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Original Article

Bidara Upas (*Merremia mammosa* Hall) Antiviral Activity Against SARS-COV-2

Neny Purwitasari[1,2], Mangestuti Agil[2,*], Siswandono Siswodihardjo[2], Reviany Vibrianita Nidom[3]

[1]*Doctorate Program, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia*
[2]*Department of Pharmaceutical Sciences, Faculty of Pharmacy, Universitas Airlangga, Surabaya, Indonesia*
[3]*Professor Nidom Foundation, Surabaya, Indonesia*

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ABSTRACT

The world was startled in December 2019 by the emergence of an unprecedented pneumonia outbreak in Wuhan, China, with an unknown cause. However, on January 7, 2020, researchers successfully identified the responsible agent as a novel coronavirus. Meanwhile, the medicinal properties of bidara upas (*Merremia mammosa* Hall), a plant belonging to the Convolvulaceae tribe and commonly found in Indonesia and Malaysia, garnered attention due to its long history of use in traditional medicine for treating respiratory ailments. As a result, a study was conducted to investigate the antiviral potential of the ethanol extract of bidara upas against SARS-CoV-2. The research incorporated various methods such as cytotoxicity tests on the ethanol extract of bidara upas tuber, inhibition tests, RT-PCR, and plaque assays. Through toxicity tests, it was determined that a concentration of 500 ppm exhibited no harmful effects on cells. In the virus inhibition test using plaque assay, concentrations of 62.5 ppm, 125 ppm, 250 ppm, and 500 ppm demonstrated inhibition powers of 59.54%, 74.7%, 74.68%, and 79.27%, respectively. RT-PCR results varied across different concentrations, using the E and RdRp genes as references. CT values were increasing as compared to the negative control, indicating a decrease in viral load due to the sample treatment. Based on the findings of the virus inhibition test, RT-PCR, and plaque assay, it can be concluded that the ethanol extract of bidara upas tuber has potential as an antiviral agent against SARS-CoV-2.

* Corresponding author: Mangestuti Agil
✉ E-mail: mangestuti@ff.unair.ac.id
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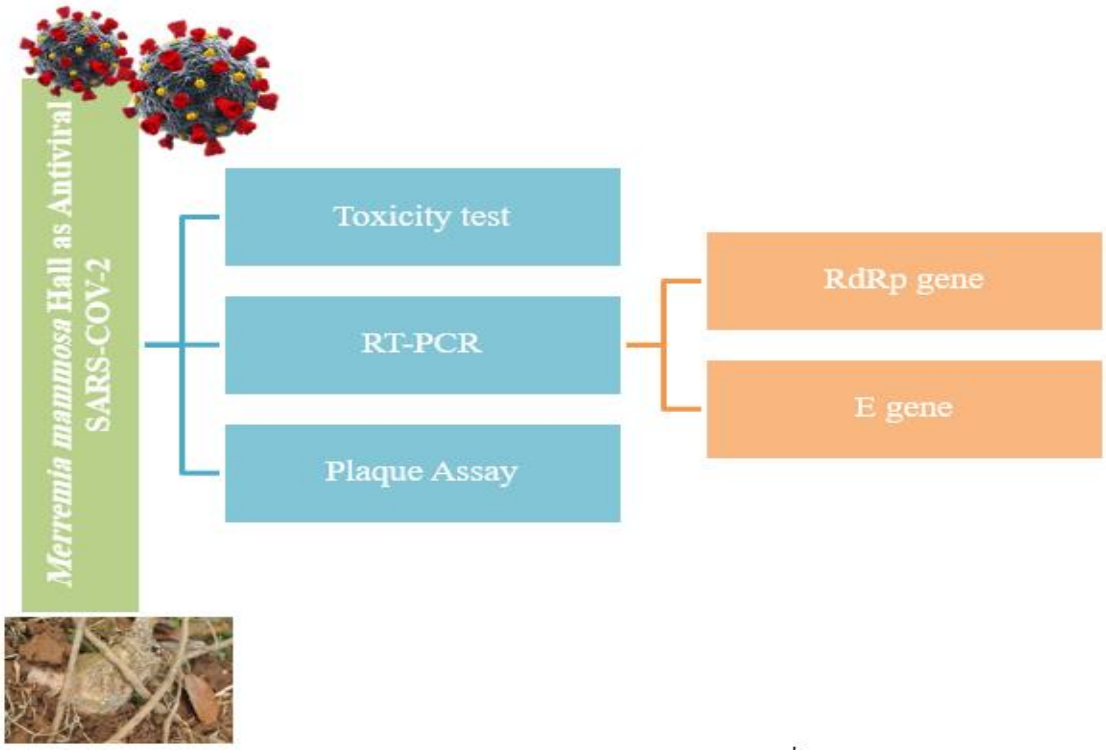
GRAPHICAL ABSTRACT



Introduction

The news of the emergence of a pneumonia outbreak with no known precise cause shocked the world at the end of 2019. This outbreak was first discovered in the city of Wuhan, Hubei Province, China. Most of these pneumonia patients come from traders at the Huanan market, which sells live animals in the city of Wuhan. On January 7, 2020, researchers succeeded in identifying the cause of this pneumonia, namely a type of novel coronavirus. Officially, WHO named this disease Corona Virus Disease 2019 (COVID-19), and the name of the virus was Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). Positive for COVID-19 after the test results came out of the laboratory in the city of Wuhan. Some of the sufferers have congenital diseases such as cardiovascular disease, diabetes mellitus, and hypertension. The spread of this virus is increasing and has spread to almost all countries in the world, so much so that on March 11, 2020, the World Health Organization (WHO) declared COVID-19 a pandemic [1-4]. Until May 7, 2023, there were more than 200 million cases spread throughout the world. In Indonesia, more than 6 million people are infected, and more than 150 thousand people are infected.

SARS CoV-2 is highly pathogenic in humans and can spread rapidly through both symptomatic and asymptomatic means [5]. Furthermore, high rates of viral infection are a worldwide issue, and the effectiveness of antiviral drugs and vaccines is still being tested in clinical trials [6]. Herbal plants are being used in the development of new antiviral therapies as a curative and preventative option for the spread of COVID-19 [7].

Conventional propagation of bidara upas (*Merremia mammosa* Hall) plants uses tubers. According to Cahyaningsih *et al.* [8], propagation with tubers has a rather long dormancy period of around 4-5 months before buds emerge. In addition, if the tubers are injured, the possibility of growing shoots is very small.

When using stem cuttings, the success rate is around 50%, many of which affect the cuttings, including the fact that there should be no shaking so that the plant shifts to the sand media, which ultimately reduces growth. Due to the low ability to grow cuttings, it is rare for plant propagation. When using the micro cutting technique, there are constraints on limited seeds and difficulties in obtaining them because they are included in a rare type of medicinal plant.

According to the National Development Planning Agency [9], the bidara upas is included in the rare criteria based on the Indonesia Biodiversity Strategy and Action Plan. The demand for simplicia is high as an herb sold as herbal medicine for the traditional medicine industry amidst the difficulty in obtaining this plant in society and nature. The bidara upas belongs to the Convolvulaceae tribe. This plant contains various secondary metabolite compounds that can be used as medicine. In Indonesia and Malaysia, this plant is used to treat various respiratory and digestive disorders, wounds caused by snake bites or burns, and diabetes [10, 11]. In addition, the active compounds in the bidara upas extract can actually overcome typhoid fever caused by the bacterium *Salmonella typhimurium*, that upas bidara also has antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* [12, 13]. According to Sayyidah *et al.* [14], based on the results of a phytochemical study, bidara upas leaf extract contains flavonoids, quinones, phenolic compounds, terpenoids, and steroids. Tran *et al.* [15], most plants containing bioactive compounds such as glycosides, alkaloids, terpenoids, flavonoids, and carotenoids have antidiabetic activity. Kurniasih [16] reported that bidara upas contains flavonoids that can inhibit the inflammatory process, so it can be used as an anti-inflammatory drug. There have not been many studies of the lotus root as an antiviral against SARS CoV2. Hence, a study of the bidara upas tuber as an antiviral against SARS CoV2 is needed.

Materials and Methods

Material

Bidara upas was identified in UPT Materia Medica, Batu, Indonesia, in October 2022 with Letter of Determination no. 074/674/102.20-A/2022 and specimen code 1a-2a-3b. Vero E6 cell line and penicillin-streptomycin-ampotericin B mix were purchased from PAN Biotech GmbH (Aidenbach, Germany), Fetal Bovine Serum was purchased from RMBio (Missouri, USA), and Dulbecco's Modified Eagle's Medium was purchased from Sigma-Aldrich (Missouri, USA).

Extraction

As much as 5 kg of Bidara upas tubers were chopped and dried, and then powdered and extracted by maceration using 96% ethanol solvent for 3 × 24 hours. After that, the extract was filtered to separate the filtrate and residue. The extraction process was repeated three times. Next, the filtrate obtained from the extraction results was concentrated with a rotary evaporator until a thick 96% ethanol extract was obtained, and then heated in an oven to obtain a constant weight.

Viruses and cell culture

This study was conducted at the Molecular Laboratory, Professor Nidom Foundation (LM-PNF), Surabaya, Indonesia, using the BSL-3 facility to conduct the experiment. Sars-CoV-2 used in this experiment was strain JI-PNF-211352 (Wuhan-like), originally from Indonesia. The virus was propagated in the Vero E6 cell line.

Cytotoxicity test

Patients' or animals' SARS-CoV-2 isolates should be frequently propagated to generate high-titer viral stocks. The African Green Monkey cell line and its derivatives were the most susceptible to SARS-CoV-2 infection when compared to other cell types. The next cytotoxicity test followed the Case method [17].

The MTT assay was done using the MTT cell proliferation assay kit (Abnova #KA1334, Taiwan). Several concentrations (500, 1000, 2000, and 4000 ppm) were prepared, and then each sample was run in triplicate and measured according to the procedure in the kit. The result

of the test was the average percentage of live cells.

RT-PCR

To detect SARS-CoV-2, a real-time PCR assay was developed. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) with TaqMan probes for the detection of viral RNA is a highly sensitive and specific method for quantifying viral load in different specimens. Since CoV uses a subgenomic RNA template for translation, the amount of viral RNA available for each gene varies according to its location in the genome. Compared to genes at the 5′ end of the sense (+) genome, those at the 3′ end will produce more transcripts. Primers and probes should take into account the fact that the "gene N" transcript will be more abundant than the genomic RNA copy when measuring RNA abundance. Some primer/probe combinations are already in use for clinical diagnosis [18, 19]. Therefore, a lot of work has already gone into the design and validation of these sets. Sensitivity is more important than absolute accuracy when measuring viral RNA in a clinical setting. Quantitative tests, however, are preferred for research purposes and may prove useful in longitudinal studies of infected human subjects. By creating RNA standard curves, RT-qPCR cycle threshold values (Ct) can be translated into transcript or genomic copy number equivalents.

The application of RT-qPCR with QuantStudio 5 Applied Biosystem PCR machine (Applied Biosystems, USA). A total of 20 µL of the reaction mixture was prepared by adding 4 µL of each primer and probe mixture, 11 µL of the One-Step RT-PCR Premix included, and 5 µL of the template RNA. The detection of positive criteria was validated when two genes (E and RdRp) were found to be less than 36. If the results were only displayed for the E gene, they would be inconclusive. Conversely, any negative criteria would be confirmed if neither E nor RdRp were detected.

Plaque assay

The most commonly used method for measuring infectious viruses in a sample is the plaque test. The plaque assay measures "plaque," which is the zone of cellular death that occurs after an infectious unit enters a cell and spreads to adjacent cells over an incubation period. This test does not require the use of the virus or reagents, making it useful when reagents are unavailable. Typically performed in 6-well plates, these cell-based assays are relatively inexpensive, labour-intensive, and unreliable if the sample is cytotoxic (e.g., homogeneous from a given tissue) or if the virus is bad cytotoxic to specific cell types. Therefore, it is crucial to choose highly permissive cell types (such as the Vero E6 cell line) in which SARS-CoV-2 induces substantial cell death. The subsequent method is based on Case *et al.* [17].

Table 1: The results of the cytotoxic test of the ethanol extract of the Bidara Upas (*Merremia mammosa* Hall)

No.	Concentration (ppm)	Absorbance (OD)	Average % live cells ± SD
1	4000	0.101	-8.78±0.6235
		0.101	
		0.103	
2	2000	0.104	-7.53±0.5350
		0.105	
		0.103	
3	1000	0.110	-1.61±2.4637
		0.116	
		0.119	
4	500	0.187	41.40±4.5939
		0.194	
		0.204	

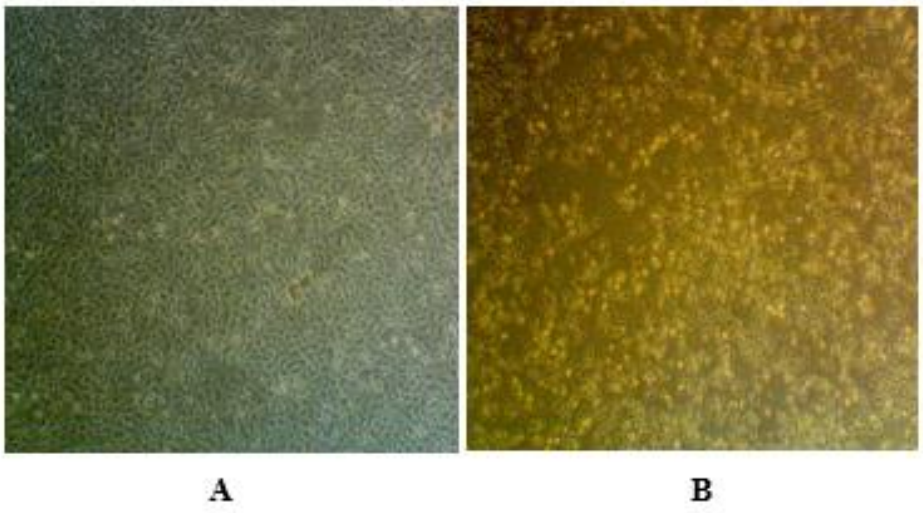


Figure 1: Comparison of normal vero E6 cells (A) and vero E6 cells with cytopathic effect of SARS-CoV2 (B). The morphological changes of the cell indicate the viral infection on cells

Figure 2: Plaque formed due to the SARS-CoV2 presence on the plate

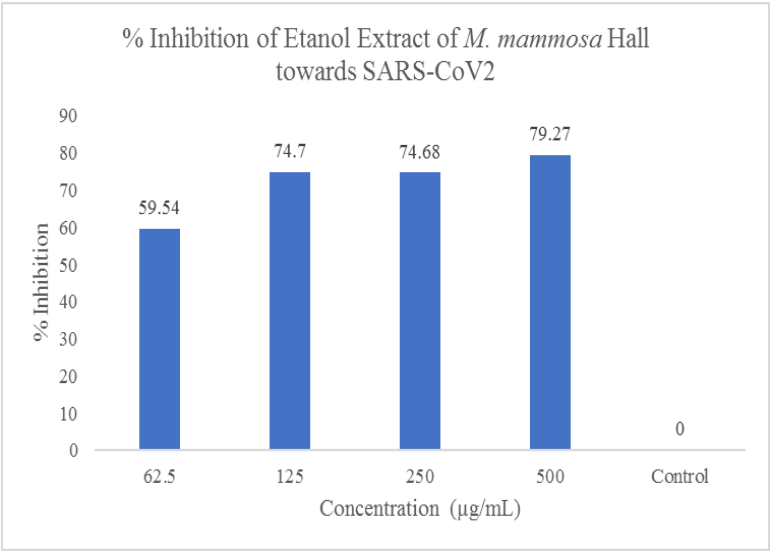


Figure 3: Inhibition of viruses from the ethanol extract of Bidara Upas (*Merremia mammosa* Hall) tuber

Results and Discussion

The results of research conducted using toxicity tests on cells found that the concentration of the ethanol extract of bidara upas on Vero E6 cells was 500 ppm non-toxic (Table 1). Therefore, the maximum concentration that will be used for viral inhibition assay is 500 ppm.

The cytopathic effect (CPE) of SARS-CoV2 on the vero E6 cell compared to the normal cell is performed in Figure 1.

Following the plaque formed on the cells (Figure 2). From the results of research conducted using the virus inhibition test, it was found that the inhibition power of the ethanol extract of the upas tuber with concentrations of 62.5 ppm, 125 ppm, 250 ppm, 500 ppm were 59.54%, 74.7%, 74.68%, and 79.27%, respectively (Figure 3).

From the results of research conducted using RT-PCR, the results were based on the E gene and the RdRp gene. The results of each concentration can be seen in Table 2 and Figure 4.

Pharmacological potential exists in bidara upas due to the presence of a rich variety of chemical compounds found in the plant. Metabolite profiling shows that various chemicals in the extract and fractions of *Merremia mammosa* Hall have antiviral, antioxidant, and anti-inflammatory effects [17], and that these compounds are essential to the pharmacological activity of the plant. *Merremia mammosa* Hall was found to have action in suppressing the spike protein of SARS-CoV2 with the PDB id. 6M0J protein, as determined by an *in silico* investigation of the chemical analysis using LC-MS/MS QTOF [11].

The cytotoxic test with Vero E6 cells (host of the SARS-CoV2 virus) from the ethanol extract of bidara upas using the MTT method showed that the maximum non-toxic concentration (MNTC) is 500 µg/mL, as determined as the maximum concentration of the extract at which the cells developed normally, therefore the viral inhibition assay used 500 µg/mL as highest concentration [18, 19]. The concentration that determined a cell viability above 80% [20]. However, in this research, the viability of cell is 41%. Therefore, the concentrations used in viral inhibition assay were 500, 250, 125, and 62.5 ppm.

The RT-PCR test showed an increase in the CT (cycle threshold) value of the virus treated from the E gene and the RdRp gene, an increase in dose above 100 ppm showed a high CT value compared to the positive control, this means that the viral load was smaller so that it can be mentioned that the increase *Merremia mammosa* Hall concentrations can reduce the amount of virus. Due to the fact that a low CT value indicates a high concentration of genetic material, it is typically associated with an elevated risk of infection [21]. A high CT value indicates a lower risk of infectivity because it reflects a low viral genetic material concentration [22]. Low viral load can be further caused by the incubation period, the recovery phase, or the virus's primary replication elsewhere in the body (i.e. lower respiratory tract (LRT)) [21, 23].

Table 2: CT PCR values

Concentration	E Gene	RdRp Gene
62.5 ppm	26.81	26.13
125ppm	27.07	27.93
250ppm	33.33	32.74
500ppm	32.41	33.42
Negative Cont.	18.34	17.21
Positive Cont.	23.7	24.32

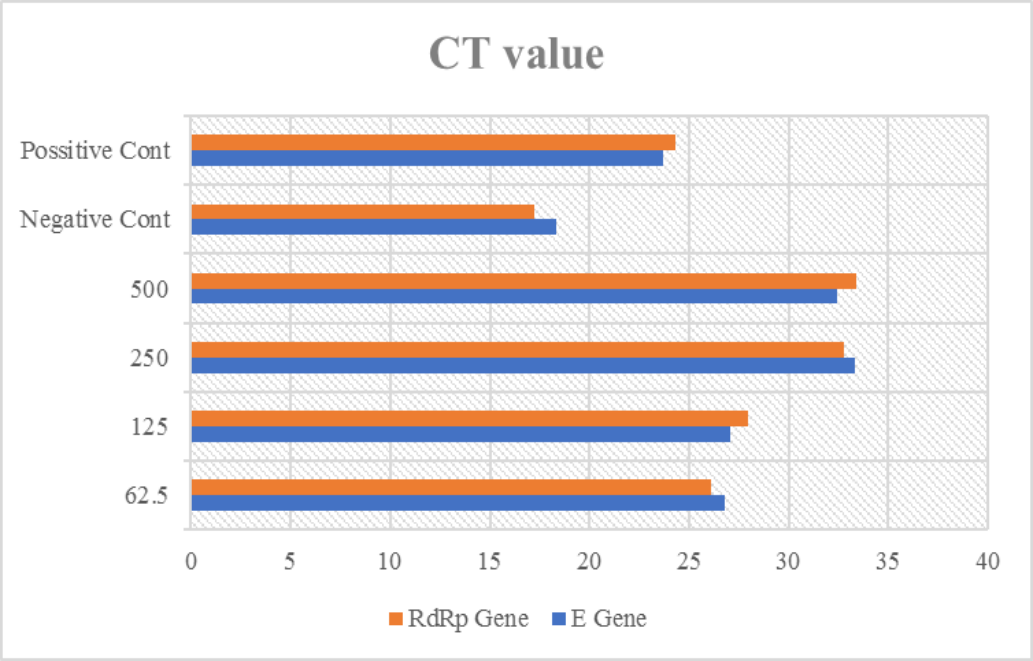


Figure 4: CT value of RdRp and E genes from the supernatant (containing treated virus and of each concentration of the extract)

CT values derived from semiquantitative RT-PCR provide a valuable surrogate for the detection of infectious viruses and can inform decisions regarding infection control [24]. The absolute sensitivity of a reverse transcription polymerase chain reaction (RT-PCR) test is difficult to determine due to the lack of a true "gold standard" and is dependent on a number of variables, such as the type of specimen, method of collection, and the specific test. Several studies comparing the performance of available RT-PCR assays using the split-specimen method reported 96% to 100% positive agreement (representing sensitivity) based on consensus test results [25].

Plaque assays are a quantitative method for measuring infectious SARSCoV2 by quantifying the plaques formed in cell culture following infection with serial dilutions of the virus specimen. Plaque assays continue to be the gold standard for determining the concentrations of replication-competent lytic viruses. New techniques for viral titration continue to evolve, plaque assays continue to be the gold standard for the quantification of infectious virus [26, 27]. Titers were expressed as plaque-forming units (PFU) per millilitre [28]. In this study, the results of the plaque test showed that the higher the concentration given, the % inhibition increased, which means that the cell death zone was getting bigger. This activity is because *Merremia mammosa* Hall contains Gemixanthone A which provides inhibiting activities to SARS-COV2 Spike Protein [11].

Agil *et al.* [29], examined the rhizome of the bidara upas used for research as an anti-tuberculosis drug by the Madurese community. In this study, a test was carried out for the antibacterial activity of the terpenoid ethyl acetate fraction derived from the n-hexane extract of *Merremia mammosa* Hall rhizome against *Mycobacterium tuberculosis*, ethyl acetate fraction. Separation was carried out using vacuum column chromatography and 11 subfractions were obtained, 2 of which indicated the presence of terpenoid compounds which had the potential to act as antituberculosis because they did not show the development of *Mycobacterium tuberculosis* in Midlebrook 7H10 medium which was stained with acid-fast Zielh-Neelsen stain. Another study also explained that the anti-influenza activity of Merremia was carried out using the hemagglutinin test, the ethyl acetate fraction of the methanol extract of *Merremia mammosa* Hall had anti-influenza A activity (subtype H1N1) [30].

Conclusion

From the results of research conducted using the virus inhibition test using RT-PCR and plaque assay, *Merremia Mammosa* Hall Extract needs to be continued *in vivo* studies to be developed as anti SARS-CoV2.

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Authors' Contributions

All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

ORCID

Neny Purwitasari
https://orcid.org/0000-0003-0817-7065
Mangestuti Agil
https://orcid.org/0000-0002-2300-9214
Siswandono Siswodihardjo
https://orcid.org/0000-0002-9579-8929
Reviany Vibrianita Nidom
https://orcid.org/0000-0003-1433-6026

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