Antiviral potential of cinnamon oil from *Cinnamomum iners* Reinw. ex Blume and *Cinnamomum burmannii* Blume against porcine reproductive and respiratory syndrome virus

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Abstract

Cinnamaldehyde, an aldehyde found in Cinnamomum bark was used as flavoring and in medicinal treatments. Cinnamaldehyde is the main organic constituent of cinnamon oil found in *Cinnamomum sp.* bark. It was used as flavoring and in medicinal treatments according to its diverse pharmacological properties. In line with this, development and exploitation of medicinal plants that possess antiviral property is an alternative way to control pathogenic viruses. Porcine reproductive and respiratory syndrome virus (PRRSV) is one of the significant causes of pig morbidity resulting in severe economic loss in swine industry worldwide. Thus, this study evaluated the antiviral effect of cinnamaldehyde from the essential oil of C. *iners* and C. *burmannii* against PRRSV grown in MARC-145 cells. The average percentage yield of cinnamon oil of C. *iners* was 0.75 % and 0.92% from C. *burmannii* respectively. FT–IR spectroscopy showed the functional groups confirming the high presence of cinnamaldehyde on both cinnamon oil extracted by hydrodistillation. After cinnamaldehyde analysis of the cinnamon oil, treatment on pre–infection and post–infection of virus was evaluated. In pre–virus entry, results showed that cinnamaldehyde from C. *iners* reduced moderately the viral titer for about 10% to 40% and 13.75% to 25% viral reduction in post–virus entry, respectively. These results suggest the potential of cinnamaldehyde from essential oil as an effective anti–PRRSV but further studies on the enhancement of its efficacy should be conducted.

Keywords: cinnamaldehyde, cinnamon oil, pig, porcine reproductive and respiratory syndrome virus

Introduction

Porcine reproductive and respiratory syndrome is one of the most significant and economically important infectious disease that affects swine industry worldwide. The disease is caused by porcine reproductive and respiratory syndrome virus (PRRSV) which infects pigs of all ages, resulting in abortion, stillbirth and weak piglets. PRRSV is a positive single–stranded and enveloped RNA virus which contains 10 open reading frames (ORFs). It was a member of genus *Arterivirus*, order *Nidovirales*, family *Arteriviridae*. (Lunney et al., 2010). The primary target cell for PRRSV replication is alveolar macrophages as revealed by in situ detection of PRRSV antigen and genome. As the PRRSV replicates in alveolar macrophages in the lungs and lymphoid organs, it
compromises the cellular immune response causing to the damaged mucosal surfaces and leads to interstitial pneumonia resulting death and illness. Transmission of PRRSV by the infected sows was up to 157 days post initial infection (Wills et al., 1997). Sows can transmit PRRSV transplacentally to their unborn piglets. It was reported that PRRSV in the lymph organs was detected up to 132 days post initial infection when the piglets were infected in the uterus (Rowland et al., 2003).

Cinnamaldehyde (3-phenyl-2-propenal) is the aldehyde responsible for the aromatic flavor of cinnamon. It is the main organic flavonoid content of essential oil found in cinnamon isolated from Cinnamomum trees (e.g. Cinnamomum iners Reinw. ex Blume, Cinnamomum burmannii Blume) belonging to the family Lauraceae. With chemical formula C6H8O, it has a molecular weight of 132.16 g/mol on which chemical stability is fair to poor. Biological activities of cinnamaldehyde such as antiviral (Fabra et al., 2016) antimicrobial (Yossa et al., 2014), anti-inflammatory (Muhammad et al., 2015), antioxidant (Naveena et al., 2014), antispasmodic (Jaafarpour et al., 2015), anti-urease (Lee et al., 2005), anti-cancer (Vangalapati et al., 2013) and hyperglycemic (Camacho et al., 2015) properties have been well documented. The antipathogenic property of cinnamaldehyde extracted from Cinnamomum verum J. Presl and Cinnamomum cassia Blume has been proven by several reports conducted (Ooi et al., 2006; Ouattara et al., 1997; Wong et al., 2008). Cinnamaldehyde from Cinnamomum verum J. Presl and Cinnamomum osmophloeum inhibits pro-inflammatory IL-1β (interleukin-1beta) and IL-6 (interleukin–6) production, suppresses iNOS (nitric oxide synthase) and COX–2 (cyclooxygenase–2). These findings conclude the anti-inflammatory effects of cinnamaldehyde (Koh et al., 1998, Zhao et al., 2011).

Trans-cinnamaldehyde, one of the major components of essential oil derived from Cinnamomum cassia Blume inhibited the Influenza A/PR/8 virus grown in vitro on Madin–darby canine kidney cells in a dose dependent manner, in vivo with mice by nasal inoculation and inhalation, showed significant survival rates. Real-time polymerase chain reaction confirmed that cinnamaldehyde inhibited viral protein synthesis at the post transcriptional level (Hayashi et al., 2007). In addition, volatile oil from dry branches of Cinnamomum cassia Presl. containing 66.85% cinnamaldehyde showed anti-influenza virus A/PR/8/34 (H1N1) (Liu et al., 2015). In contrast, trans-cinnamaldehyde alone has low antiviral property on human viruses (Goswani and Rahman, 2010). Generally, the chemical property of cinnamaldehyde is unstable in vivo hence unstable in rat blood with a half-life of 4–minutes (Yuan et al., 1992, Li et al., 2016). To overcome this problem, cinnamaldehyde derivatives were synthesized. Li et al. (2016) demonstrated the antiviral potential of α-bromo-4-methyl-cinnamaldehyde and α-bromo-4-chloro-cinnamaldehyde effectively reduced the viral titer of coxsackievirus B3 (CVB3) in HeLa cells. Antiviral activities of cinnamaldehyde were significantly increased when cinnamaldehyde was brominated and chlorinated also resulting with low toxicity.

Gene expression revealed the antiviral potential of cinnamaldehyde. Cinnamaldehyde derivatives such as 2-hydroxycinnamaldehyde and 2-benzyloxycinnamaldehyde inhibited IL2Rα and IFN–G (interferon gamma) expression but no effect on IL–2 (Interleukin–2) expression in mouse splenocytes culture (Koh et al., 1998). Volatile oils of cinnamom increased the expression of IFN–β (Interferon–beta), TLR7 (Toll–like receptor 7), IRAK–
4 (interleukin-1 receptor–associated kinase 4), in Madin–Darby kidney cell infected with influenza virus A/PR/8/34 (H1N1) (Liu et al., 2015). In contrast, Youn et al. (2008) reported that cinnamaldehyde suppressed the activation of NFKB and IRF3 (Interferon regulatory factor 3) induced by a TLR4 agonist (i.e. lipopolysaccharide), leading to the decreased expression of target genes such as COX–2 and IFN-β.

The emergence of drug–resistant viruses was led by the augmented availability and use of antiviral drugs like essential oils from plants. However, search for new drugs, vaccine and therapeutics on porcine viral diseases were still required to manage with this emerging worldwide problem in swine industry. Studies on the anti–PRRSV of cinnamaldehyde have not yet been investigated in spite of several reports documenting on its antiviral properties against various viruses. Thus, the present study reports on the antiviral potential of cinnamaldehyde against PRRSV grown in vitro on MARC–145 cells.

Methods

Essential Oil Extraction

Hydrodistillation was done to extract the essential oil of *Cinnamomum iners* Reinw. ex Blume and *Cinnamomum burmannii* Blume. (Singh et al., 2007). Dried cinnamon bark obtained from *C. iners* and *C. burmannii* (derived from Indonesia) was purchased at Warorot market, Chiang Mai, Thailand. One kg of cinnamon was mixed with 3 L of distilled water, and heated at 100 ºC for 8 h (Wong et al., 2014). Using methylene dichloride, the volatile products were extracted thrice from the water phase (Singh et al., 2007). Essential oil structure was characterized by FT-IR spectroscopy.

Cinnamaldehyde analysis by thin layer chromatography and Fourier–Transform IR spectroscopy

IR spectra of the cinnamon oil were recorded on Perkin Elmer FT–IR spectrometer with KBr pellets. IR spectra were reported in % transmittance. Cinnamon oil IR spectra were compared with cinnaldehyde IR spectrum standard. The wave number region for the analysis was 4000–400 cm⁻¹ (in the mid infrared range). The purity of the compound was checked by TLC (thin layer chromatography) on precoated SiO2gel (HF254 200 mesh) aluminum plates (E Merck) (Kankeaw and Masong, 2015).

Source of MARC–145 cells and PRRSV culture

Virus culture and cell line were obtained from Veterinary Diagnostic Laboratory, Faculty of Veterinary Science, Chulalongkorn University. It was maintained in MEM++ comprising minimum essential medium (Caisson Laboratories, Utah, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Capricorn Scientific GmbH, Germany), antibiotics/antimycotic penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (250 ng/ml) (all from Gibco, New York, USA) to avoid microbial contamination in the culture. PRRSV was propagated in sub cultured MARC–145 cells grown in MEM++ at 37°C in a 5% CO₂ incubator. After four days of incubation, the frozen overnight virus cultures were thawed twice, centrifuged, and harvested. Supernatant was filtered through 0.22 µM filter (Minisart®, Sartorius, France) to remove unnecessary particles, cells and microbes. Harvested virus were stored at –80°C. Viral titer was determined by 50% cell culture
infectious dose (TCID50) endpoint dilution assay after 96 h inoculation and adjusted to 10^6 TCID50/ml prior to anti-PRRSV culture assay.

**Cinnamaldehyde cytotoxicity test on MARC–145 cells**

Cytotoxicity test of cinnamaldehyde was done by crystal violet assay and 3-(4,5-dimethylthiazol–2-yl)-2,5-diphenyltetrazolium bromide (MTT) method as previously described (Sun et al., 2012). Approximately 1x10^5 cells/ml MARC–145 cells were sub cultured in flat-bottomed 96–well plate (Nunc, Denmark). Test extract were serially 2-fold diluted, added onto the wells and cultivated at 37°C in humidified 5% CO2 atmosphere for 96 h. After completion of incubation, the media was removed and washed with PBS thrice. Cells were fixed with acetone:methanol (40:60) solution at 4°C for 30 min. Then, 0.5% crystal violet solution was added in each well and incubated at room temperature for 5 min followed by washing with water. Wells the received Sorenson’s buffer and incubated at room temperature for 15 min. The optical density (OD) absorbance was determined at 570 nm (Feoktistova et al., 2016). On the other hand, another cell culture set up was prepared for MTT method. MTT solution was added to each well and the plates were incubated for 4 h at room temperature. Then, 100 µl of DMSO was added to dissolve formazan crystals. The plates were gently shaken for five min until the crystals were fully dissolved. The OD was measured at 595 nm on a microplate reader (Thirabunyanon et al., 2009). Percentage of cell viability was calculated using the formula [(A−B)/A]x100, where A and B are the OD of treated and control cells, respectively.

**Anti-PRRSV assay**

In this study, cytopathic effect was observed. Using 50% tissue culture infectious dose (TCID50) endpoint dilution assay, pre-treatment and post-treatment antiviral effect were investigated. Three concentrations of the volatile product were based on the results of cytotoxicity assay described above prior to the testing of their activity against PRRSV.

To examine the pre-treatment effect, cultured MARC–145 cells in 25–ml flask was incubated (37°C, 5% CO2, 16 h). The medium was removed and fresh MEM + was added prior to the addition of the selected concentration of cinnamaldehyde. Five hundred µl of PRRSV with approximately10^6 TCID50/ml was added onto the flask and the culture was incubated at 37°C in humidified 5% CO2 atmosphere for 96 h. Supernatant was collected and stored at –80°C. Subsequently, PRRSV titration was performed by the addition of serially 10-fold diluted supernatant in MEM + and inoculated in 96 well–plate containing 100 µl of confluent MARC–145 cell. The cultures were incubated for 96 h at 37°C in a 5% CO2 incubator. TCID50 values were determined after uninfected and infected cells were observed under inverted microscope.

To investigate post-treatment effect, the sub cultured MARC–145 cells in 25–ml flask was cultivated. Media was removed and 500 µl of PRRSV (10^5 TCID50/ml) was added, followed by fresh MEM +. Then, cinnamaldehyde was added and incubation was carried out for 16 h at 37°C in 5% CO2 incubator. Supernatant was collected and serially 10-fold diluted. The serially diluted supernatant in MEM + was added to the wells that contains confluent MARC–145 cells. The cells were incubated for 96 h at 37°C in a 5% CO2 incubator. TCID50 values were determined under inverted microscope.
Percentage virus inhibition analysis

The calculation of virus inhibition percentage was based on the formula \( (100\% \times (A - B)/A) \), where A and B denotes to logarithmic number of virus titer in the absence and presence of test sample, respectively.

Results and discussion

Yield of essential oil and cinnamaldehyde composition

<table>
<thead>
<tr>
<th>Table 1 Essential oil extraction of cinnamon bark of <em>Cinnamomum iners</em> Reinw. ex Blume from Thailand</th>
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</thead>
<tbody>
<tr>
<td><strong>Weight of Cinnamon bark (kg)</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
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<tr>
<td><strong>Average</strong></td>
</tr>
</tbody>
</table>

The average percentage yield of essential oil from *C. iners* bark obtained from Thailand was shown in table 1. After extraction of volatile oil by hydrodistillation, the average yield was 0.75%.

<table>
<thead>
<tr>
<th>Table 2 Essential oil extraction of cinnamon bark of <em>Cinnamomum burmannii</em> Blume from Indonesia</th>
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<tbody>
<tr>
<td><strong>Weight of Cinnamon bark (kg)</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td><strong>Average</strong></td>
</tr>
</tbody>
</table>

The table 2 showed the percentage yield of essential oil from the cinnamon bark of *C. burmannii* originated from Indonesia. The average percentage yield of cinnamon oil after hydrodistillation was 0.92%.

The yield of cinnamon oil in different species varies. Li et al., 2013 observed the differences of essential oil harvested in every *Cinnamomum* species and it was due to the environmental factors such as type of weather or climate, geographical distribution, growth conditions and site of cultivation. This implies that results of essential oil isolation from cinnamon bark was correlated with ecological factors mentioned above on which difference between the essential oil percentage yield of *C. iners* and *C. burmannii* was due to species diversity and cultivation site. In addition, Li et al., 2013 found out that thicker bark has higher cinnamon oil content and the most essential oil was concentrated in the phloem.
Figure 1. FT–IR spectrum of cinnamaldehyde standard

Figure 2. FT–IR spectrum of cinnamon oil of *C. iners*

Figure 3. FT–IR spectrum of cinnamon oil of *C. burmannii*
FT-IR spectroscopy revealed the functional groups of the cinnamaldehyde chemical structure. The characteristics of cinnamon oil extracted from C. iners (fig. 2) and C. burmannii (fig. 3) were compared to the reference FT-IR spectra of cinnamaldehyde. It was found out that both volatile oil extract contained high quality of cinnamaldehyde. There were present peaks of 1677 cm\(^{-1}\) (C=O stretching of aldehyde), 2814, 2741 cm\(^{-1}\) (C-H stretching of aldehyde), 1624 cm\(^{-1}\) (C=C stretching of alkene) and 1449 cm\(^{-1}\) (C=C stretching of aromatic) (Awang et al., 2013; Kankeaw and Masong, 2015; Gende et al., 2008).

Cinnamaldehyde is the major compound of cinnamon oil. About 90% cinnamaldehyde was revealed by HPLC (high-performance liquid chromatography) from the steam-distilled essential oil (Wong et al., 2014). Adinew, 2014 also identified 87% cinnamaldehyde analyzed by GC–MS and FT–IR spectroscopy from the essential oil of cinnamon bark. In addition, 85% of cinnamaldehyde in hydro-distilled cinnamon oil from C. cassia and C. verum was revealed by GC–MS (Gas chromatography–mass spectrometry) (Ooi et al., 2008). Moreover, essential oil from three species of Cinnamomum (i.e. C. loureirii, C. verum, C. cassia) analysed by GC–MS and FT–IR spectroscopy have 66.28–81.97% of cinnamaldehyde (Li et al., 2013).

### Antiviral assay

**Table 3 Antiviral activity of cinnamaldehyde**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Concentration (nl/ml)</th>
<th>%Virus inhibition</th>
<th>Pre-infection</th>
<th>Post-infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamaldehyde 1</td>
<td>4</td>
<td>10%</td>
<td>13.75%</td>
<td></td>
</tr>
<tr>
<td>(C. iners)</td>
<td>0.4</td>
<td>10%</td>
<td>21.25%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.04</td>
<td>40%</td>
<td>25%</td>
<td></td>
</tr>
<tr>
<td>Cinnamaldehyde 2</td>
<td>2</td>
<td>-1.25%</td>
<td>-5%</td>
<td></td>
</tr>
<tr>
<td>(C. burmannii)</td>
<td>0.2</td>
<td>21.25%</td>
<td>17.5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.02</td>
<td>-1.25%</td>
<td>-5%</td>
<td></td>
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</tbody>
</table>

The antiviral activity of cinnamaldehyde from two cinnamon bark sources was summarized in Table 3. The selected concentrations of each extract were non–cytotoxic to MARC–145 cells. The pre–infection entry of virus showed low to moderate anti–PRRSV effect of cinnamaldehyde 1 (C. iners from Thailand) ranging from 10% to 40% virus inhibition. While 0.2 ng/ml cinnamaldehyde 2 (C. burmannii from Indonesia) showed only 21% inhibition in pre–infection of virus. On the other hand, in post virus entry, only cinnamaldehyde 1 reduced PRRSV with 13.75% to 25% viral reduction. Conversely, cinnamaldehyde 2 reduced 21% viral titer at the pre–infection treatment. The moderate antiviral activity of cinnamaldehyde is due to its instability. The natural occurring trans-cinnamaldehyde from essential oils has low antiviral property alone against viruses but can increase its efficacy when synthesized with derivatives and carrier. (Li et al., 2016, Goswani and Rahman, 2010). This result is in
line with the study reported by Liu et al. (2016) that cinnamaldehyde from the volatile oil of *Cinnamomum* has the ability to suppress IFN–B, TLR7 and IRAK–4 which have the significant roles in antiviral mechanism of the immune cells.

This study has revealed the anti–PRRSV property of cinnamaldehyde from two *Cinnamomum* essential oil. This study showed the moderate to no antiviral activity of cinnamaldehyde. Further studies on synthesizing cinnamaldehyde structure have to be carried out to enhance the efficacy of these organic compounds on their anti–PRRSV properties.

**Reference**


