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Coral plant *Jatropha multifida* L leaf extracts inhibit dengue virus-2 (DENV-2) growth through NS5

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ABSTRACT

The incidence of dengue fever (DF) increases drastically from year to year, especially in tropical countries like Indonesia. In contrast, antiviral against dengue virus (DENV) is not available in clinics, yet. *Jatropha multifica* L, locally named *tanaman yodium*/coral plant, is a medicinal plant that is traditionally used to treat dengue hemorrhagic fever (DHF). However, its scientific evidence is limited. This study aimed to investigate the antiviral activity of *J. multifida* L leaf extracts against DENV-2 and evaluate the effect on NS5 RNA expression. The leaf extracts were prepared by multilevel extraction using chloroform and methanol. The study was conducted *in vitro* using DEN-2 and Vero cells. The antiviral activity was assessed by using qRT-PCR to assess the number of virus copies and then used to calculate the inhibitory concentration of 50% (IC_{50}). The effect of the most active extract on NS5 DENV-2 RNA expression was then evaluated by using qRT-PCR. Among 4 extracts tested, the methanolic insoluble chloroformic extract (MIS) is the most active with an IC_{50} value of 124.3 µg/mL. Furthermore, the MIS (0.02) strongly inhibited NS5 DENV-2 RNA expression compared to control (1.0). In conclusion, the MIS of *J. multifida* is active against DENV-2 through inhibition of NS5 RNA expression.

ABSTRAK

Angka kejadian demam berdarah (DB) meningkat drastis dari tahun ke tahun, terutama di negara tropis seperti Indonesia. Sebaliknya, antivirus untuk virus dengue (DENV) belum tersedia di klinik. *Jatropha multifica* L dengan nama lokal jarak tintir merupakan tanaman obat yang secara tradisional digunakan untuk mengobati penyakit demam berdarah dengue (DBD). Namun, bukti ilmiahnya terbatas. Penelitian ini bertujuan untuk mengkaji aktivitas antivirus ekstrak daun *J. multifida* L terhadap DENV-2 dan mengevaluasi pengaruhnya terhadap ekspresi RNA NS5. Ekstrak daun dibuat dengan ekstraksi bertingkat menggunakan kloroform dan metanol. Penelitian dilakukan secara *in vitro* menggunakan sel DEN-2 dan Vero. Aktivitas antivirus dinilai dengan menggunakan qRT-PCR untuk menilai jumlah salinan virus dan kemudian digunakan untuk menghitung konsentrasi hambat 50% (IC_{50}). Pengaruh ekstrak paling aktif terhadap ekspresi RNA NS5 DENV-2 kemudian dievaluasi dengan menggunakan qRT-PCR. Diantara 4 ekstrak yang diuji, ekstrak kloroform tidak larut metanol (MIS) merupakan ekstrak paling aktif dengan nilai IC_{50} sebesar 124,3 µg/mL. Selain itu, MIS (0,02) menghambat dengan kuat ekspresi RNA NS5 DENV-2 dibandingkan dengan kontrol (1,0). Kesimpulannya, MIS *J. multifida* aktif melawan DENV-2 melalui penghambatan ekspresi RNA NS5.

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INTRODUCTION

Dengue fever (DF) is an infectious disease caused by the dengue virus (DENV) that affects 400 million people annually worldwide.[1] Of the 129 countries reporting dengue cases, 70% of patients are in the Asian region.[2] The mortality rate for DF varies, from <1% if handled properly to 15% if not treated properly.[3] The spectrum of clinical symptoms of DF also varies, from asymptomatic to severe manifestations called as dengue shock syndrome (DSS). The DENV consists of 4 serotypes, namely dengue virus -1 (DENV-1), DENV-2, DENV-3, and DENV-4. Dengue virus-2 is a serotype of the DENV that has the potential to manifest more severely than the other three serotypes.[4]

Dengue virus is a positive single-stranded RNA virus. The DENV genome encodes 3 structural proteins, namely capsid (C), envelope (E), and membrane (M) and 7 non-structural proteins (NS), namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. The NS5 protein is the largest protein consisting of an RNA-dependent RNA-polymerase (RdRp) domain at the C-terminal end and methyltransferase (MTase) and guanylyltransferase (GTase) domains at the N terminus. The NS5 protein has an important role in viral replication during infection of host cells, so it is often used as an antiviral target.[5]

Currently, the treatment for DF is still supportive to reduce fever symptoms. There is no effective antiviral in dealing the dengue infection. There are already antiviral candidates that have entered clinical trials such as chloroquine and balapiravir, however the results obtained could not reduce viraemia.[6] The search for the dengue antivirus is still ongoing. The most drug discovery and development comes from natural products.[7] One of the herbs that have been used by Indonesian people for a long time to treat DF is *Jatropha multifida* L. leaves.[8] *Jatropha multifida*, locally name *jarak tintir*, contains groups of diterpene compounds, flavonoids, phenolics, cyclic peptides, and glucosides.[9,10] A previous study reported that *J. multifida* ethanolic extract at a concentration of 500 µg/mL inhibits the growth of DENV-2. However, it has not been reported, yet the most active of extract type against DENV-2 and its effect on NS5.

MATERIAL AND METHODS

Plant preparation and extraction

Jatropha multifida leaves were obtained in Sleman District, Yogyakarta and harvested in April 2022. The plant was determined at the Laboratory of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta. The leaves were dried and then powdered. 500 g of the powdered leaves were macerated in 1500 mL chloroform with intermittent shaking for three days and filtered. The residue was further macerated two times by using chloroform. All the filtrates were pooled together and evaporated by using rotary evaporator to obtain dried chloroformic extract. The chloroformic extract was then partitioned by using methanol to obtain methanolic-soluble and methanolic-insoluble extracts. Whereas, the remaining residue was then macerated by using methanol similar to the procedure carried out for the chloroform maceration. The yield of each extract was weighed and stored at 4 °C until analyzed.

Cell and virus preparation

The *in vitro* DENV-2 inhibition test was undertaken using *Aedes albopictus* C6/36 cells and Vero cells (African green monkey kidney cell line). C6/36 cells were cultured in DMEM medium supplemented with 5% FBS at 33°C. Vero cells were cultivated in M-199 medium supplemented with 5% FBS at 37°C. Dengue virus DENV-2 strain was propagated on C6/36 cells in DMEM medium supplemented with 2% FBS and incubated at 33°C in 5 d. The supernatant was harvested and utilized as viral stock.[11-2, -3, 4]

Viral infectivity optimization on Vero cells

The virus stock that was obtained from propagation and carried out by testing optimization of virus infection on Vero cells to determine the effective volume of viral stock that can optimally infect Vero cells. A total of $1x10^5$ Vero cells were grown on 24-well plates and incubated for 24 hr. After that, the viral stock was made with serial dilutions, that is 1x, 10x and 100x dilutions. The virus was placed into a 24-well plate containing Vero cells. Vero cells that had given viral stock, then, were incubated for one hr at 37°C CO2 5% and every 15 min the well plate was shaken so that the infection spread evenly. Afterwards, the supernatant was discarded and treated with the methanol extract of *J. multifida* 500 µg/mL. The treated cells were subsequently incubated for 24 hr. Later, the supernatant and cells were harvested and RNA extraction was carried out according to the kit protocol. The resulting RNA samples were then performed qRT-PCR using one-step qRT-PCR to determine the Ct value. Ct-values below 25 at virus-level dilutions were used to test antidengue activity based on the efficiency level of DENV-2 infection in Vero cells.

DENV-2 inhibition assay

The antidengue activity test was conducted by using the viral growth inhibition assay method with slight modifications.[12] Vero cells were infected with the DENV2 virus with the most effective viral dilution to infect Vero cells obtained from optimization and incubated for one hour. Then, added media containing *J. multifida* leaves extracts with serial concentrations of 500, 250, 125, 62.5, and 31.25 µg/mL and incubated for 24 hr at 37°C. The negative control group was given DMSO treatment. After incubation, the supernatant was harvested and the RNA copy was quantified using qRT-PCR. The primer sequences applied for DENV-2 are listed on TABLE 1. The antidengue activity was expressed by the 50% inhibitory concentration (IC_{50}) which is defined as the extract concentration needed for a 50% reduction of DENV-2 RNA expression.

NS5 expression

For the most active extract, its effect on NS5 DENV-2 RNA expression was examined by qRT-PCR using supernatants from cells used on the DENV-2 inhibition assay. The primer sequences applied for NS5 are listed on TABLE 1.

Statistical analysis

The data was analyzed using IBM SPSS Statistics. Inhibitory concentration 50% (IC_{50}) was determined using Probit analysis. RNA quantification was presented in the form of relative fold change normalized by housekeeping gene (GAPDH). Analysis of differences between the two groups was analyzed using an independent t-test. A p value <0.05 was considered significant.

TABLE 1. Primer sequences of DENV-2, GAPDH and NS5[13,14]

Primer	Sequence
Forward primer (DENV2)	5'-GARAGACCAGAGATCCTGCTGTCT-3'
Reverse primer (DENV2)	5'-ACCATTCCATTTTCTGGCGTT-3'
Forward primer (GAPDH)	5′ GTG GAC CTG ACC TGC CGT CT 3′
Reverse primer (GAPDH)	5′ GGA GGA GTG GGT GTC GCT GT 3′
Forward primer (NS5)	5′ GGA AGG AGA AGG ACT GCA CA-3′
Reverse primer (NS5)	5′ ATT CTT GTG TCC CAT CCT GCT 3′

RESULTS

The powdered leaves of *J. multifida* was successively extracted with different organic solvents in the increasing polarity order, i.e. with chloroform followed by methanol. The yield of each extract was weighed and stored at 4 °C until analysis (FIGURE 1).

The DENV-2 inhibition test was performed after optimizing the effective viral stock to infect Vero cells. The results of virus stock optimization were presented in FIGURE 2. It could be seen that at 10x dilution of the viral stock, the Ct value was <25. This indicated that the dilution of the viral stock had been effective and efficient in infecting Vero cells. Therefore, the DENV-2 inhibition test was carried out with 10x diluted viral stock.

The antidengue activity of various *J. multifida* leaves extracts against DENV-2 are presented in FIGURE 3. Among 4 extracts tested, the methanolic insoluble chloroformic extract (MIS) is the most active with an IC_{50} value of 124.3 µg/mL.

For the most active extract, MIS extract, its effect on NS5 DENV-2 RNA expression was evaluated. The NS5 expression of DENV-2 after 24 hr incubation with MIS extract (1.0) was significantly lower than control (0.02; $p<0.005$) as presented in FIGURE 4.

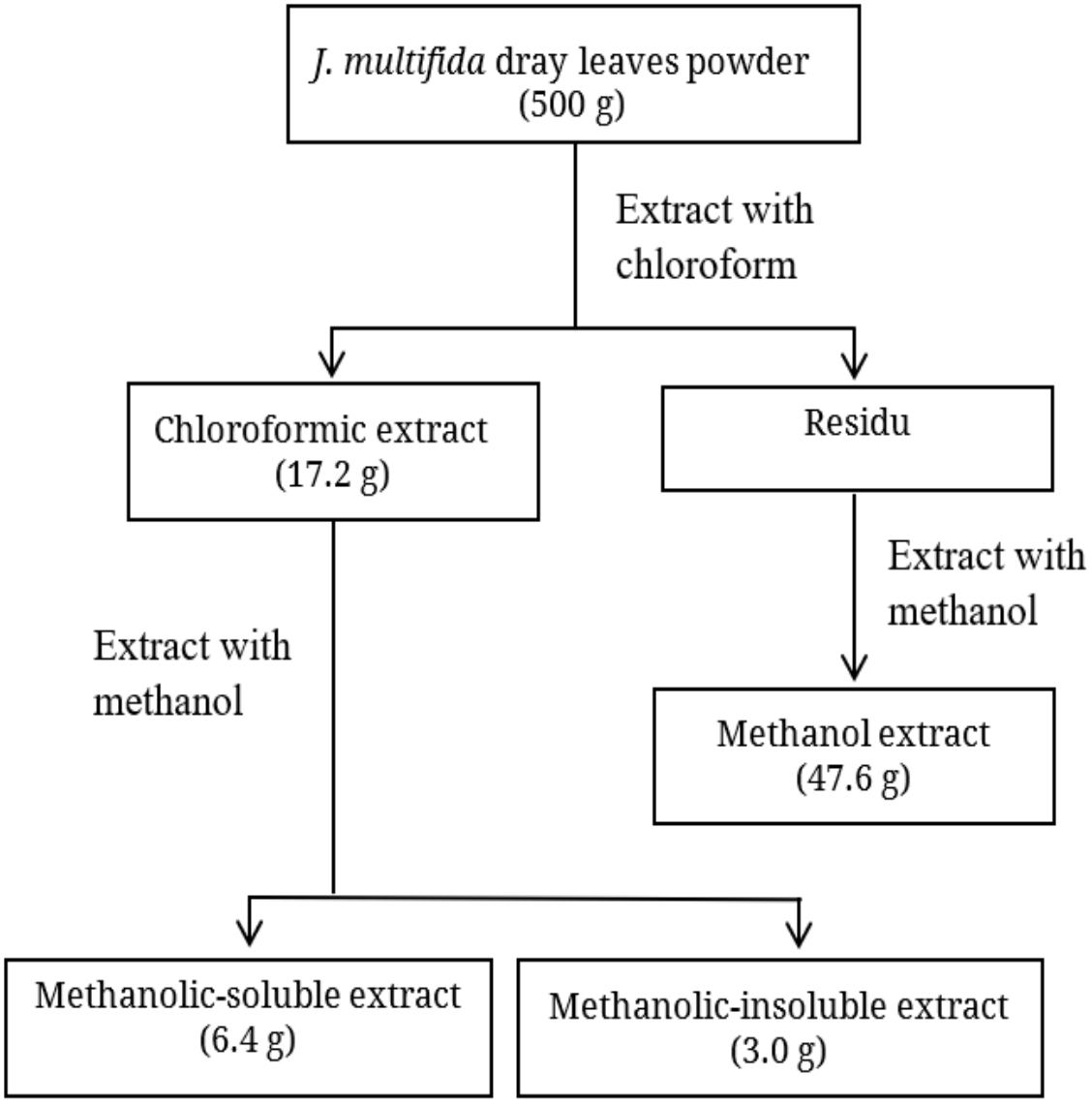

FIGURE 1. *Jatropha multifida* extract preparation process

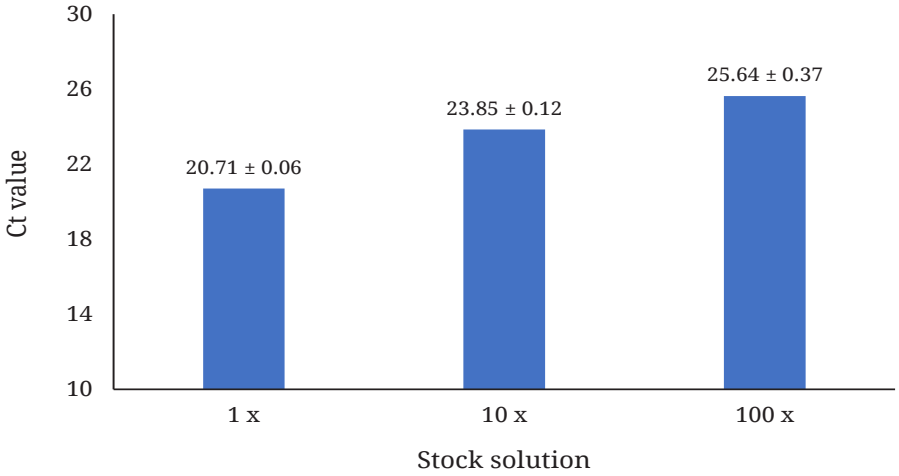

FIGURE 2. Ct value and DENV-2 stock solution

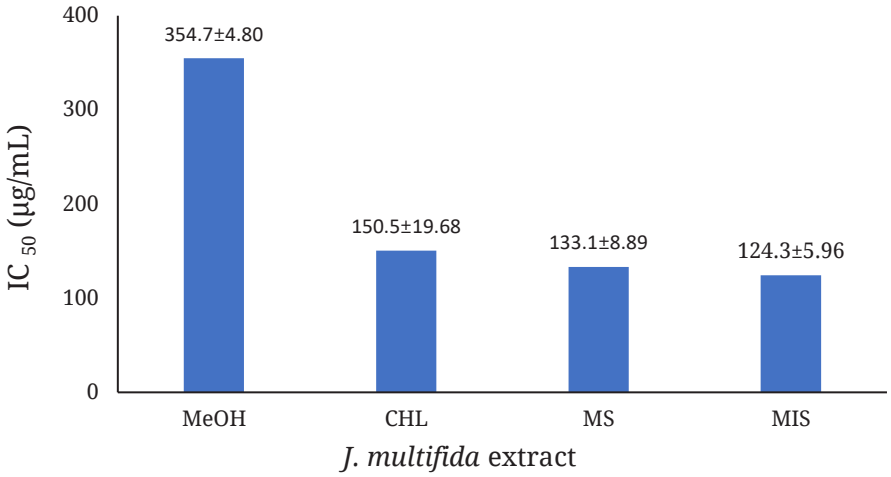

FIGURE 3. IC_{50} value (µg/mL) of various *J. multifida* leaves extract against DENV-2 growth. MeOH: methanolic extract; CHL: chloroformic extract; MS: methanolic soluble chloroformic extract; MIS: methanolic insoluble chloroformic extract.

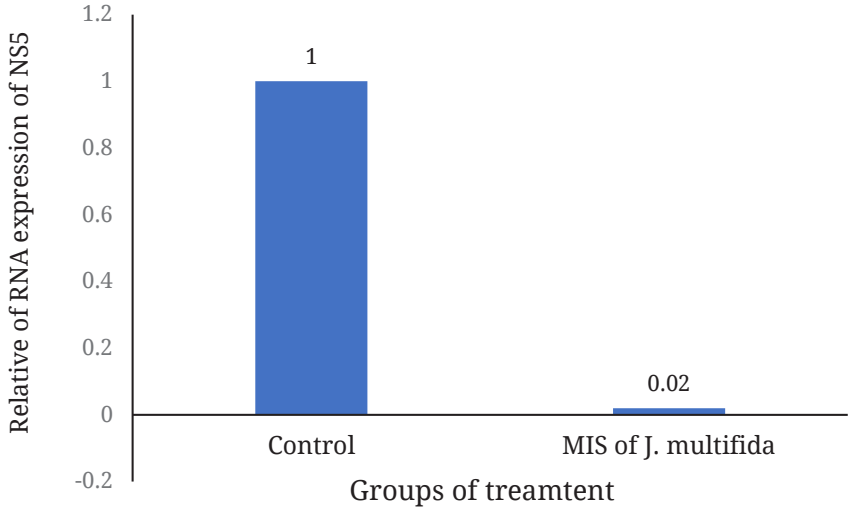

FIGURE 4. Relative RNA expression of NS5 DENV-2 after treatment with MIS of *J. multifida* compared to control.

DISCUSSION

The search for new drugs to overcome DF is still being developed, including drug candidates derived from herbal ingredients. This study was conducted to investigate the effect of *J. multifida* extract against the DENV-2 strain. In this study, it was found that the most active extract was the methanolic insoluble chloroformic extract with an IC_{50} value of 124.3 µg/mL. A previous study reported that the *J. multifida* at a concentration of 500 µg/mL can inhibit the DENV-2 growth.[8] Furthermore, Shoji *et al.*[15] reported that *J. multifida* chloroformic extract has strong activity in inhibiting the H_1N_1 virus growth in MDCK cells. One active compound isolated J. multifida, 4E-jatrogrossidentadione, has anti-HSV-1 activity with an IC_{50} of 2.05 µg/mL.[16]

The effect of *J. multifida* methanolic insoluble chloroformic extract on NS5 DENV-2 RNA expression was also analyzed. This extract stronger inhibit the RNA expression compared with the untreated group. Dengue virus is a single-stranded RNA virus that encodes 3 structural proteins and 7 non-structural proteins. Nonstructural protein 5 (NS5) is the largest protein and the most conserved protein of DENV-2. About 70% of the sequence is identical to the four serotypes of dengue virus. The NS5 protein is expressed in the DENV during infection. NS5 plays an important role in viral RNA capping and replication.[15] Therefore, NS5 is one potential targets for the antidengue discover and development.[16]

It was reported that *J. multifida* contains active compounds including terpenoids, flavonoids, phenolics, cyclic peptides, glucosides.[9,10] Flavonoids and terpenoids of *J. multifida* are believed to play a role in its antidengue activity.[8] Flavonoids isolated from various plants have been proven to have antidengue activity. Naringin and catechin inhibit DENV-2 in Vero cells at concentration of 47.59, and 33.7 µg/mL, respectively.[19] *In silico* analysis showed that quercetagetin has a strong affinity with RNA capping MTase.[20] Apigenin belonging to a flavone class, and hesperidin belonging to a flavanon class demonstrate strong interaction with NS5 RdRp of DENV *in silico.*[21] Meanwhile, quercetin belonging to a flavonol class exhibits RdRp NS5 inhibitory activity with an IC_{50} value of

3.6 µM.[22] Some diterpenes have been isolated from plants and evaluated their antidengue activity. Andrographolide from *Adrographis paniculata* can inhibit DENV-2 with an EC_{50} value about 22 µM.[23] The terpenoid compounds malacitanolide, reissantin E, and paclitaxel have been proven as potential inhibitors against target proteins of DENV.[24]

Although the flavonoids and terpenoids are thought to be responsible for antidengue of the *J. multifida* leaf extract. However, in this study, these active constituents have not been identified and isolated, yet. Further study will be conducted to isolate these active constituents.

CONCLUSION

Methanol-insoluble chloroform extract of *J. multifida* is the most active extract against DENV-2 growth with an IC_{50} value of 124.3 ± 5.96 µg/mL through inhibition of NS5 RNA expression.

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REFERENCES

1. Roy SK, Bhattacharjee S. Dengue virus: Epidemiology, biology, and disease aetiology. Can J Microbiol. 2021;67(10):687-702. https://doi.org/10.1139/cjm-2020-0572
2. WHO. Dengue and Severe Dengue [Internet]. 2023 [cited 2023 Aug 21]. https://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue
3. Adams L. Dengue Epidemiology: Globally and in the United States. In: Advisory Committee on Immunization Practices: Dengue Vaccine Workgroup. United States: Centers for Disease Control and Prevention; 2022.
4. Jiang L, Liu Y, Su W, Cao Y, Jing Q, Wu X, *et al.* Circulation of genotypes of dengue virus serotype 2 in Guangzhou over a period of 20 years. Virol J 2022; 19(1):1-9. https://doi.org/10.1186/s12985-022-01773-7
5. Norazharuddin H, Lai NS. Roles and prospects of dengue virus nonstructural proteins as antiviral targets: An easy digest. Malaysian J Med Sci 2018; 25(5):6-15. https://doi.org/10.21315/mjms2018.25.5.2
6. Low JGH, Ooi EE, Vasudevan SG. Current status of dengue therapeutics research and development. J Infect Dis 2017; 215(Suppl 2):S96-102. https://doi.org/10.1093/infdis/jiw423
7. Atanasov AG, Zotchev SB, Dirsch VM, Orhan IE, Banach M, Rollinger JM, et al. Natural products in drug discovery: advances and opportunities. Nat Rev Drug Discov 2021; 20(3):200-16. https://doi.org/10.1038/s41573-020-00114-z
8. Yuniyanti MM, Nugrahaningsih DAA, Wahyuningsih MSH. Phytochemical screening and antidengue activity of *Jatropha multifida* extract against DENV-2. Maj Obat Tradis 2022; 27(1):75. https://doi.org/10.22146/mot.73368
9. de Carvalho C, Vieira Mariano L, S Negrão V, Passarelli Gonçalves C, Cristina Ribeiro Marcucci M. Phenols, flavonoids and antioxidant activity of *Jatropha multifida* L. collected in Pindamonhangaba, Sao Paulo State, Brazil. J Anal Pharm Res 2018; 7(5):581-4. https://doi.org/10.15406/japlr.2018.07.00286
10. Zhu JY, Zhang CY, Dai JJ, Rahman K, Zhang H. Diterpenoids with thioredoxin reductase inhibitory activities from Jatropha multifida. Nat Prod Res 2017; 31(23):2753-8. https://doi.org/10.1080/14786419.201

7.1297441
11. Medina F, Medina JF, Colon C, Vergne E, Santiago GA, Munoz-Jordan JL. Dengue virus: isolation, propagation, quantification, and storage. Curr Protoc Microbiol 2012; (SUPPL.27):1-24. https://doi.org/10.1002/9780471729259.mc15d02s27
12. Tohma D, Tajima S, Kato F, Sato H, Kakisaka M, Hishiki T, *et al.* An estrogen antagonist, cyclofenil, has antidengue-virus activity. Arch Virol 2019; 164(1):225-34. https://doi.org/10.1007/s00705-018-4079-0
13. Ellan K, Thayan R, Raman J, Hidari KIPJ, Ismail N, Sabaratnam V. Anti-viral activity of culinary and medicinal mushroom extracts against dengue virus serotype 2: an *in vitro* study. BMC Complement Altern Med 2019; 1 9(1):1-12. https://doi.org/10.1186/s12906-019-2629-y
14. Gurukumar K, Priyadarshini D, Patil J, Bhagat A, Singh A, Shah P, *et al.* Development of real time PCR for detection and quantitation of Dengue Viruses. Virol J 2009; 6:1-8. https://doi.org/10.1186/1743-422X-6-10
15. Shoji M, Woo SY, Masuda A, Win NN, Ngwe H, Takahashi E, *et al.* Anti-influenza virus activity of extracts from the stems of *Jatropha multifida* Linn. collected in Myanmar. BMC Complement Altern Med 2017; 17(1):1-7. https://doi.org/10.1186/s12906-017-1612-8
16. Chokchaisiri R, Srijun J, Chaichompoo W, Cheenpracha S, Ganranoo L, Suksamrarn A. Anti-herpes simplex type-1 (HSV-1) activity from the roots of *Jatropha multifida* L. Med Chem Res 2020; 29(2):328-33. http://dx.doi.org/10.1007/s00044-019-02484-5
17. Sahili A El, Lescar J. Dengue virus non-structural protein 5. Viruses 2017; 9(4):1-20. https://doi.org/10.3390/v9040091
18. Lim SP, Noble CG, Shi PY. The dengue virus NS5 protein as a target for drug discovery. Antiviral Res 2015; 119:57-67. http://dx.doi.org/10.1016/j.antiviral.2015.04.010
Trujillo-Correa AI, Quintero-Gil DC, Diaz-Castillo F, Quiñones W, Robledo SM, Martinez-Gutierrez M. *In vitro* and *in silico* anti-dengue activity of compounds obtained from *Psidium guajava* through bioprospecting. BMC Complement Altern Med 2019; 19(1):1-16. https://doi.org/10.1186/s12906-019-2695-1
19. Manjula S, Kumaradhas P. Evaluating the suitability of RNA intervention mechanism exerted by some flavonoid molecules against dengue virus MTase RNA capping site: a molecular docking, molecular dynamics simulation, and binding free energy study. J Biomol Struct Dyn 2020; 38(12):3533-43. http://dx.doi.org/10.1080/07391102.2019.1666744
20. Sivaraman D, Pradeep PS. Exploration of bioflavonoids targeting dengue virus NS5 RNAdependent RNA polymerase: *in silico* molecular docking approach. J Appl Pharm Sci 2020; 10(5):16-22. https://doi.org/10.7324/JAPS.2020.10503
21. Coulerie P, Maciuk A, Eydoux C, Hnawia E, Lebouvier N, Figadère B, *et al.* New inhibitors of the DENV-NS5 RdRp from *Carpolepis laurifolia* as potential antiviral drugs for dengue treatment. Rec Nat Prod 2014; 8(3):286-9.
22. Panraksa P, Ramphan S, Khongwichit S, Smith DR. Activity of andrographolide against dengue virus. Antiviral Res 2017; 139:69–78. https://doi.org/10.1016/j.antiviral.2016.12.014
23. Ragavan RM, Purushothaman I, Swaminathan R, Almutairi SM, Hussein DS, Rasheed RA, *et al.* Malacitanolide, reissantin E and paclitaxel compounds as inhibitors of envelope, NS5 and NS2B/NS3 target proteins of dengue virus: Computational docking and molecular dynamics simulations studies. J King Sud Univ Sci 2023; 35:102868. https://doi.org/10.1016/j.jksus.2023.102868