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Short communication

# Antiviral activity of luteolin against Japanese encephalitis virus

Wenchun Fan, Suhong Qian, Ping Qian, Xiangmin Li*

*State Key Laboratory of Agricultural Microbiology, Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, PR China*

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ABSTRACT

Japanese encephalitis virus (JEV), a member of family *Flaviviridae*, is a neurotropic flavivirus that causes Japanese encephalitis (JE). JEV is one of the most important causative agents of viral encephalitis in humans, and this disease leads to high fatality rates. Although effective vaccines are available, no effective antiviral therapy for JE has been developed. Hence, identifying effective antiviral agents against JEV infection is important. In this study, we found that luteolin was an antiviral bioflavonoid with potent antiviral activity against JEV replication in A549 cells with $IC_{50}$ = 4.56 μg/mL. Luteolin also showed extracellular virucidal activity on JEV. With a time-of-drug addition assay revealing that JEV replication was inhibited by luteolin after the entry stage. Overall, our results suggested that luteolin can be used to develop an antiviral drug against JEV.



Japanese encephalitis virus (JEV): genus *Flavivirus*, family *Flaviviridae*, is one of the most important causative agents of viral encephalitis in humans. In Asia, JEV causes 68 000 clinical viral encephalitis cases every year (WHO, 2015). The fatality rate of JEV could be as high as 30%. The number of encephalitis patients with permanent neurologic or psychiatric sequelae is estimated at approximately 30% to 50% (Solomon et al., 2000). Nowadays, Japanese encephalitis (JE) is considered a global public-health issue. With 24 countries having more than 3 billion people at risk to JEV infection (WHO, 2015). JEV is an enveloped virus with a single-stranded and positive-sense RNA 11 kb long. Its genome formesd a single long open-reading frame (ORF) flanked by the 5′- and 3′-untranslated regions (UTRs). The translation of the JEV genome by host cellular machinery produces a polyprotein that is cleaved by viral and cellular proteases to yield three structural proteins named capsid (C),pre-membrane (prM), and envelope (E), as well as seven nonstructural (NS) proteins called NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Chambers et al., 1990; Sumiyoshi et al., 1987).

At present, vaccination is the most common method to prevent JEV infection. Currently, safe and effective JE vaccines are available to prevent JEV infection, including inactivated mouse brain-derived vaccines, inactivated BHK-21 cell-derived vaccines, live attenuated vaccines, Vero cell derived inactivated vaccines and chimeric-live attenuate vaccine (Kim et al., 2014; Miyazaki et al., 2014; Paulke-Korinek and Kollaritsch, 2008; Wang et al., 2014; Yun et al., 2015). However, no effective antiviral agent has been developed to absolutely cure JE. The treatment must to relieve symptoms and stabilize patients. Therefore, finding effective antiviral agent against JEV infection is necessary (Gould et al., 2008).

Luteolin, 3′,4′,5,7-tetra- hydroxyflavone, which is a member of the flavone family, is a nontoxic and nonmutagenic dietary flavonoid found in camomile tea, perilla leaf, green pepper, and celery (Kim et al., 2003). Luteolin possesses various pharmacological benefits, such as anti-inflammatory (Hytti et al., 2015; Jia et al., 2015; Kim et al., 2003; Rafacho et al., 2015; Sung and Lee, 2015; Tsilioni et al., 2015), anticancer (Abdel Hadi et al., 2015; Bai, 2015; Chian et al., 2014; Lin et al., 2015; Ma et al., 2015), antioxidant properties (An et al., 2016 Fazio et al., 2016; Rui-qu et al., 2015), and antiviral function (Bai et al., 2015; Lv et al., 2014; Mehla et al., 2011; Ryu et al., 2010; Xu et al., 2014). Some bioflavonoids were reported to exhibit antiviral activity against JEV *in vitro* (Johari et al., 2012a,b; Zhang et al., 2012). In this study, we aimed to evaluate the antiviral activity of luteolin against JEV replication in A549 cells.

First, the cytotoxicity of luteolin (Fig. 1 A) (Shanghai Yuanye Bio-Technology Co., Ltd.) to A549 cells were determined using CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega, Beijing, China). As shown in Fig. 1B, the $CC_{50}$ of luteolin was 54.4 μg/mL in A549 cells. The cell viability is 98.7%, 90.6%, 83.3% at a concentration of 10, 20, and 40 μg/mL, respectively. Thus 5, 10, 15, 20, 30, and 40 μg/mL luteolin were used for the potential antiviral effect assay. For the antiviral effects assay, A549 cells were seeded in 24-well plates and left overnight to attach. On the next day, the cells were infected with JEV SX09S01 strain (Li et al.,

* Corresponding author at: Laboratory of Animal Virology, College of Veterinary Medicine, Huazhong Agricultural University, Wuhan, Hubei, PR China.
*E-mail address:* lixiangmin@mail.hzau.edu.cn (X. Li).

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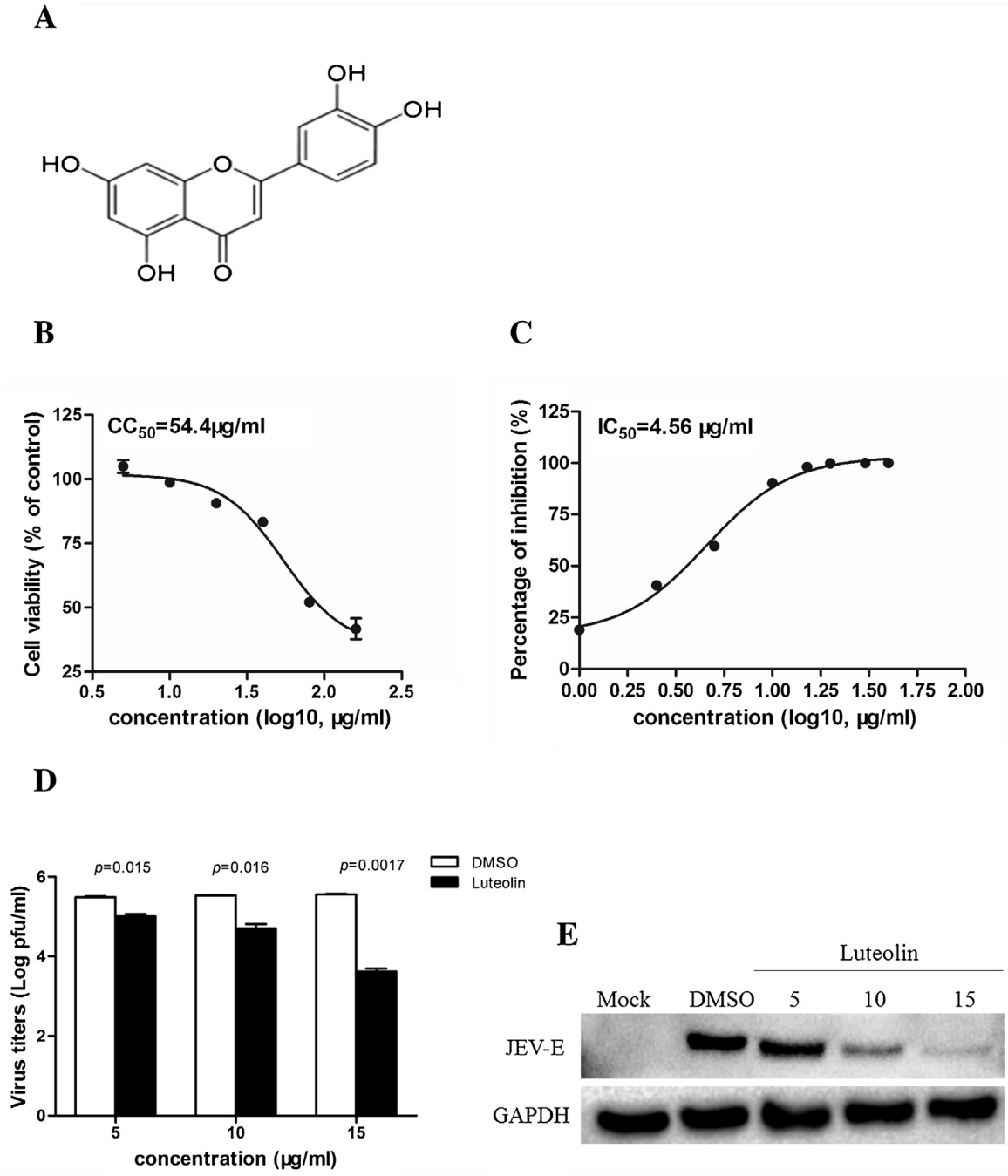


**Fig. 1.** Identification of luteoin against JEV infection. (A) Molecular structures of luteolin. (B) Determination of $CC_{50}$ of luteolin. A549 cells were seeded in 96-well plates and left to attach. Next day, the cells were treated with or without luteolin at specified concentrations. Cell viability was determined after 24 h of post-treatment. (C) Evaluation of the $IC_{50}$ of luteolin. A549 cells in 24-well plates were infected with JEV at an MOI of 1. 2 h later. The infection mixtures were removed and replaced with a medium containing luteolin at specified concentrations. The supernatants were collected at 24 hpi, and progeny virus titers were determined using plaque forming assay. (D, E) Confirmation of luteolin antiviral effect against JEV by titration and Western blot analysis for viral E protein. A549 cells in 24-well plates were infected with JEV at an MOI of 1. The cells were treated with or without luteolin at indicated concentration throughout the duration of experiment. The supernatants were harvested at 24 hpi. The production of infectious virion production was determined using plaque forming assay (D). The viral E protein expression was detected using western blot assay (E). All the experiments were performed three times independently, and the representative results are presented. GraphPad Prism software Version 5 was utilized in this study. The statistical analysis was performed using two-tailed student *t*-test. The statistical significance is $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

2014) at an MOI of 1. Cell culture mixtures with luteolin were collected after 24 h of post-infection (hpi). The progeny virus titer was determined by plaque forming assay in BHK-21 cells. The $IC_{50}$ of luteolin on JEV was determined. The result was showed in Fig. 1C, the IC50 was evaluated to be 4.56 μg/mL. Fig. 1D indicates that the JEV infection was inhibited significantly with the treatment of luteolin at 15 μg/mL. To confirm the reduction of virus titer induced by luteolin, the expression of JEV E protein was detected using Western blot analysis. The results showed that the JEV E protein level was significantly reduced with luteolin treatment, and the reduction occurred in a dose-dependent manner (Fig. 1E). These results suggested that luteolin exerted antiviral activity against JEV infection.

Previous studies reported that flavonoids inhibited JEV replication at all different stages of the JEV life cycle (Johari et al., 2012a,b; Zhang et al., 2012). We examined whether luteolin con-