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Phytochemical Screening and Anti-dengue Activity of *Jatropha multifida* Extract against DENV-2

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ABSTRACT

Cases of dengue infection are still high in various parts of the world and no antiviral has been found to treat dengue infection. *Jatropha multifida* is one of the herbs used by the community to treat dengue infection, but pre-clinical and clinical scientific evidence has not been carried out. Phytochemical screening of ethanol extract of *Jatropha multifida* leaves was carried out using thin layer chromatography. Cytotoxic assay was performed on Vero cells using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide). The cytotoxic concentration 50 (CC50) was determined using probit analysis. Anti-dengue test on dengue virus serotype 2 (DENV-2) was performed on Vero cells and the RNA (ribonucleic acid) copy number was quantified using quantitative polymerase chain reactions ((qPCR). Phytochemical screening results showed that the ethanol extract of *Jatropha multifida* leaves contains flavonoid and terpenoid compounds. CC50 of the extract was found to be 651.8 µg/mL. The RNA copy number of the treated group was lower than the control group and the difference was significant. The ethanol extract of *Jatropha multifida* leaves has anti-dengue activity against DENV-2.
Keywords: *Jatropha multifida*; dengue; phytochemical screening; DENV-2

INTRODUCTION

Dengue infection is still a burden disease worldwide. WHO reports that dengue cases in the world in 2019 were 4.2 million, an increase of almost two times compared to 2010 which was 2.4 million. Of the total number of cases, about 70% of cases were contributed from Asian region(WHO, 2020). Dengue infection is caused by dengue virus (DENV), a single-stranded RNA virus from the family Flaviviridae and consists of 4 serotypes, namely DENV-1, DENV-2, DENV-3, and DENV-4. Dengue infection caused by DENV-2 causes more severe clinical manifestations than other serotypes (Vaughn *et al*., 2000). Until now, there is no effective antiviral to treat dengue virus infection. Several antiviral candidates intended as anti-dengue have entered clinical trials such as balapiravir and celgosivir, but have not shown satisfactory results(Low *et al*., 2017). Herbal products can be an alternative that can be developed as a therapeutic agent to treat this dengue virus infection. One of the herbal ingredients that Indonesians have commonly used to treat dengue hemorrhagic fever is *Jatropha multifida* L leaves or 'yodium' leaves by drinking boiled water from the leaves.

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The effect of *Jatropha multifida* L that has been studied is that it has anti-inflammatory activity (Anani *et al*., 2016), antifungal (Hamza *et al*., 2006) and has an effect on wound healing (Juniarti *et al*., 2013). *Jatropha multifida* extract can increase the number of platelets in experimental animal models of dengue hemorrhagic fever (Sundaryono *et al*., 2019). *Jatropha multifida* L contains alkaloids, flavonoids, steroids, tannins, phenols, terpenes (monoterpenes, sesquiterpenes, diterpenoids, triterpenes), coumarins, and lignans (Rampadarath *et al*., 2014; Sabandar *et al*., 2013). Various flavonoid compounds have been proven *in vitro*, *in vivo,* and *in silico* to reduce virus load by acting on various proteins in DENV. Luteolin can inhibit the growth of DENV and can reduce the virus load as much as ten times in mice infected with the dengue virus (Boniface & Ferreira, 2019). One class of diterpenoids isolated from *Trigonostemon cherrieri* can inhibit NS5 from DENV with IC50 ranging from 3.1-16 µM (Lim *et al*., 2021). This study aims to identify the effectiveness of *Jatropha multifida* leaf ethanol extract as an anti-dengue agent *in vitro*.

METHODOLOGY

Materials. TLC silica gel 60 F254 (Merck, Germany), benzene:ethyl acetate, chloroform: methanol, Dragendorff, sitroborate,

cerium sulphate, DMEM, M-199, FBS, Penicillin-streptomycin (Capricorn scientific, Germany), mycoXpert (Capricorn Scientific, Germany), tripsin, DMSO, MTT, SDS, PBS, triptan blue, primer, Viral nucleic acid extraction kit (Favorgen, Taiwan), Sensifast SYBR Lo-Rox *One Step Kit* qRT-PCR (Bioline, UK), oven, UV 254 nm, UV 366 nm, LAFCO_2 incubator, inverted microscope (Zeiss, Germany), 96 well plate, 24 well plate, Elisa reader(Bio-rad, USA), freezer -80^o C, nanodrop, qRT-PCR (Applied Biosystem, US).

Methods

Extract preparation

The leaves of *Jatropha multifida* L were obtained from Sleman, DIY (July, 2021). The leaves were dried in an oven at 50-70 ^{o}C. The dried leaves were ground using a grinder. Fifty hundred gram powder of dried leaves were extracted by maceration using 1 liter of absolute ethanol for 24 hours. Then the macerate was filtered to obtain the supernatant and sediment. The supernatant was evaporated while the sediment was re-macerated with the solvent and the same method until three times maceration. All supernatants were combined and evaporated until a thick mass occurred or called extract.

Phytochemical screening

The phytochemical screening of the ethanolic extract of *Jatropha multifida* was tested using thin-layer chromatography. The ethanol extract of *Jatropha multifida* leaves was dissolved in chloroform: methanol (1:1, v/v). The solution was placed on a silica gel plate, then dried. After drying, the plate was put into a chamber that had been given a mobile phase, benzene: ethyl acetate (1:1, v/v) solution. Detection was carried out under UV light of 254 nm, 366 nm and specific reagents (Dragendorff, cerium sulfate and citroborate). Qualitative analysis was determined by the color changes of the spots and the Rf value.

$$\text{Rf value: } \frac{\text{Distance traveled by substance}}{\text{Distance traveled by the solvent}}$$

Virus and cell preparation

The anti-dengue test was carried out in vitro using cell clone *Aedes albopictus* C6/36 and Vero cells (African green monkey kidney cell line). C6/36 cells were cultured in DMEM media supplemented with 10% FBS at 28 ^{o}C while Vero cells were cultured in M-199 supplemented with 10% FBS, 1% HEPES, 1% sodium bicarbonate at 37 ^{o}C. Dengue virus DENV-2 strain was propagated on C6/36 cells in DMEM media supplemented with 3% FBS and incubated at 28°C for seven days until C6/36 cells showed a cytopathic effect. The supernatant was harvested and made into virus stock which was stored at -80 ^{o}C.

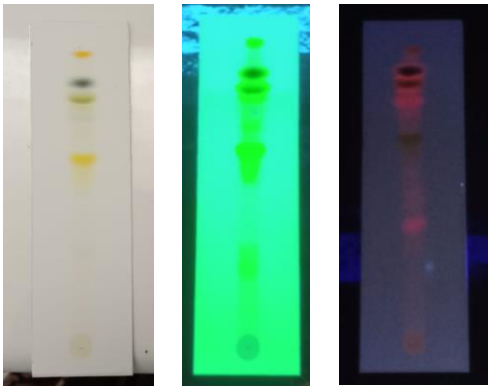
Cytotoxic assay

The ethanol extract of *Jatropha multifida* L was tested for its cytotoxic effect on Vero cells using MTT assay. Vero cells were grown in 96-well plates 1 x 10^4 cells/ml. After being incubated overnight, the ethanol extract of *Jatropha multifida* L was added in Vero cells with serial concentrations so that the final concentration of the extract was 2000 µg/mL; 1000 µg/mL; 500 µg/mL; 250 µg/mL; 125 µg/mL; 62.5 µg/mL, 31.25 µg/mL; 15.625 µg/mL; 7.813 µg/mL and 3.906 µg/mL. DMSO with the same concentration as the highest concentration in the treatment group was given to Vero cells to know whether DMSO as extract solvent was toxic to cells. Vero cell without any treatment was set as a negative control. Vero cells that had been treated were incubated for 24 hours at 37°C, 5% CO_2. MTT solution was added to the treated cells and incubated for 4 hours. The MTT reaction with the cells was stopped with a stopper solution of 10% SDS. After incubation overnight, the absorbance was read at 595 nm. CC50 was determined using Probit analysis. CC50 is the concentration of extract that produces a cytotoxic effect in 50% of cells compared to the negative control. The highest concentration of extract that did not produce a cytotoxic effect (MNCC) on Vero cells was used for the anti-dengue assay.

Anti-dengue assay

The anti-dengue assay was carried out using the viral growth inhibition assay method with slight modifications (Tohma *et al*., 2019). As much as 1x10^5/well, Vero cells were infected with DENV2 virus with a volume of 200 µL of stock solution and incubated for 1 hour. Furthermore, media containing ethanol extract of *Jatropha multifida* with MNCC concentration was added and incubated for 24 hours and 48 hours at 37 ^{o}C. After incubation, the supernatant was harvested and the RNA copy was quantified using RT-PCR. In detail, the harvested supernatant was extracted for DENV virus RNA using the Viral nucleic acid extraction kit according to the protocol in the kit. After getting the viral RNA, the purity of the RNA was calculated using Nanodrop with a purity value of 1.6-2.0. Then the viral RNA will be quantified using a one-step RT PCR master mix. The primary sequences used are as follows (Ellan *et al*., 2019; Gurukumar *et al*., 2009).

Statistical analysis was carried out using SPSS Version 26 software. CC50 was determined



(a) (b) (c)

Figure 1. Overview of spots of ethanol extract of *Jatropha multifida* leaves after immersion in the mobile phase, a. seen in the bright light; b. viewed under UV light at 254 nm; c. viewed under UV light at 366 nm.

		Sekuence
DENV	Forward primer	5'-GARAGACCAGAGATCCTGCTGTCT-3'
	Reverse primer	5'-ACCATTCCATTTTCTGGCGTT-3'
GAPDH	Forward primer	5′ GTG GAC CTG ACC TGC CGT CT 3′
	Reverse primer	5′ GGA GGA GTG GGT GTC GCT GT 3′

RNA copy was compared between DENV-2 RNA in the treatment group compared to the control group.

Statistical analysis

Table I. Rf values of each spot produced from the ethanol extract of *Jatropha multifida* leaves after being immersed in the mobile phase

No	**Light source**	**Rf value**
1	Bright light	0,63; 0,83; 0,89; 0,98
2	UV 254 nm	0,70; 0,76; 0,87; 0,91; 0,98
3	UV 366 nm	0,43; 0,68; 0,80; 0,84; 0,88; 0,92; 0,99

Table II. Changes in spot color from spraying plates with specific reagents

No	**Specific reagent**	**Color changes**
1	Dragendorff	-
2	Citroborate	Yellow, greenish yellow
3	Serium sulfate	Dark brown

using probit analysis. Comparison of copy RNA numbers between treatment control groups was tested using ANOVA with post hoc analysis. Significance was set at p-value < 0.05.

RESULTS AND DISCUSSION

Phytochemical screening

The results of the spots that were seen after the ethanol extract of *Jatropha multifida* leaves were dipped in the mobile phase are shown in Figure 1. The spots that were visible in room light were 4 spots. While the spots visible under UV light at 254 nm were 5 spots. There were 7 spots visible under UV light at 366 nm. The Rf values of each spot are presented in Table I.

After being immersed in the mobile phase, the plate is sprayed with specific reagents to detect the class of compounds. The results of spot color interpretation are presented in Table II.

Cytotoxic assay

Cytotoxic assay was carried out by the MTT assay method. The percentage of Vero cell viability after being given ethanol extract of *J. multifida* leaves with various serial concentrations is presented in Figure 3.

Vero cells that were given an extract concentration of 500 µg/mL showed 100% cell viability, which means that all Vero cells did not show death when given concentration of

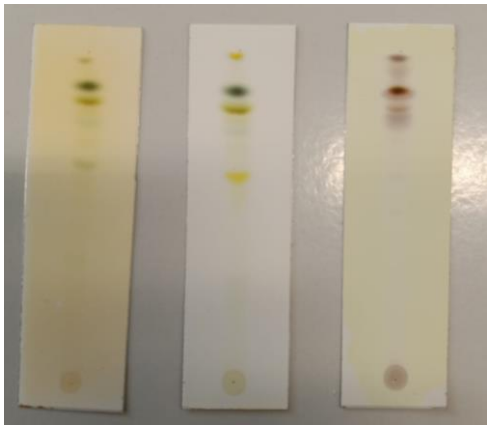


Figure 2. The image of the spots after being sprayed with specific reagents. a. Dragendorff; b. Citroborate; c. Serium sulfate. Description: An arrow indicates a color changes

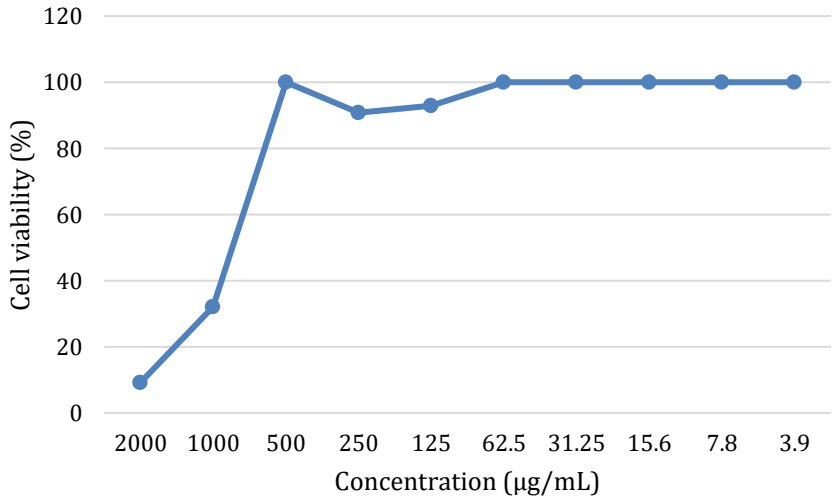


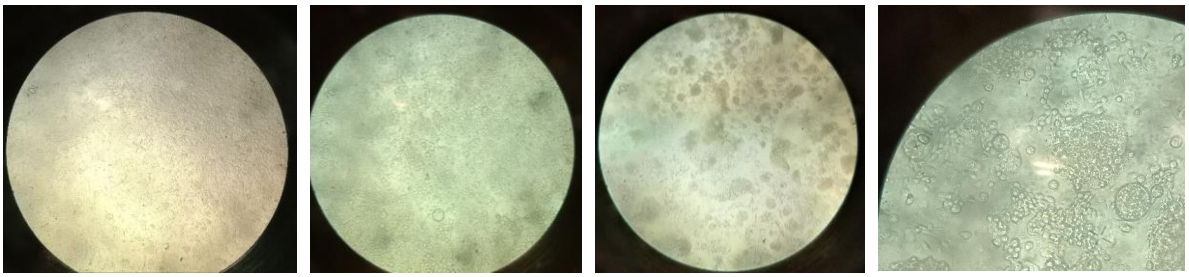
Figure 3. Percentage of cell viability of Vero after administration of ethanol extract of *J. multifida* leaves with various concentrations for 24 hours using the MTT assay method.

500 µg/mL. The cell viability results were then further analyzed using probit analysis to determine CC50, the concentration of extract required to cause cell death of 50%. The cytotoxic concentration 50 (CC50) of the ethanolic extract of the leaves of *J. multifida* was 651.8 µg/mL. So the concentration used to test the anti-dengue activity is 500 µg/mL.

Propagation of DENV-2 on C6/36

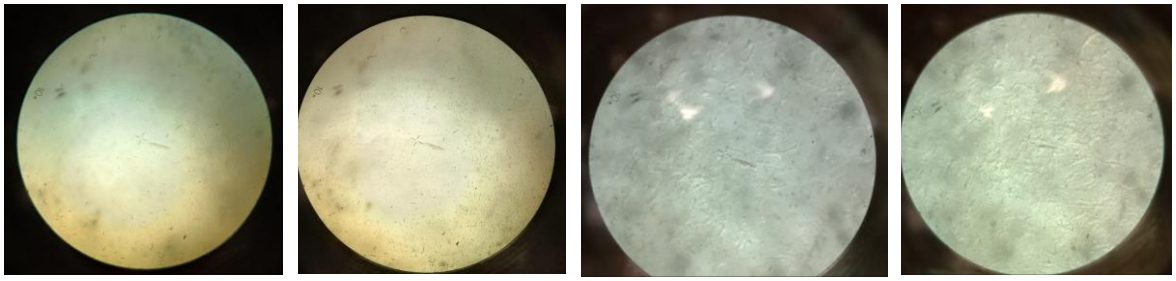
C6/36 cells were cultured in DMEM medium at 28-29 ^{o}C to produce optimal cell growth. After C6/36 confluent, DENV-2 was infected to C6/36 cells to produce viral stocks. Morphological changes of C6/36 cells before and after DENV-2 infection is depicted in Figure 4.

Normal C6/36 cells and not infected with DENV-2 were spherical with uniform size. On the first post-infection day, C6/36 cells did not change their morphology. On the third day after infection, C6/36 cells began to show cytopathic characteristics, the cells began to turn into giant cells, larger than normal cells, and prominent vacuoles. Changes in the morphology of C6/36 cells will be more clearly seen at seven days post-infection, with more cells that change shape into giant cells, these cells break and merge with surrounding cells. On the seventh day, the supernatant was harvested and used as a virus stock.



(a) (b) (c) (d)

Figure 4. Morphological changes of C3/36 cells before and after DENV-2 infection. a. Prior to infection with DENV-2; b. 3 days post-DENV-2 infection; c. 7 days post-infection DENV-2 100x magnification; d. 7 days post-infection DENV-2 400x magnification.



(a) (b) (c) (d)

Figure 5. Morphological changes of Vero cells before and after DENV-2 infection. a. Prior to infection with DENV-2; b. 24 hours post-DENV-2 infection 100x magnification; c. 24 hours post-infection DENV-2 400x magnification; d. 48 hours post-infection DENV-2 400x magnification.

Anti-dengue assay

Vero cells were infected with DENV-2 for 1 hour and treated with *Jatropha multifida* extract for 24 hours and 48 hours. Morphological changes of DENV-2-infected vero cells at 24 and 48 hours are presented in Figure 5. The morphology depicted is not too different between DENV-2-infected Vero cells at 24 and 48 hours. Infected vero cells will shrink and vacuoles are visible more prominent.

Comparison of DENV RNA copy when given *Jatropha multifida* extract is depicted in Figure 6. There was a significant decrease in DENV RNA copy given *Jatropha multifida* extract after 24 hours of administration and after 48 hours of administration compared to controls. However, there was no significant difference between the duration of *J. multifida* administration and the decrease in RNA copy number.

Discussion

This study aims to examine the anti-dengue effect of *Jatropha multifida* extract and explore the content of groups of compounds in the ethanol extract of *Jatropha multifida* leaves. The *Jatropha multifida* TLC plate after being sprayed with citroborate reagent showed a color change to greenish-yellow at an Rf of around 0.63 and 0.83. It shows that *Jatropha multifida* contains a group of flavonoid compounds. It is in accordance with previous studies which showed that the flavonoid compounds found in *Jatropha multifida* include the flavones apigenin, 4'-OMe apigenin, acacetin and luteolin (Thomas & Sallykutty Thomas, 2016). Besides flavonoids, *J. multifida* also contains terpenoid compounds as evidenced by the change in color of the TLC plate to purple after spraying with cerium sulfate reagent. The terpenoid compounds are located at Rf around 0.88 and 0.92. The terpenoids that have been found in *Jatropha multifida* include jatrogrossidentadione, multidione, multifidone, multifolone, multifidanol and multifidenol; dinorditerpenoids; jatromulone A; jatrointelone A; jatrophodione A and podocarpane diterpenoids (Sabandar *et al*., 2013; Zhu *et al*., 2017). On spraying with Dragendorff's solution, no changes were found on the TLC plate. It means that the ethanolic extract of *Jatropha multifida* used in this study does not contain alkaloids. Another thing that can be seen in the results of this TLC examination is the presence of spots at Rf 0.43 which are only visible on observations using 366 nm UV light. The spots were probably caused by the presence of

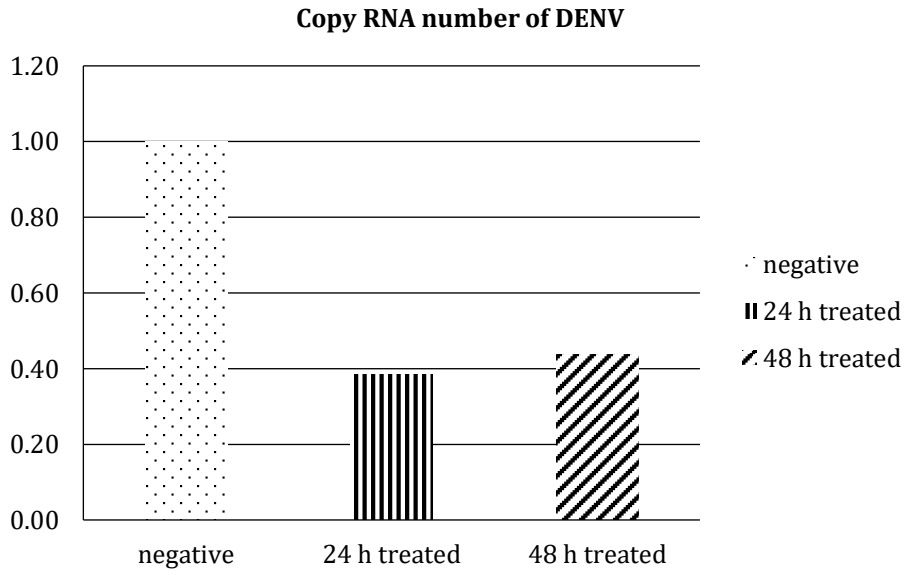


Figure 6. Copy RNA number of DENV 2 on negative group and treated group

compounds with long conjugate bonds, because they were only detected at 366 nm UV light and not visible at 254 nm UV light.

Cytotoxic test on normal cells such as Vero cells is intended to see the extract's safety. The results obtained were CC50 of 651.8 µg/mL, which means that the ethanol extract of *Jatropha multifida* leaves is not toxic to normal cells. The cut-off concentration considered safe for a healthy cell is above 50 µg/mL (Phuwajaroanpong *et al*., 2020). It is in line with previous research that the ethanol extract of *Jatropha multifida* showed a non-toxic effect on normal cells compared to extracts obtained with other solvents (Werdyani *et al*., 2020).

Dengue virus infection in cell lines will cause changes in cell morphology due to cytopathic effects. In C6/36 cells infected with DENV-2, there was a change in the shape of the cells from the initial round, small and uniform to enlarged, visible vacuoles were more prominent and finally ruptured. C6/36 cells will show cytopathic effects such as prominent vacuoles and syncytia formation after being infected with DENV-2 virus for 5-7 days (Rivera *et al*., 2018). In contrast to C6/36 cells, the cytopathic effect that appears is that the cells become irregular, elongated, and have prominent vacuoles. There is also a blebbing phenomenon due to cells undergoing apoptosis (Zargar *et al*., 2011).

Anti-dengue test was carried out by examining the effect of *J. multifida* extract on the RNA copy number of the dengue virus. After administration of *J. multifida* extract for 24 hours and 48 hours, the treated group had lower RNA copy compared to the untreated group and the difference was significant. It indicates that the extract of *Jatropha multifida* has anti-dengue activity by affecting the life cycle of DENV in the late stages (Peng *et al*., 2017). The compounds thought to be responsible for this anti-dengue activity are compounds from the flavonoid and terpenoid groups. Some of these group compounds extracted from several plants have anti-dengue activity. Diterpenes compounds named trigocherrins A, trigocherriolide A and B isolated from *Andrographis paniculata* can reduce virus load in cell line C6/36 infected with DENV-2 and in silico andrographolide has an interaction with NS5 protein (Kaushik *et al*., 2021). One of the triterpene compounds that show inhibitory activity against dengue virus is celastrol. Celastrol is a compound isolated from *Tripterygium wilfordii* that can inhibit DENV-2 replication through NS5 protein with an EC50 of 0.12 µM (Lim *et al*., 2021). Luteolin as one of the flavonoid compounds can inhibit the growth of DENV in vitro with EC50 ranging from 4.36 µM-8.38 µM and can reduce virus load by ten times in mice infected with dengue virus (Boniface & Ferreira, 2019).

CONCLUSION

The ethanol extract of *Jatropha multifida* leaves contains secondary metabolite flavonoid and terpenoid. *Jatropha multifida* extract possesses no toxicity against Vero cells and has significant

anti-dengue activity against DENV-2 compared to control group. In future research, it is necessary to explore the mechanism of action of *Jatropha multifida* which plays a role in anti-dengue activity.

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