extracts was added to the cells and incubated under 5 % CO₂ at 37 °C for 72 h. The CPE was determined under light microscopy and

graded as follows: 0 = 0 %, 1 = 0 to 25 %, 2 = 26 to 50 %, 3 = 51 to 75 %, and 4 = 76 to 100 % CPE. The percentage CPE inhibition was calculated using the following formula: % inhibition = $(CPE_{\text{exp}}/CPE_{\text{control}}) \times 100$, to determine the 50 % effect concentration (EC_{50}) of the extracts where CPE_{exp} = CPE of extract and CPE_{control} = CPE of virus control.

Plaque reduction assay

Plaque reduction assay was conducted according to the method described by Hsuan et al. [18]. Briefly, confluent monolayers of Vero cells were grown in 24-well plates. 100 μL of extracts at either 62.5, 125, 250, 500 $\mu\text{g}/\text{mL}$ or 100 μL of 100pfu PrV were added to the monolayer Vero cells and incubated for 90 min under 5 % CO_2 at 37 °C. Cells treated virus but not with plant extract served as the positive control while those not treated with either virus or extract served as the negative control. The incubating solution contain virus and extract was discarded and 1 mL 1 % methylcellulose in DMEM containing 2 % FBS added to the cells and the plates incubated under 5 % CO_2 for 48 h at 37 °C. The infected cells were fixed with 10 % formal saline, stained with 0.5 % crystal violet solution, and the plaques counted. The 50 % plaque inhibition concentration (IC_{50}) was determined by the following formula [19]: % inhibition = $[1 - (pfu_{\text{exp}}/pfu_{\text{control}})] \times 100$, where pfu_{exp} = plaque formation by extract-and virus-treated cells and pfu_{control} = plaque formation by virus-treated cells.

Inhibition assay on viral replication

The viral replication inhibition assay was according to that described previously [20]. 100 μL of 100pfu PrV were incubated with the monolayer Vero cells in a 24-well flat-bottom plate under 5 % CO_2 at 37 °C for 90 min. The incubating solution was discarded, the cells treated with 0.5 mL of either 25, 50, 100, or 200 $\mu\text{g}/\text{mL}$ extract in 1 % methylcellulose and reincubated under 5 % CO_2 at 37 °C for 48 h. The infected cells were fixed with 10 % formal saline and stained with 0.5 % crystal violet solution. The plaques were counted and the IC_{50} calculated as described in plaque reduction assay.

Determination of extracellular virucidal assay

The extracellular virucidal activity of extracts on PrV was performed according to the method described by Carlucci et al. [21]. Monolayer Vero cells were treated with 5×10^6 pfu PrV and 100 μL of either 62.5, 125, 250, or 500 $\mu\text{g}/\text{mL}$ extract and incubated at 25 °C for 6 h, followed by 10-fold serial dilution. 100 μL mixture of equal amounts of virus (50 μL of $1 \times 10^8/\text{mL}$) and extract (50 μL) in 2 % FBS/DMEM was incubated under 5 % CO_2 for 90 min at 37 °C. The incubating solution containing virus and extract was discarded, 0.5 mL of 1 %

methylcellulose in 2 % FBS/DMEM was added to the cells and the plate incubated under 5 % CO_2 at 37 °C for 48 h. The plaques formed were fixed with 10 % formal saline and stained with 0.5 % crystal violet solution for 30 min and counted. Residual virus infectivity was determined according the following formula: plaque formation (pfu) = Number of plaques \times [1/viral inoculation] \times [1/dilution fold]. The IC_{50} of extracts of plaque inhibition was calculated as follows: $IC_{50} = (pfu_{\text{exp}}/pfu_{\text{control}}) \times 100$, where pfu_{exp} = plaque formation by extract- and virus-treated cells and pfu_{control} = plaque formation by virus-treated cells.

Data analysis

All values are expressed as mean \pm standard deviation. The CC_{50} , EC_{50} , and IC_{50} values were estimated by extrapolating the graph plotted with software Microsoft Excel 2007. Mean values were compared using multiple comparison Tukey test with the SPSS 6.0 software. The difference was statistically significant when $p < 0.05$.

Results

Cytotoxicity assay

The CC_{50} of 37 L EA and 37 L H on Vero cells was 213 and 813 $\mu\text{g}/\text{mL}$, respectively. For 37 L ET, the CC_{50} was >1000 $\mu\text{g}/\text{mL}$ with <50 % cytotoxicity (Table 1, Figs. 1 and 2). The 37 L H and 37 L EA extracts were significantly ($p < 0.05$) more cytotoxic than 37 L ET extract to Vero cells.

Antiviral assay

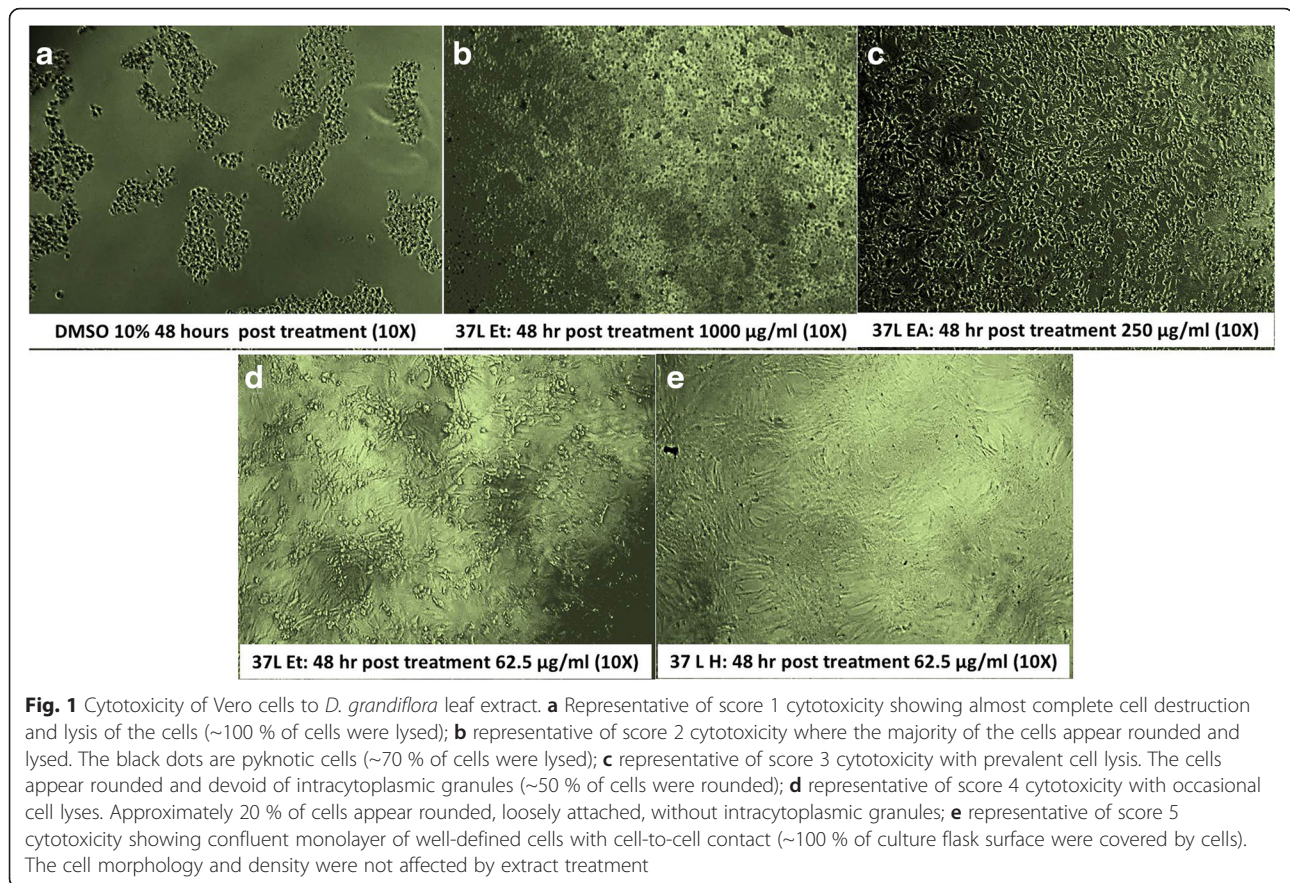
CPE reduction assay

The anti-PrV activity of *D. grandiflora* leaf extracts were evaluated by the CPE reduction assay after 72 h of incubation. The 37 EA and 37 ET extracts showed a dose-dependent effect in PrV-induced CPE on Vero cells with EC_{50} of 75 and 150 $\mu\text{g}/\text{mL}$, respectively (Fig. 3). The 37 L H extract did not show the same effect.

Table 1 Cytotoxicity scoring of

Conc. ($\mu\text{g}/\text{mL}$)	<i>D. grandiflora</i> leaf extract		
	37 L H	37 L ET	37 L EA
62.5	3	3	3
125	3	3	3
250	3	2	2 ^a
500	2	2	2
1000	2 ^a	2 ^a	2

^aScored for concentration near to CC_{50} . Score 1 = severely cytotoxic. Almost complete cell destruction and lysis of the cells (~ 100 % of cells were lysed), 2 = significantly cytotoxic (~ 70 % of cells were lysed), 3 = moderately cytotoxic (~ 50 % of cells were rounded), 4 = mildly cytotoxic. Approximately 20 % of cells appear rounded, loosely attached, without intracytoplasmic granules, 5 = non-cytotoxic (~ 100 % of culture flask surface were covered by cells). 37 L H, 37 L ET, and 37 L hexane, ethanol, and ethyl acetate extract, respectively



Plaque reduction assay

In the plaque reduction assay, the Vero cells were infected with 100 pfu of PrV and 100 µL of either 62.5, 125, 250, or 500 µg/mL plant extract. Extracts 37 L ET and 37 L EA caused reduction in plaque formation with 37 L ET and 37 L EA causing 100 % reduction at 250 and 500 µg/mL, respectively (Fig. 4). However, 37 L H did not produce any effect on the Vero cells.

Viral replication inhibition assay

The Vero cells were infected with PrV for 90 min and then treated with either 25, 50, 100, or 200 µg/mL *D. grandiflora* leaf extracts to determine their effect on virus replication. The results showed that 37 L H did not inhibit plaque formation. However, 200 µg/mL 37 L Et and 37 L EA caused complete inhibition in plaque formation indicating total inhibition of viral replication in Vero cells (Fig. 5).

Virucidal activity

D. grandiflora leaf extracts were incubated with PrV to determine their effect on the virus and its residual infectivity. The results showed that 37 L EA at 62.5 µg/mL had no residual viral infectivity in the Vero cells indicating total viral inactivation. The 37 L ET only had slight

residual infectivity of 1.3 % that is translated as 98.7 % viral inactivation. On the other hand, 37 L H produced 100 % residual infectivity indicating no inhibitory effect on the viral replication in Vero cells (Fig. 6).

The selective index (SI) [CC_{50}/IC_{50}] was estimated by the viral inhibition and CPE assays. Based on the SI, 37 L ET extract has the highest antiviral potential while 37 L H extract had the lowest.

These results showed that ethanol is the best solvent for the extraction of compounds with virucidal properties from *D. grandiflora* leaves (Fig. 7).

Discussion

The study showed that the cytotoxicity of *D. grandiflora* leaf extracts increased with concentration and this is attributed to their bioactive compound content. It was earlier shown that chloroform *D. grandiflora* extract contains 3 triterpenes, 3 flavones, 4 phenolic compounds and 1 steroids [22]. Some of these compounds and their derivatives possess antimicrobial properties [7]. In this study, we determined the antiviral activity of the *D. grandiflora* leaf extract obtained by using hexane, ethanol, and ethyl acetate as solvents. It appears that the ethanol *D. grandiflora* leaf extract had the highest antiviral activity while being less toxic to Vero cells than either the hexane or ethanol extract.

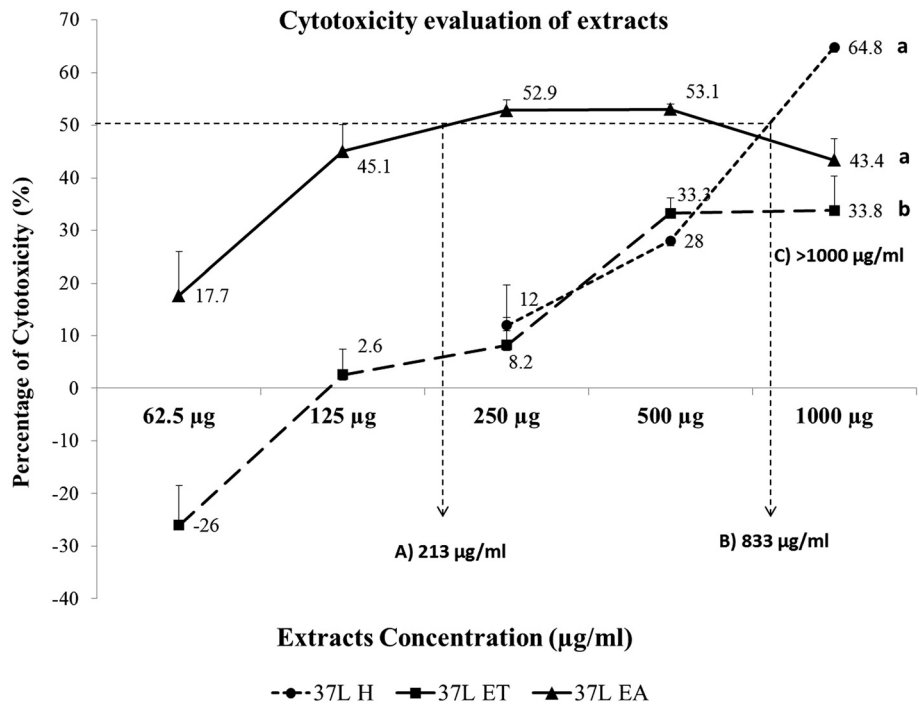


Fig. 2 Determination of IC₅₀ of *D. grandiflora* leaf extract on Vero cells. The CC₅₀ of Vero cell treated with 37 L EA, 37 L H, and 37 L ET was 213, 833, and >1000 µg/mL, respectively. "a and b" letter indicate significant difference between individual extracts at $p < 0.05$

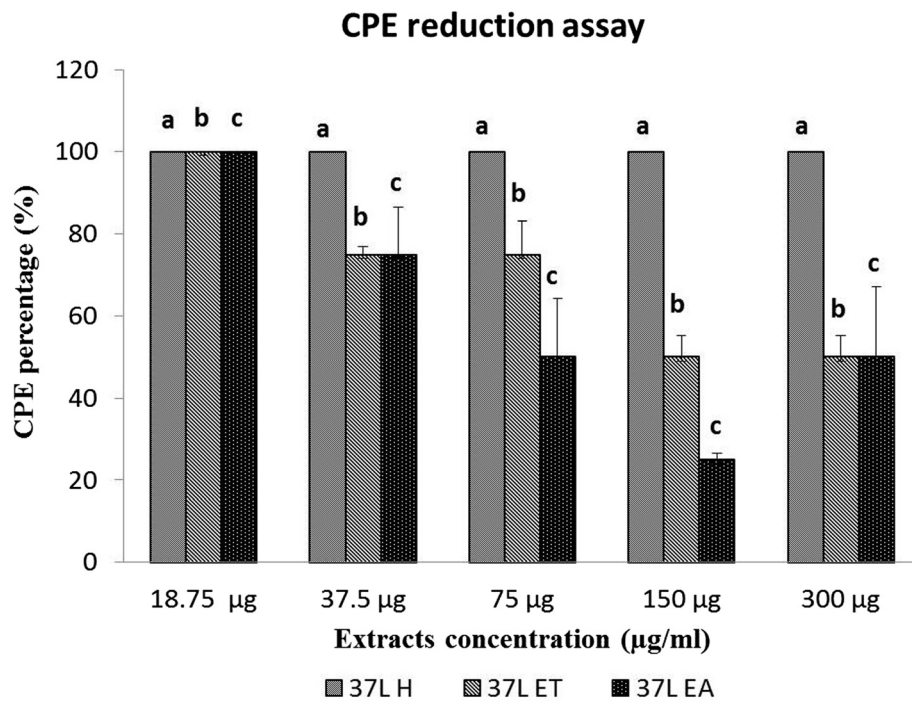


Fig. 3 Effect of *D. grandiflora* leaf extracts on PrV-induced cytopathic effect on Vero cells. a,b,c indicate significant difference between individual extracts in each concentration at $p < 0.05$

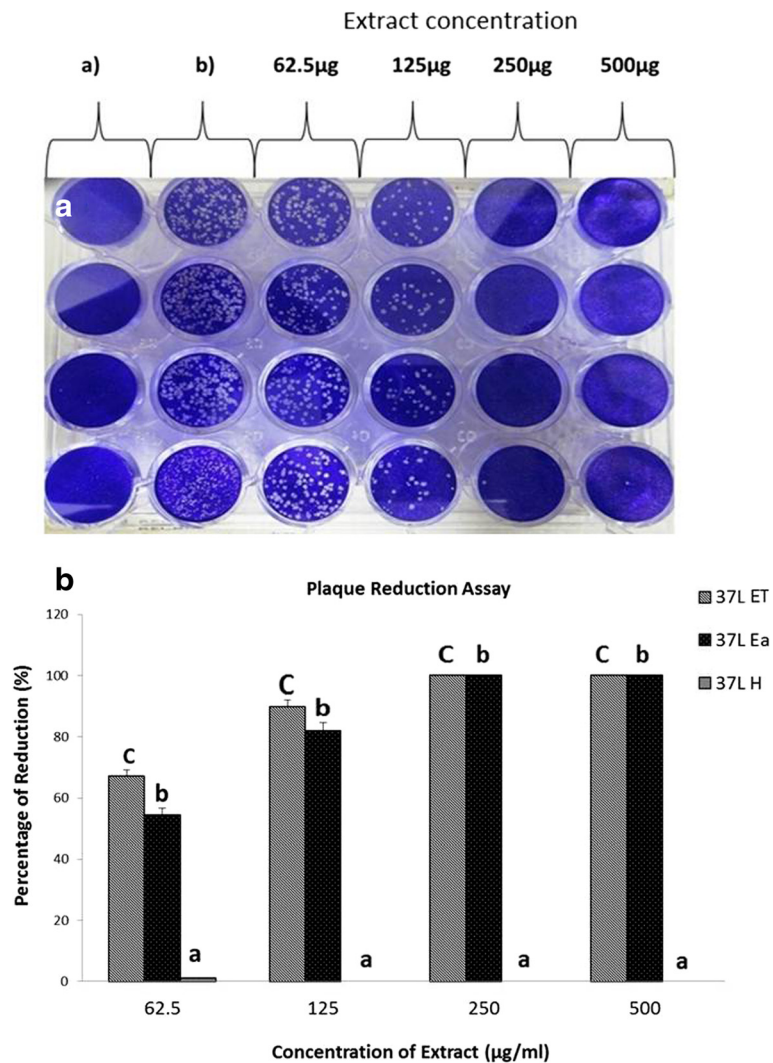


Fig. 4 Plaque reduction assay of Vero cells infected with PrV pre-treated with *D. grandiflora* leaf extracts. (A) Plates show reduction in plaque formation; a) Negative control, not treated. b) Positive control treated with PrV only. Greater reduction in plaque formation with increase in extract concentration. No plaque formation at 250 and 500 µg/mL extract. (B) Quantification of plaque reduction. a,b,c indicate significant difference between individual extracts in each concentration at $p < 0.05$

DMSO usage as diluent of extracts at concentrations of less 10 % is not cytotoxic [23]. Our study showed that 0.1 % DMSO produced approximately 3 % Vero cell toxicity. Thus, at this concentration, DMSO did not contribute significantly to the cytotoxic effect of *D. grandiflora* leaf extract.

Many plants extracts have been shown to inhibit or inactivate virus replication. Among extracts with antiviral properties is the *Isatis indigotica* extract that produced a dose-dependent inhibition of PrV replication in vitro [18] and the *Geranium sanguineum* extracts that inhibit herpesvirus infection by causing perturbations in the early stages of virus replication [17]. *D. grandiflora* leaf extracts seems to have similar antiviral potentials. It was shown in this study that the extract inhibits viral

infection via the cytopathic effect, plaque reduction, viral replication, and virucidal activities. However, the antiviral activities of the *D. grandiflora* leaf extracts are dependent on the solvent used in the preparation of the extract. The hexane extract did not have any inhibitory activity towards PrV. Based on the CPE, virus inhibition and virucidal assays, the ethanol and ethyl acetate *D. grandiflora* leaf extracts have equal virucidal and inactivation activity before the PrV could infect Vero cells [24]. The CPE of the extracts is dose-dependent with the most effective antiviral concentrations assumed to be from 150 to <300 µg/mL. Increasing the extraction concentration to 300 µg/mL did not seem to improve the antiviral effect of either the ethanol or ethyl acetate *D. grandiflora* leaf extract.

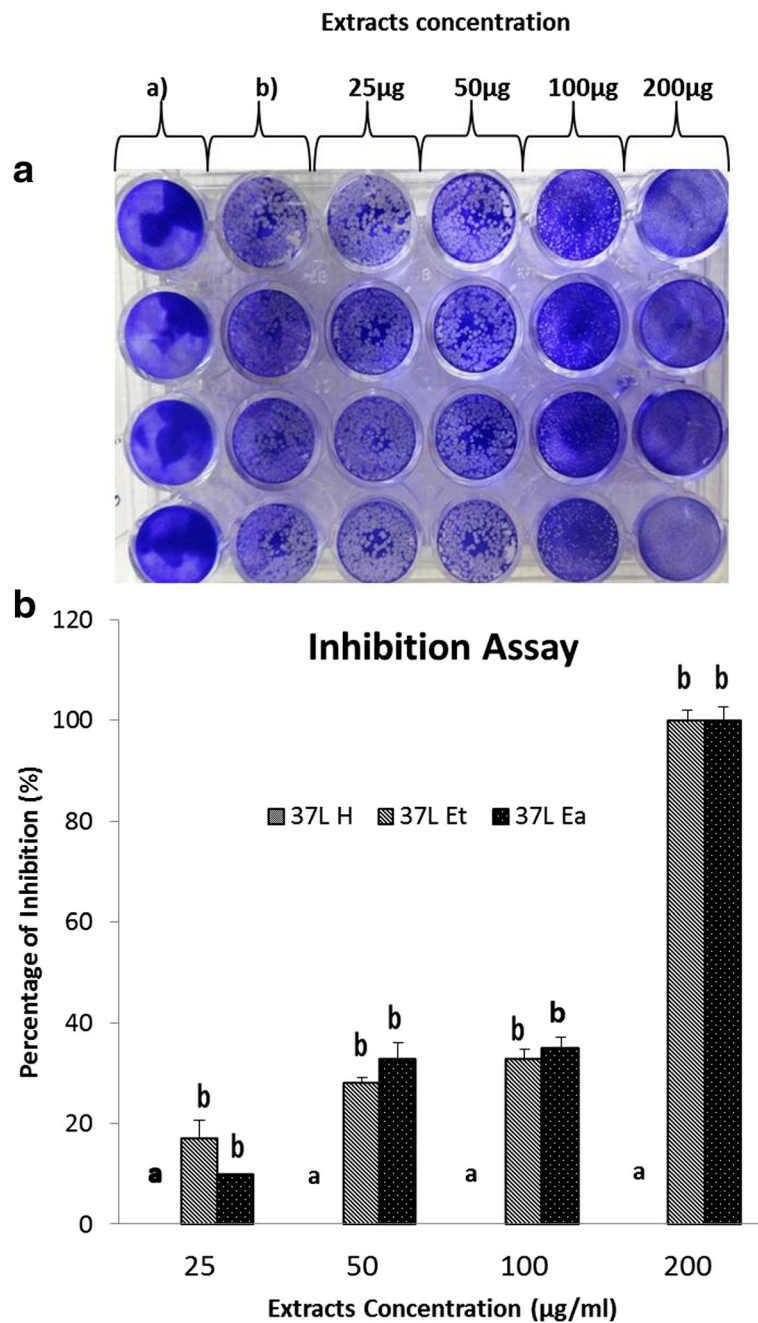
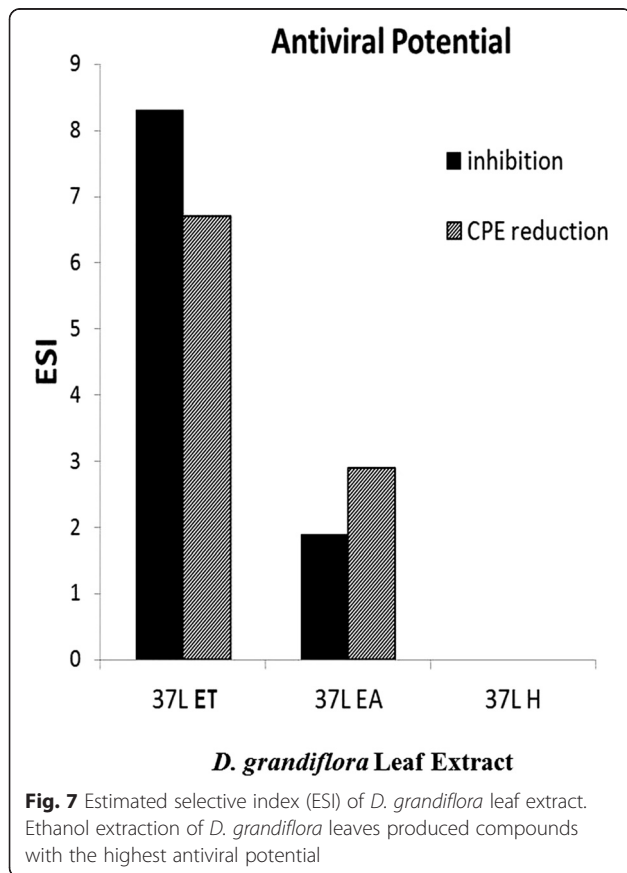
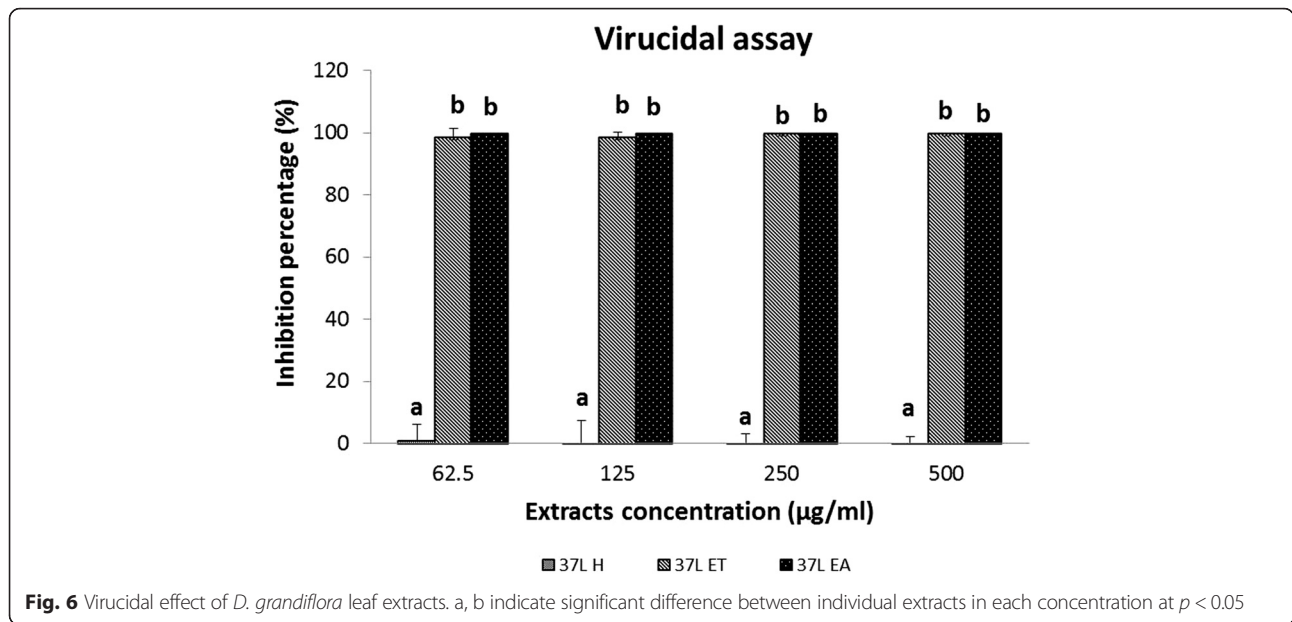


Fig. 5 Inhibition of viral replication in PrV-infected Vero cells after treatment with *D. grandiflora* leaf extract. **a** Plates show reduction in plaque numbers in extract-treated PrV-infected cells; a) Negative control, untreated, without plaque formation, b) Positive control, PrV-treated only. The number of plaque decreased with increase in extract concentration. **b** Quantification of plaque inhibition by *D. grandiflora* leaf extract by difference solvents on PrV replication. a,b indicate significant difference between individual extracts in each concentration at $p < 0.05$

The *D. grandiflora* leaf extracts seemed to inhibit PrV infection of Vero cells. This is clearly evident by the plaque reduction assay where extract treatment prevented Vero cell plaque formation, particular at concentrations of $\geq 250 \mu\text{g/mL}$. In fact, all *D. grandiflora* leaf extracts prevented PrV infection of Vero cells. However, it should be noted that in in vitro experiments, viral

infection of cells is dependent on factors such as virus concentration and incubation period for viral infection. In this study, the virus concentrations and incubation periods for CPE and plaque reduction assays were variable. In the CPE assay, although the incubation period was longer, less virus was used than in the plaque reduction assay. For that reason, the results from the two



assays were not completely complimentary. There was total inhibition of plaque formation in infected Vero cells treated with high concentrations of ethanol and ethyl acetate *D. grandiflora* leaf extracts while the cytopathic effect was between 20 and 50 %.

In the evaluation of virucidal activity, the PrV were pre-incubated with *D. grandiflora* leaf extracts for 6 h before infecting Vero cells. The antiviral effect of the extracts was determined by the viral residual infectivity in Vero cells. The ethanol *D. grandiflora* leaf extract at all concentrations used in the study almost completely inhibited plaque formation. To confirm the antiviral potential of the extracts, the SI was calculated. A high SI suggests that the extract would have good antiviral properties. In this study, the ethanol had at least twice as high SI than the ethyl acetate *D. grandiflora* leaf extract. Based on these results, the ethanol *D. grandiflora* leaf extract is most appropriate to be developed into a therapeutic compound for viral diseases.

The efficacy of anti-viral drugs can be determined by their IC_{50} on Vero cells. The anti-herpesvirus drug, Acyclovir for example, has an IC_{50} of 0.8 to 0.9 µg/mL on Vero cells [25, 26]. The ethanol and ethyl acetate *D. grandiflora* leaf extracts were shown to have much lower IC_{50} than Acyclovir at 0.12 and 0.112 µg/mL, respectively suggesting that the *D. grandiflora* leaf extracts are more efficacious in the treatment of herpesvirus infections than Acyclovir.

The antiviral activities of the ethanol and ethyl acetate *D. grandiflora* extracts are attributed to their phytochemical contents [3], particularly tannins, phenolic compounds, flavonoids, and alkaloids that may act synergistically. Among the advantages of crude extracts as

antiviral agents are their greater potency, fewer side effects and toxicity, and cost-effectiveness [27–29]. Although, further studies would be of interest to determine whether the components of the *D. grandiflora* leaf extracts have synergistic effects, this does not detract from fact that the extracts have effective anti-herpesvirus properties.

Conclusion

The ethanol *D. grandiflora* leaf extracts while showing the least cytotoxic effect towards Vero cells, is the most efficacious anti-viral agent among the *D. grandiflora* leaf extracts investigated in this study. The wide safety margin, low cytotoxicity and high selective index make the ethanol *D. grandiflora* leaf extract a prime candidate for development as an anti-viral agent.

Additional file

Additional file 1: Supplementary document DMSO Cytotoxicity assay. (JPG 40 kb)

Abbreviations

ATCC: American Type Culture Collection; CC₅₀: the cytotoxic concentrations; CPE: cytopathic effect; DMEM: Dulbecco modified Eagle medium; DMSO: dimethyl sulfoxide; EA: ethyl acetate; EC₅₀: 50 % effect concentration; ET: ethanol; FBS: fetal bovine serum; H: hexane; IC₅₀: 50 % inhibitory concentration; MRSA: methicillin-resistant *Staphylococcus aureus*; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; PBP2a: penicillin-binding protein; PFU: plaque forming unit; PrV: Pseudorabies virus; RPMI: Roswell Park Memorial Institute; SI: selective index; TCID₅₀: 50 % tissue culture infective dose.

Acknowledgments

The authors would like to thank and acknowledge the Ministry of Science and Technology of Malaysia (MOSTI) for providing financial support under the research Project number 03-02-04-0563-SR0008/05-06. In addition, we thank Universiti Putra Malaysia for providing the research environment and necessary facilities.

Funding

Financial support under the research Project number 03-02-04-0563-SR0008/0506 by the Ministry of Science and Technology of Malaysia (MOSTI).

Availability of data and materials

The data set was deposited in publicly available repositories. The data set was deposited in publicly available repositories (<http://dx.doi.org/10.6070/H4CV4FSP>).

Authors' contributions

FZAM: Carried out data collection, analysis and preparation of manuscript draft. ZNA: Supervised the study, was project leader and helped to draft the manuscript. HSL: has made substantial contributions to conception, design and virological experimentation. TKN: has made substantial contributions to conception and design, has given final approval of the version to be published. HH: has been involved in drafting the manuscript and revising it critically for important intellectual content; has given final approval of the version to be published. RA: has been involved revising the manuscript critically for important intellectual content; has given final approval of the version to be published. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

This information is not relevant.

Ethics approval and consent to participate

This information is not relevant.

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Received: 13 November 2015 Accepted: 13 May 2016

Published online: 23 May 2016

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