Journal of Medicinal Plants Research Vol. 5(23), pp. 5534-5539, 23 October, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

In vitro antiviral activity of Fisetin, Rutin and Naringenin against Dengue virus type-2

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Accepted 25 August, 2011

***In vitro* antiviral activities of three flavonoids; fisetin, naringenin and rutin against DENV-2 (NGC strain) were evaluated. Inhibitory effects of each compound at the different stages of DENV-2 infection were examined using foci forming unit reduction assay (FFURA) and quantitative real-time polymerase chain amplification (qRT-PCR). Fisetin, rutin and naringenin showed cytotoxic effects against Vero cells with 50% cytotoxicity (CC_{50}) values of 247, >1000, and 87 µg/mL, respectively. Fisetin when added to Vero cells after virus adsorption inhibited DENV replication with a half maximal inhibition concentration (IC_{50}) value of 55 µg/mL and selectivity index (SI) of 4.49. The IC_{50} value of fisetin was 43.12 µg/mL with SI=5.72 when Vero cells were treated for 5 h before virus infection and continuously up to 4 days post-infection. There was no direct virucidal activity or prophylactic activity of fisetin against DENV-2. Rutin and naringenin did not inhibit DENV-2 replication in Vero cells. Naringenin however, exhibited direct virucidal activity against DENV-2 with IC_{50} = 52.64 µg/mL but the SI was <1. The present study suggests that among the flavonoids examined, only fisetin showed significant *in vitro* anti dengue virus replication activity.**

Key words: Infectious disease, virology, dengue, antivirals, flavonoid.

INTRODUCTION

Dengue virus (DENV) belongs to the *Flaviviridae* family which consists of several important human pathogens. There are four different DENV genotypes; DENV-1, DENV-2, DENV-3 and DENV-4. All four endemic DENV genotypes are transmitted by the mosquitoes, *Aedes agypti* or *Aedes Albopictus*. Most primary DENV infections are asymptomatic or manifests as dengue fever (DF), a mild febrile illness with some vascular leakage tendencies. The severe forms of dengue usually occur in secondary infections which could manifest as dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) with severe vascular leakage. Currently, there is no effective antiviral drug or licensed vaccine against dengue. Finding effective antiviral compound(s) against the virus is crucial as the disease is rapidly spreading worldwide and causing many deaths. Among the different types of compounds and chemical derivatives studied for antiviral activities against DENV, include those from natural resources such as plant extracts and their purified compounds (Jain et al., 2008). Plant-derived compounds are desired due to the potential of low side effects and their easy accessibility from the nature. Among these plant-derived compounds, flavonoids have received considerable attentions as possible sources of new therapeutics for viral infections (Asres et al., 2005; Wang et al., 2006). Flavonoids are phenolic compounds found in many plants (Tapas et al., 2008). There have been many studies reporting the beneficial effects of flavonoids on general health such as their anti-oxidant activities, anti-tumor activities and also their anti-microbial activities (Chukwujekwu et al., 2011; Kale et al., 2008; Middleton, 1996; Tim Kushnie et al., 2005). Antiviral activities for a number of flavonoids have also been described against many different viruses (Lyu et al., 2005; Arena et al., 2008; Xu et al., 2010). The

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flavonoids have been shown to possess antiviral activities against HSV-1, HSV-2, Sindbis virus, parainfluenzavirus-3 and HCMV (Hayashi et al., 2007; Lyu et al., 2005; Orhan et al., 2010; Paredes et al., 2003). A recent study reported the potential of flavonoids as antivirals against DENV (Muhamad et al., 2010). In the present study, we examined the *in vitro* antiviral activity of the flavonoids, fisetin, naringenin and rutin against DENV-2.

MATERIALS AND METHODS

Chemicals

Fisetin and naringenin were purchased from Sigma Chemical Company (Sigma, St, Louis, USA) and rutin was purchased from Indofine Chemical Company (Indofine, NJ, USA). All compounds were initially dissolved in dimethyl sulfoxide (DMSO) (Sigma, St, Louis, USA) to prepare the stock solution (20 mg/mL) and stored at -20°C until needed. To prepare the different concentrations of each compound, the stock solution was serially diluted in serum free cell culture medium and sterile-filtered using syringe filter with 0.2 µm pore size. Fetal bovine serum (FBS) was added to the treatment medium to a final concentration of 2% when needed for the treatment of the infected cells.

Cells and virus

Dengue virus type-2 (DENV-2) New Guinea C strain (NGC) was used for the study. Virus was propagated in *A. albopictus* C6/36 monolayer cells at 28°C in the presence of 3% CO_2 and harvested on day 7 post-infection (PI). Virus stock was prepared and titrated as previously described (Wong SS et al., 2007) and stored at -70°C until needed.

African green monkey kidney cells (Vero) was used for the foci forming unit reduction assay (FFURA). Cells were cultured in Eagle's minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS; Gibco, NY, USA) at 37°C in the presence of 5% CO_2. For maintenance medium, serum concentration was reduced to 2%.

Cytotoxicity assay.

In vitro cytotoxicity of compounds used in the study was determined using MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) method performed strictly following the manufacturer's protocol and as previously described (Mosmann, 1983). Briefly, Vero cells were cultured in 96-well cell culture microplate. Confluent cells were treated by increasing concentration of each compound in triplicate wells. The final volume of cell culture medium and compound in each well was 90 µl. Treated cells were incubated for 4 days at 37°C. After 4 days, 15 µl of MTT solution (Promega, WI, USA) was added to each well. The microplate was kept at 37°C for an additional 4 h in a humidified 5% atmosphere. After the incubation period, 100 µl of the solublization/stop solution was added to each well. The absorbance value of each well was measured using a 96-well plate reader (TECAN, Mannendorf, Switzerland) at 570 nm.

In vitro antiviral assays.

The *in vitro* antiviral assay was performed using three different treatment conditions. In the first instance, Vero cells were plated into 24 wells cell culture microplate. After attaining 80% confluency, virus inoculum consisting of 200 FFU DENV-2 was added to each well and virus was allowed to absorb to the cells for 1 h at 37°C. Unabsorbed viruses were removed by rinsing cells with sterile PBS twice. Different concentrations of each compound were mixed with 1.5% carboxymethylcellulose (CMC) containing cell-growth medium supplemented with 2% FBS and the plates were incubated at 37°C for 4 days. After 4 days, cells were fixed with 4% paraformaldehyde for 20 min and subsequently permeabilized with 1% NP40 solution (Sigma, St, Louis, USA) for 10 min at room temperature. Cells were washed 3 times with PBS and blocked with 3% skim milk solution prepared in PBS, for 2 h at room temperature. Dengue hyper-immune serum prepared in rabbit, diluted at 1:500 using 1% skim milk solution was added to each well. The cells were incubated at 37°C for 1 h. After incubation, cells were washed 3 times with PBS and similarly incubated with goat anti-rabbit IgG conjugated with horse-radish peroxidase (HRP) with final concentration of 1:250 in 1% skim milk solution (Sigma, St. Louis USA). Finally, 3' di-amino-benzidine (DAB) peroxidase substrate (Pierce, Illinois USA) was added to each well to stain the virus foci (Okuno et al., 1979). Foci were counted under a stereomicroscope and expressed as foci-forming-unit (FFU). Antiviral activities of the compounds were determined by calculating the percentage of foci reduction (%RF) compared against the controls maintained in parallel using the following formula; RF (%) = (C-T) × 100/C, where, C is the mean of the number of foci from triplicates treatment without compound added and T is the mean of the number of foci from triplicates of each treatment measures with the respective compound (Laille et al., 1998).

In a separate study, the effects of prophylactic treatment of cells with the compounds prior to DENV infection were examined. Vero cells were treated with the different concentrations of each compound for 5 h before viral infection. The treatment medium was aspirated after 5 h and cells were rinsed twice with sterile PBS and then infected with 200 FFU of DENV-2 in the presence of the various concentrations of the compounds. The microplate was kept at 37°C for 1 h to allow virus adsorption. After virus adsorption, the infected cell monolayer was rinsed twice with sterile PBS in order to remove the unabsorbed viruses and incubated in 2% FBS containing EMEM with 1.5% CMC and the different concentration of the compounds. The plates were incubated at 37°C for 4 days in the presence of 5% CO_2. Viral foci were stained and counted as described in the foregoing.

The potential direct virucidal effects of the compounds on DENV were examined by treating virus suspension containing 200 FFU of DENV-2 with increasing concentration of each of the compound for 2 h at 37°C. Vero cell monolayers in 24 wells cell culture microplate were infected with the compound-treated virus suspensions. After 1 h adsorption at 37°C, cells were rinsed twice with PBS to remove unabsorbed viruses. Then 1.5% CMC containing growth medium was added to each well and the microplate was incubated for 4 days in a humidified 37°C incubator in the presence of 5% CO_2. Viral foci staining were performed as described in the foregoing.

Quantitative RT-PCR

DENV genome replication was estimated using the quantitative RT-PCR method described previously with some modifications (Wong et al., 2007). Briefly, DENV-2 RNA was extracted from the DENV-2 infected cells and cell culture supernatant using RNA extraction kit (Qiagen, Hilden, Germany). Quantitative RT-PCR was performed using SensiMix SYBR green mixture (Quantace, Watford, United Kingdom) in a total reaction volume of 20 µl consisting of ddH2O (7.4 µl), 2× SensiMix One-Step (10 µl), 50× SYBR Green solution (0.4 µl), RNase Inhibitor (10 units), 50 pmol each of forward (DNF) and reverse (D2R) primers, and the extracted DENV RNA (1 µl) (Seah et al., 1995). All amplifications were performed in triplicates

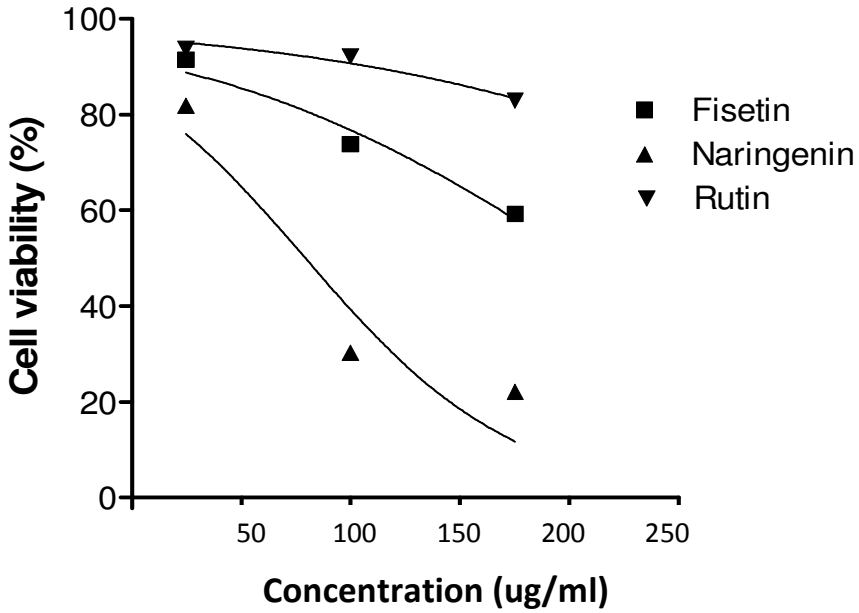

Figure 1. Cytotoxicity of flavonoids against Vero cells. MTT assay was used to evaluate the cytotoxicity of the flavonoids. All experiments were conducted in triplicates.

Table 1. *In vitro* cytotoxicity effects of flavonoids against Vero cells.

Flavonoid	CC_{50}(µg mL^{-1})
Fisetin	247
Rutin	>1000
Naringenin	83

using the DNA engine opticon system® (MJ Research/Bio-Rad, Hercules, CA) and the following amplification cycles: reverse transcription at 5°C for 30 min, initial denaturation at 95°C (10 min), followed by 45 cycles of 95°C for 15 s, 59°C for 30 s and 72°C for 30 s. Melting curve analysis was performed at temperature from 60 to 98°C to verify the assay specificity. For absolute quantitation of the viral RNA, a standard curve was established with a serially diluted RNA extracted from virus inoculum with known infectious virus titer.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism for Windows, version 5 (Graph Pad Software Inc., San Diego, CA, 2005). Selectivity index value (SI) was determined as the ratio of CC_{50}/IC_{50} of each compound.

RESULTS AND DISCUSSION

Cytotoxicity of bioflavonoids

Potential cytotoxic effects of the compounds were determined using the MTT assays (Figure 1). Results from the assays suggested that the 50% cytotoxicity (CC_{50}) values for fisetin, rutin and naringenin were 247, >1000 and 87 µg/mL, respectively (Table 1). Naringenin was the most cytotoxic compound to Vero cells among the three examined compounds.

Rutin showed no significant cytotoxicity against Vero cells. There were no measurable cytotoxic effects of 0.5% DMSO against Vero cells examined at the highest possible concentration as solvent for the compounds used in the study.

Antiviral assays with different compounds against DENV-2

Foci forming unit reduction assay (FFURA) was used to evaluate the *in vitro* anti-dengue virus activities of the flavonoids examined in the study. All the compounds were examined (i) for their prophylactic activity (ii) when added subsequent to virus infection of cells, (iii) when added to cells at 5 h prior to infection and then all throughout the assay period and (iv) directly to virus suspension to examine their direct virucidal effects.

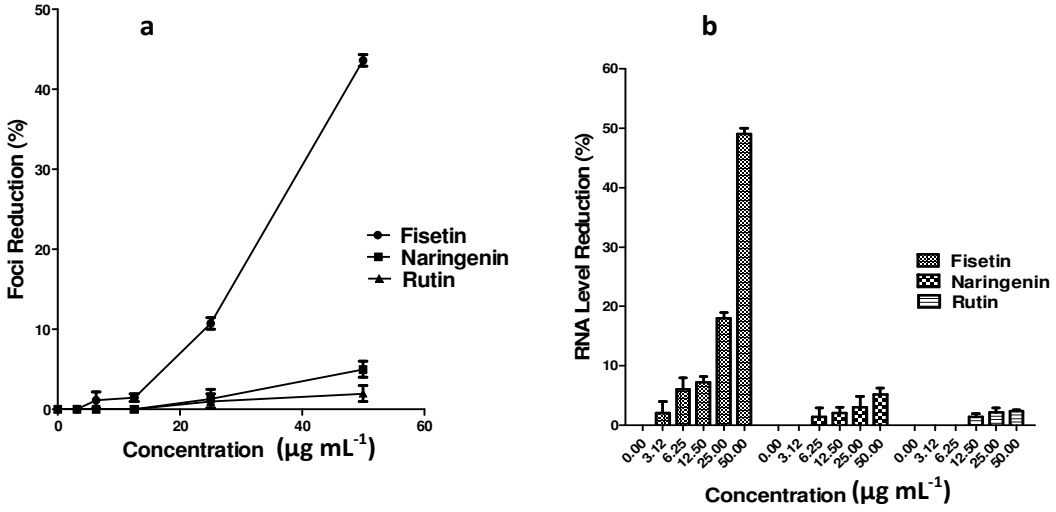

Figure 2. Assay of flavonoids against DENV-2 intracellular replication. Foci forming unit reduction assay (FFURA) was used to evaluate the *in vitro* anti-dengue virus activities of the tested flavonoids after viral adsorption (a) and the respective DENV2 RNA copies were quantified using QRT-PCR (b). All experiments were conducted in triplicates. The percentages of foci reduction (%RF) were obtained by comparing against the controls maintained in parallel. Data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.).

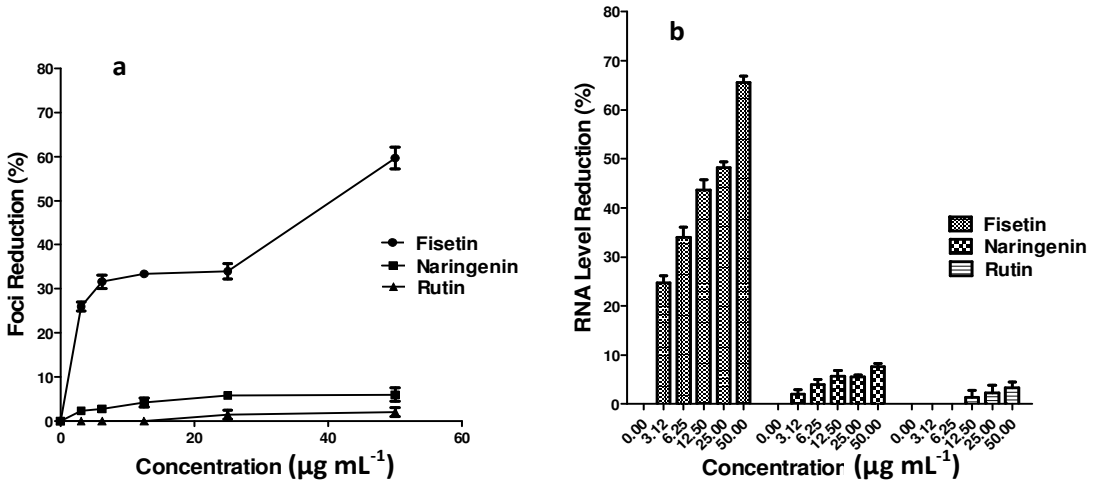

Figure 3. Evaluation of flavonoids continous flavoniods treatment against DENV-2 replication. Foci forming unit reduction assay (FFURA) was used to evaluate the *in vitro* anti-dengue virus activities of the tested flavonoids 5 h before infection to 4 days post infection (a) and the respective DENV2 RNA copies were quantified using QRT-PCR (b). All experiments were conducted in triplicates. The percentages of foci reduction (%RF) were obtained by comparing against the controls maintained in parallel. Data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.).

Fisetin showed a dose-dependent inhibition of DENV-2 replication in the FFURA with a half maximal inhibition concentration (IC_{50}) value of 55 µg/mL when added subsequent to infection of the Vero cells (Figure 2a). Comparable IC_{50} inhibition value (IC_{50} = 50 µg/mL) was obtained for inhibition of 49% ± 1 DENV-2 genome copy number relative to the untreated Vero cell cultures, determined by qRT-PCR (Figure 2b). There was no significant direct virucidal activity of fisetin against DENV-2 (Figure 4). Fisetin when continuously present exhibited anti-dengue activity with IC_{50} = 43.12 µg/mL (Figure 3a). This finding was supported by the qRT-PCR results which showed that the DENV-2 RNA level was decreased by more than 65% ± 1.3 when Vero cells were similarly treated with 50 µg/mL fisetin (Figure 3b). Naringenin and rutin when added to cells subsequent to

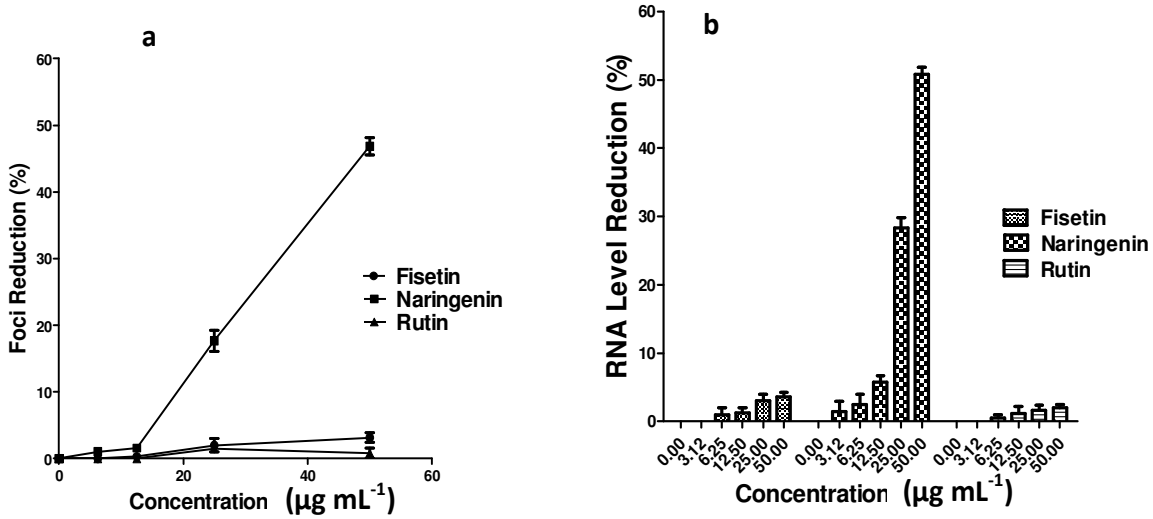

Figure 4. Direct virucidal activity of flavonoids against DENV-2. Foci forming unit reduction assay (FFURA) was used to evaluate the direct anti-dengue virus activities of the tested flavonoids by treatment of viral suspension with flavonoids prior to infection (a) and the respective DENV2 RNA copies were quantified using QRT-PCR (b). All experiments were conducted in triplicates. The percentages of foci reduction (%RF) were obtained by comparing against the controls maintained in parallel. Data were plotted using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.).

DENV-2 infection did not exhibit any anti-DENV-2 activities (Figure 2). No antiviral activity was also recorded when naringenin and rutin was added to cells continuously from 5 h before virus infection up to 4 days post infection (Figure 3). These findings were corroborated by results obtained from qRT-PCR which indicated no significant reduction in the amount of total DENV-2 genomic RNA levels (Figures 2b and 3b).

Naringenin, however, exhibited direct virucidal activity against DENV-2 with IC_{50} = 52.64 µg/mL when added directly to the virus suspension (Figure 4a). Naringenin at 50 µg/mL decreased the DENV-2 RNA level by 50% ± 1.3 when compared to the non treated virus inoculum (Figure 4b). In an earlier study, it was described that the flavonoids, glabranine and 7-O-Methyl-Glabranine exhibited significant anti-dengue activity in cell culture (Sanchez et al., 2000). It was also described in an *in vitro* study that pinostrobin could inhibit DENV-2 NS2B/NS3 protease (Kiat et al., 2006).

Results from the present study suggest that fisetin ($C_{15}H_{10}O_6$), a flavonol commonly found in *A. greggii*, *A. berlandieri*, *Gleditschia triacanthos*, *Quebracho colorado* and also in mangoes, parrot tree and Euroasian smoketree, possesses anti-DENV activities. The anti-DENV activity is notable when cells were treated continuously for 5 h before infection until 4 days post-infection as well as when added subsequent to virus infection. Naringenin ($C_{15}H_{12}O_5$), a plant flavonone found in grapefruit, orange and tomato skin, on the other hand, showed no inhibition of DENV-2 replication. Naringenin however, exerted direct virucidal effects on DENV even though it is highly cytotoxic with selectivity index (SI) of <1. Rutin ($C_{27}H_{30}O_{16}$), one of the flavonoid glycosides found in buckwheat, asparagus, citrus fruits and some berries showed no inhibition of virus adsorption and DENV-2 replication as well as no prophylactic effect against virus infection. The mechanism of how fisetin affects DENV virus replication is unclear.

Fisetin is not likely to act directly on the virus, as there was no inhibition of the virus when fisetin was added directly the virus inoculum. These results also suggest that fisetin does not affect DENV-2 binding to cells. Results from the qRT-PCR assay suggest that fisetin could affect DENV genome copy number. One possibility is that fisetin could interfere with DENV-2 replication by binding directly to virus RNA, forming a flavonoid-RNA complex (Nafisi et al., 2009) or affecting the RNA polymerases (Ono et al., 1990) resulting in inhibition of virus replication. Prophylactic treatment of cells with fisetin could result in accumulation of intracellular fisetin, which in-turn increases inhibition of dengue virus replication. Further study to improve the efficacy of the anti-DENV properties of fisetin could be undertaken utilizing fisetin as a base molecule for further modifications.

ACKNOWLEDGEMENTS

The authors thank Ministry of Science, Technology, and Innovation of Malaysia (Malaysia Genome Institute initiative grant 07-05-MGI-GMB015) Ministry of Higher

Education for Long Range Grant Scheme (LRGS) cycle 1/2011 and the University of Malaya for financial support.

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