Research Article

# Cell Viability and Cytotoxic Testing of Seventeen Asian Plant Extracts toward MARC-145 Cells by MTT Assay

**Kraijak Kaewprom[1,3,*], Chao-Nan Lin[1,2], Ming-Tang Chiou[1,2]**

*[1]Department of Veterinary Medicine, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan*

*[2]Animal Disease Diagnostic Center, College of Veterinary Medicine, National Pingtung University of Science and Technology, Pingtung, Taiwan*

*[3]Program of Veterinary Technology, Faculty of Agricultural Technology, Rajabhat Maha Sarakham University, Maha Sarakham, Thailand*

## Abstract

Porcine reproductive and respiratory syndrome (PRRS) is characterized by reproductive failure of sows and respiratory problems of nursery and growing pigs. Present management strategies mainly focus on the prevention of infection using vaccination but are not sufficient to prevention. Previous studies have discovered a few natural compounds and compositions that have antiviral activities on PRRSV. Therefore, the aim of this study is to evaluate the cell cytotoxicity effect of 17 Asian plant extracts on MARC-145 cells using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assaybefore application to the anti-PRRSV evaluation *in vitro*.The maximum non-cytotoxic concentration (MNCT) was used to determine the 90% cell viability detection. The results showed that cytotoxicity of tested compounds were different compounds on the same cell varied remarkably. Three compounds were*Houttuyniacordata*, *Artemisia argyi* and *Pogostemoncablin*had a low toxicity with MNTC of $2^{-2}$ dilution. Eleven compounds were presented a moderate toxicity witch MNTC ranged from $2^{-3}$-$2^{-6}$ dilution. While three compounds were*Curcuma longa*, *Acorusmacrospadiceus* (stem) and *Acorusmacrospadiceus*(leaf) had a high toxicity with MNTC of ≥ $2^{-11}$ dilution. This study indicates that some compounds had not cytotoxicity on MARC-145 cells and may be useful for future application to the anti-PRRSV evaluation *in vitro*.

**Keywords:** Plant extracts, MTT assay and cytotoxicity

*Corresponding author:E-mail.:kkraijak@yahoo.com

# การทดสอบความเป็นพิษของพืชสมุนไพร 17 ชนิดของเอเชียต่อเซลล์เพาะเลี้ยงชนิด MARC-145 cells ด้วยวิธี MTT Assay

**ไกรจักร แก้วพรม[1,3,*], ฉาว หนาน หลิน[1,2], หมิง ตัง โฉว[1,2]**

*[1]ภาควิชาสัตวแพทยศาสตร์ วิทยาลัยสัตวแพทยศาสตร์ มหาวิทยาลัยวิทยาศาสตร์และเทคโนโลยีแห่งชาติผิงตุง ผิงตุง ประเทศไต้หวัน*

*[2]ศูนย์ชันสูตรและวินิจฉัยโรคสัตว์ วิทยาลัยสัตวแพทยศาสตร์ มหาวิทยาลัยวิทยาศาสตร์และเทคโนโลยีแห่งชาติผิงตุง ผิงตุง ประเทศไต้หวัน*

*[3]สาขาวิชาเทคนิคการสัตวแพทย์ คณะเทคโนโลยีการเกษตร มหาวิทยาลัยราชภัฏมหาสารคาม มหาสารคาม ประเทศไทย*

**บทคัดย่อ**

โรค PRRS ในสุกรเป็นโรคที่ก่อให้เกิดความล้มเหลวของระบบสืบพันธุ์ในแม่สุกรและก่อให้ความผิดปกติของระบบทางเดินหายใจของลูกสุกรในระยะอนุบาลและกำลังเจริญเติบโต ปัจจุบันแนวทางการในการจัดการส่วนใหญ่มุ่งเน้นไปที่การป้องกันการติดเชื้อโดยการฉีดวัคซีนแต่ไม่สามารถที่จะกำจัดไวรัสและกระตุ้นการสร้างภูมิคุ้มกันให้แก่ร่างกายสุกรได้อย่างมีประสิทธิภาพ การศึกษาวิจัยก่อนหน้านี้ได้ค้นพบว่ามีสารสกัดจากธรรมชาติและองค์ประกอบของสารบางชนิดมีฤทธิ์ในการต้านเชื้อไวรัส PRRSV ได้ ดังนั้นจุดมุ่งหมายของการศึกษาครั้งนี้ คือ ประเมินความเป็นพิษของพืชสมุนไพรจากธรรมชาติที่พบในเอเชีย จำนวน 17 ชนิด ต่อเซลล์เพาะเลี้ยง MARC-145 ด้วยวิธี MTT assay ก่อนที่จะนำไปประยุกต์ใช้ในการต้านเชื้อไวรัส PRRSV ต่อไป โดยประเมินระดับความเข้มข้นสูงสุดที่ไม่เป็นพิษต่อเซลล์เพาะเลี้ยง (MNCT) ที่ระดับการมีชีวิตของเซลล์ 90% ผลการศึกษาพบว่าความเป็นพิษของสารสกัดจากพืชสมุนไพรมีความแตกต่างกันที่ระดับความเข้มข้นต่างๆ กัน โดยพืชสมุนไพร 3 ชนิด ได้แก่ *Houttuyniacordata*, *Artemisia argyi* และ *Pogostemoncablin* มีความเป็นพิษต่ำที่ระดับความเข้มข้น $2^{-2}$. พืชสมุนไพรจำนวน 11 ชนิด มีความเป็นพิษปานกลางที่ระดับความเข้มข้น $2^{-3}$-$2^{-6}$ ในขณะที่พืชสมุนไพร 3 ชนิด ได้แก่ *Curcuma longa*, *Acorusmacrospadiceus* (stem) และ *Acorusmacrospadiceus* (leaf) มีระดับความเป็นพิษสูงที่ระดับความเข้มข้นที่มากกว่า $2^{-11}$ ดังนั้นการศึกษาครั้งนี้แสดงให้เห็นว่าสารสกัดจากพืชสมุนไพรบางชนิดไม่มีความเป็นพิษต่อเซลล์เพาะเลี้ยงชนิดMARC-145 และจำเป็นต้องมีการศึกษาค้นคว้าเพิ่มเติมในการทดสอบความสามารถในการป้องกันโรค PRRSV ในระดับห้องปฏิบัติการต่อไป

**คำสำคัญ**: สารสกัดพืชสมุนไพร, วิธีทดสอบความเป็นพิษต่อเซลล์ และ ความเป็นพิษต่อเซลล์

* ผู้เขียนที่ให้ติดต่อ: E-mail:kkraijak@yahoo.com

## Introduction

Medicinal plants have a great potential for producing new drugs for human benefit. According to a report of World Health Organization, more than 80% of world's populations depend on traditional medicine for their primary health care needs (Duraipandiyan *et al*., 2006). In general, natural compounds constitute a major source of effective pharmacological agents including, artemisinin, antitumor, alkaloids, opiates, salicylates, etc. (Rajbhandari *et al*., 2009; Mishra and Tiwari, 2011). Hence, cytotoxic level of medicinal plants must also be evaluated against host cells. The safety of plants as a potential therapeutically agents must be ascertained and the side effects should be acceptable to the host (Morobe *et al*., 2012). Therefore a determination of the cytotoxicity level of any medicinal plant will reveal its safety as a potential therapeutic agent. Bioactive compounds with no or less toxic effect to the host are the good candidates for formulation of drugs. *In vitro* cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antiviral properties for future work. Currently, the non-radioactive, calorimetric assay system using MTT assay has been widely used for evaluating cell viability *in vitro*. The assay measures the conversion of MTT into purple-colored MTT formazan by living cells and decrease in cellular MTT reduction could be an index of cell damage (Abe and Matsuki, 2000). Previous studies have discovered a few natural compounds and compositions that have antiviral activities swine disease (Cheng *et al*., 2013). Consequently this study determined the cytotoxic activities of aqueous extracts of seventeen Asian natural herb extracts on MARC-145 cells by MTT assay in order to gauge their usefulness as potential candidates for application to the anti-PRRSV evaluation *in vitro*.

## Methodology

### 1. Reagents

Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 10% or 5% heat-inactivated fetal calf serum (FCS; Hyclone, USA), 100 IU/ml Penicillin G and 50 µg/ml Streptomycin was used for cell growth or maintenance medium. A 0.25% trypsin (Amresco, USA) was prepared in pH 7.2 PBS. A 0.5% 3-(4,5-dimethyithiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Amresco) was prepared in PBS (pH 7.4). These solutions were sterilized by a 0.22 µm Millipore membrane filter and aliquots made for future use. Dimethyl sulfoxide (DMSO) (Avator, USA), methanol (Merck KGaA, Germany), aceton (Merck KGaA, Germany), fluorescein goat anti-mouse IgG (H+L) (Sigma, USA), anti-mouse IgG (whole molecule)-FITC, product in Goat, Affinity isolated antibody (Sigma, USA). The DMEM, fluorescein goat anti-mouse IgG (H+L) and anti-mouse IgG (whole molecule)-FITC were stored at 4°C, whereas MTT and trypsin were stored at -20°C. DMSO, methanol and acetone were stored at room temperature.

### 2. Preparation of the natural plant extracts

The seventeen herbs (Table 1) were cultivated in experimental field of Taichung District Agricultural Research and Extension Station. Aerial part of plants was harvested at maturity then subjected to distillation immediately. Hydrosol was collected using an Essential Oil Distiller (Kou-Chou Instrument Co.). In brief, 10 kg of fresh leaves was distilled with 30 L of water; the vapor was

condensed to collected hydrosol until the volume of hydrosol reached 2 L. Subsequently, the plant extracts aliquot, stored at the room temperature and prevent sunlight exposure.

**3. MARC-145 cells culture**

African green monkey kidney (MARC-145) cells were cryopreserved at -196°C in DMEM supplemented with a 10% fetal calf serum (FBS), and 10% DMSO. Prior to use, MARC-145 cells were suspended at concentrations of $5x10^3$ cell/well in 96-well plates for MTT assay and IFA assay and $1x10^5$ cell/ml 48-well plates for antiviral assay, maintained in DMEM supplemented with a 10% FBS, penicillin (100 U/ml) and streptomycin (50 µg/ml), were incubated 24 hours at 37°C with 5%$CO_2$ before added each compound to MARC-145 cells.

**4. Cell viability and Cytotoxicity assay**

Cell viability and cytotoxicity of 17 natural compounds was investigated using a MTT assay (Cheng *et al*., 2013). Briefly, each compound was made by a 2-fold serially dilution. The MARC-145 cells were suspended in 100 µl of 5% DMEM in 96-well plates. After 24 hours of incubation, 200 µl of medium containing different concentrations of each compounds was added to the each well, followed by incubation for 72 hours. Medium without any compound was used as a control. To evaluate cell viability, 100 µl of MTT solution (5mg/mL in NaCl) was added to the each well, then incubated for 4 h at 37°C in a humidified atmosphere containing 5% $CO_2$. Subsequently, the supernatant was removed and 100 µl of DMSO was added to each well in order to dissolve the formazan crystals. After gently shaking the plates for 30 min, the absorbance was read on an ELISA reader which is proportional to the number of viable cells, was measured at 570 nm using a ELISA reader. The maximum non-cytotoxic concentration (MNTC) was calculated as the concentration required to retain cell viability by 90% (Chen *et al*., 2010). The percentage of viability was calculated as the following formula: % cell viability = (OD of drug-treated sample/OD of untreated sample) X 100. Four replicates were carried for each compound.

## Result and discussion

**1. Morphological changes**

MTT method monitored using microscopy were used to determine the MNTC of hydrosol compounds in MARC-145 cells treated with different concentrations of hydrosol compounds 72 hours. As shown in Figure 1, the cytotoxicity to MARC-145 cells was closely related with the concentrations of hydrosol compounds. Specifically, compared with cell only control of *C. longa*, *A. macrospadiceus* (stem) and *A. macrospadiceus* (leaf) compound striated MARC-145 cells displayed remarkable high toxicity by morphological changes such as detachment of monolayer, cell disruption, particularly vacuolization, granulation, lyses, pyknosis, condensation, darkening of cell boundaries, and cell detachment, similar Cheng *et al*., (2013) study on *In vitro* screening for compounds derived from traditional Chinese medicines with antiviral activities against porcine reproductive and respiratory syndrome virus.

However, we found that *H. cordata*, *A.argyi* and *P. cablin* compounds were showed a low toxicity with MNTC from appeared a little morphological change and high cell viability. In addition, we found that some compound promoted the high cell viability more than 100%

such as *C. sinensis*, *M. spicata* (Fig. 2). These compounds have chemical contents to exhibited excellent antioxidant activity (Govindarajan *et al*., 2012; Chen *et al*., 2010).

**2. Cytotoxicity effect**

Cytotoxicity assays are essential for the initial phases of antiviral drug development. The maximum non-cytotoxic concentration (MNTC) was calculated as the concentration required to retained cell viability by 90%. In this study, we found that *H. cordata*, *A. argyi* and *P. cablin* were showed a low toxicity with MNTC of $2^{-2}$ dilution. Eleven compounds were *C. sinensis*, *A. officinarum*, *N. cataria*, *M. spicata*, *C. cassia*, *P. frutescens*, *V. negundo*, *A. gramineus* (Stem), *A. gramineus* (Leaf), *M. arvensis* and *A. galangal*, were presented a moderate toxicity witch MNTC ranged from $2^{-3}$-$2^{-6}$ dilution. In addition, some extracts of many dilutions had the % cell viability > 90 (Table 1 and Figure 2).

While *A. macrospadiceus* (stem), *A. macrospadiceus* (leaf) and *C. longa* were showed a high toxicity with MNTC of ≥ $2^{-11}$ dilution. The *C. longa* is cultivated in tropical and subtropical regions. Depending on its origin and the soil conditions where it is grown, turmeric contains 2%–9% curcuminoids indicates a group of compounds such as curcumin, demethoxycurcumin, cyclic curcumin and bis-demethoxycurcumin. Out of these, curcumin is the major component, and cyclic curcumin is the minor component (Priyadarsini, 2014). The volatiles from the leaves and rhizomes of *Acorus spp*. found that methylchavicol (54.01%) is the major constituent of *A. macrospadiceus* essential oil and Nootkatone (15.92%)is the major ketone in this essential oil (Huang *et al*., 2012). However, the other chemical components in this essential oil were different from those of the other *Acorus spp*. (Du *et al*., 2008). In this results, we suggested that the *A. macrospadiceus*and *C. longa*compounds not suitable for MARC-145 cells culture.

The cytotoxicity of hydrosol compounds were found to be in a dose-dependent manner with the concentrations employed (Figure 2). Increasing concentrations of compounds will decrease the cell viability. However, when decreasing concentrations of some hydrosol compounds will high increase the cell viability such as *C. sinensis* and *M. spicata*. *C. sinensis*contain up to 14% of total polyphenols and 7% of flavonoids (Rana *et al*., 2015), constituents have stronger antioxidant and antibacterial properties (Chen *et al*., 2011), and also *M. spicata* had good total phenolic and flavonoid contents. It exhibited excellent antioxidant activity (Kanett *et al*., 2007; Snoussi *et al*., 2015; Govindarajan *et al*., 2012). We know that an antioxidant is a molecule that inhibits the oxidation of other molecules. Oxidation is a chemical reaction that can produce free radicals, leading to chain reactions that may damage cells (Aruoma *et al*., 1999; Adiguzei *et al*., 2009).

## Conclusion

This study indicates that the 3 compounds showed strong toxicity while the 14 compounds had a low - moderate toxicity on MARC-145 cells. We suggested that the compound showed high toxicity not suitable for MARC-145 cells culture but low-moderate toxicity compounds will be used to application on anti-PRRSV in the future research. In addition, also some compounds promoted cell viability.

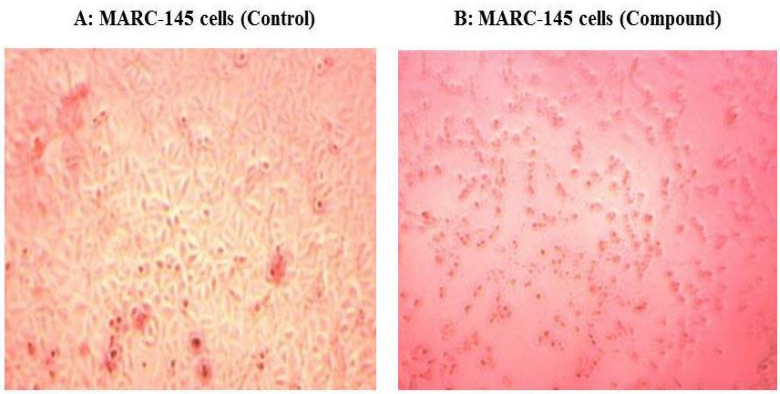


**Fig. 1** Cytotoxicity tested of extracts on MARC-145 cells; the cytotoxicity of compounds on Marc-145 cells was in a dose-dependent manner. MARC-145 morphological changes were observed daily and were photographed using a microscope at a magnification of 100x. A: control group; MARC-145 cells normal morphological shape and B: compound group; MARC-145 cells underwent more morphological changes such as lyses, granulation, pyknosis, condensation, vacuolization in the cytoplasm, darkening of cell boundaries and cell detachment

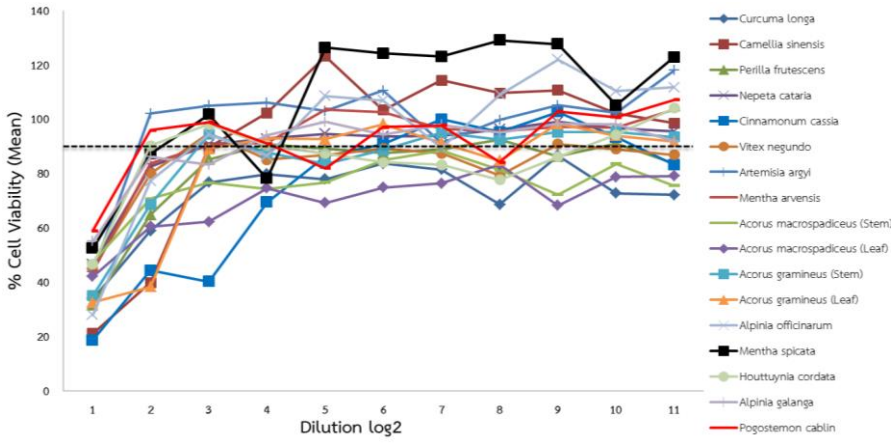


**Fig. 2** Effect of 17 Asian plant extracts on the viability of MARC-145 cells based on MTT assay. The % cell viability of Marc-145 cells was calculated as the concentration required to retained cell viability by 90% in a dose-dependent manner which clearly indicated that the cytotoxicity of different compounds on the same cell varied remarkably. The results represent mean ± SEM, n = 4

**Table 1** The cytotoxicity results of 17 Asian herb extracts at 2 fold serially dilution in MARC-145 cells after 72 hours of incubation.

| No. | Herbs | Maximum non-cytotoxicity concentration (Dilution log2) |
|---|---|---|
| 1 | *Camellia sinensis* | $2^{-4}$ |
| 2 | *Curcuma longa* | $>2^{-11}$ |
| 3 | *Alpiniaofficinarum* | $2^{-3}$ |
| 4 | *Nepetacataria* | $2^{-3}$ |
| 5 | *Menthaspicata* | $2^{-3}$ |
| 6 | *Cinnamomum cassia* | $2^{-6}$ |
| 7 | *Perillafrutescens* | $2^{-4}$ |
| 8 | *Vitexnegundo* | $2^{-3}$ |
| 9 | *Acorusmacrospadiceus*(Stem) | $>2^{-11}$ |
| 10 | *Acorusmacrospadiceus*(Leaf) | $>2^{-11}$ |
| 11 | *Acorusgramineus*(Stem) | $2^{-3}$ |
| 12 | *Acorusgramineus*(Leaf) | $2^{-4}$ |
| 13 | *Houttuyniacordata* | $2^{-2}$ |
| 14 | *Artemisia argyi* | $2^{-2}$ |
| 15 | *Menthaarvensis* | $2^{-3}$ |
| 16 | *Alpiniagalanga* | $2^{-4}$ |
| 17 | *Pogostemoncablin* | $2^{-2}$ |

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