**Dengue antiviral activity of polar extract from Melochia umbellata (Houtt) Stapf var. Visenia**

Nunuk Hariani Soekamto¹, S Liong¹, S Fauziah¹, I Wahid², Firdaus¹, P Taba¹ and F Ahmad³

¹ Department of Chemistry, Hasanuddin University, Jl. PerintisKemerdekan, Km 10, Tamalanrea, Makassar 90245, Indonesia
² Department of Parasitology, Faculty of Medicine Hasanuddin University
³ Postgraduate student in Department of Chemistry Hasanuddin University, Jl. Perintis Kemerdekan, Km 10, Tamalanrea, Makassar 90245, Indonesia

E-mail: nunukhariani@unhas.ac.id

Abstract. A research on the dengue antiviral activity test on the polar bark extract of M. umbelatta (Houtt.) Stapf var. Vicenia have been done to determine the relation to its activity against brine shrimp Artemia salina. The bark was extracted by maceration with n-hexane, chloroform, and ethylacetate. The activity of the ethyl acetate extract was then tested against A. salina and dengue virus. It was found that the ethyl acetate extract was active to A. salina with the LC₅₀ value of 101.66 μg/mL and also very active to dengue virus with the IC₅₀ value of 1.67 μg/mL. It is clear that the toxicity to brine shrimp A. salina has a positive correlation with the dengue anti virus.

1. Introduction

One of the diseases caused by the virus is dengue hemorrhagic fever (DHF). DHF is caused by dengue virus infection (DENV) transmitted by Aedes aegypti and Aedes albopictus female infected by DENV. The World Health Organization (WHO) reports that 2.5 billion (or more than 70%) of the human population is at risk for dengue fever, including in Southeast Asia and the Western Pacific [1]. An effective antiviral drug or patent vaccine against dengue virus is not currently available. One alternative approach that may be used for the prevention or treatment of dengue fever is to use natural products derived from plants.

Some species of the Malvaceae family have been tested for their efficiency as antiviral and antitumor agents, partly based on information on plants traditionally used as medicines to treat various human diseases. [2-5]. In addition, studies of the genus Melochia (Malvaceae) have also been performed, for example the roots of M. chamaedrys are used to treat various diseases such as cancer and hypertension [6]. Leaves M. corchorifolia as anti-cancer [7], M. odorata Inhibits HIV P24 [8], root wood and stem wood M. umbellata (Houtt.) Stapf var. Degrabrata K. has an activity as antitumor of murine leukemia P-388 cell [9,10].

Plants with the same family or genus will produce similar bioactivity. It can thus be assumed that the bark of M. umbellata (Houtt) Stapf var. Visenia has an antiviral bioactivity, especially dengue antiviral.
2. Experimental

2.1. Materials
The materials used in this research are plant stem bark powder *M. umbellata* (Houtt) Stapf var. Visenia, some organic solvents such as technical methanol, technical n-hexane, chloroform pa, ethyl acetate, technical acetone, Liebermann-Buchard phytochemical reagent, Dragendorff, Wagner, iron (III) chloride medium MEM, Fetal Bovin Serum (FBS), MEM medium, Vero cell (derived from the kidney of an African green monkey), cell line C6/36, DENV-2, Phosphate-Buffered Saline (PBS), 1% L-glutamine, 1.5% CMC, tetramethylbenzidine (TMB), NaHCO₃, H₂SO₄ 0.1 M.

2.2. Instruments
Instrument used in this research were tools of glass, blender, funnel, separating funnel, Buchner funnel, rotary evaporator, digital scales, Vigreux distillation device, concave tube, T25 flask, CO₂ incubator, incubator without CO₂, centrifuge 4°C, freezer, hemositometer, microwell plate, inverted microscope, ELISA reader (Benchmark).

2.3. Procedure

2.3.1. Extraction. Dried powder of *M. umbellata* stem of 50 g is macerated with n-hexane for 1 x 24 hours 4 times. The macerate collection obtained is then concentrated to obtain a concentrated n-hexane extract. The residue was macerated with chloroform solvent 4 times, then the residue was again macerated with ethyl acetate 3 times. The obtained macerate was then concentrated to obtain concentrated ethyl acetate extract as much as 85.1 mg of green color. The residue of the next partition was given a notation as methanol extract of 47.2 mg. The water extract was obtained from boiling the dried sample of the plant with water solvent and obtained the result of 6.21 g.

2.3.2. Bioassay. The extract obtained was tested toxicity to *A. salina* using Meyer method [11] and dengue Antivirus test is done by using Focus Formation Unit Reduction Assay method (FFURA)

2.3.3. Dengue Antivirus test. Dengue antivirus test is done by using Focus Formation Unit Reduction Assay (FFURA) method to know IC₅₀ (Inhibition Concentration 50%) on extract obtained and Enzyme-Linked Immunosorbent Assay (ELISA reader) method. Stages of bioactivity testing extracts of dengue virus among other:

2.3.4. Cell culture Vero Line. Cell line was added to DMEM (Gibco) medium which added nutrient in the form of 10% Fetal Bovine Serum (FBS), L-glutamine 1%, incubated at 37 °C under 5% CO₂ condition. Vero cells are taken from storage in the freezer. Then diluted at room temperature for 2-3 minutes. The cells in the cryovial were transferred into a sterile conical tube containing 5 mL MEM free, then centrifuged 800 rpm for 5-10 min and the supernatant was discarded. The pellet was added 5 mL Culture medium, resuspended, then transferred to T25 flask, incubated in CO₂ incubator at 37 °C and 5% CO₂. The medium is replaced after 72 hours and the cells are allowed to grow back until confluent. Confluent cells are harvested, the medium in the flask removed and the cell washed with 1 X PBS 2 times by rinsing it slowly. The cells in the flask were added 0.25% aqueous solution of 0.5 mL, incubated in CO₂ incubator at 37 °C and 5% and centrifuged 800 rpm for 5-10 min.
2.3.5 Cell Culture line C6/36. Cell line vero was added to the MEM (Gibco) medium which added nutrients in the form of 10% FBS, 1% glutamine, incubated at 37 °C under 5% CO₂. Cell vero is taken from storage in the freezer. Then diluted at room temperature for 2-3 minutes. The cryovial cell was transferred to a sterile conical tube containing 5 mL MEM free, then centrifuged 800 rpm for 5-10 min and the supernatant was discarded. The pellets were added 5 mL of culture medium, slowly suspended, then the cells transferred to T25 flask, incubated in CO₂ incubator at 37 °C and 5% CO₂. The medium is replaced after 72 hours and the cells are allowed to grow back until confluent (evenly filling the flask). Confluent cells are harvested, by means of medium in flask removed and cell washed with PBS 2 times by rinsing them slowly. The cells in the flask were added 0.5% aqueous trypsin solution 0.25%, incubated in CO₂ incubator at 37 °C and 5% CO₂ then centrifuged 800 rpm for 5-10 min.

2.3.6. Preparation of virus stock in cell line C6/36. Cell line C6/36 is used to multiply the DENV-2 virus (stock preparation) by 400 μL of the virus added from the previous stock diluted with MEM medium (containing 2% FBS, 1% glutamine 1 to 1 mL) into the flask which contains C6/36 cells then shaken, then incubated at 28 °C. Every 15-20 minutes (for 1 hour) gently beaten, after 1 hour added MEM medium (with nutrient FBS 2% 5 mL) then incubated until the cytopathic effect (morphological cell changes) slowly, then centrifuged 4000 rpm at 4 oC for 10 minutes, then the virus supernatant (stock virus) is stored at -80 °C.

2.3.7. Virus stock creation on cell line Vero. Virus infected by C6/36 infection was then infected again in vero cells in flask by 50 μL viral infected with diluted MEM 1 mL (containing 2% FBS, 1% glutamine L), then incubated in CO₂ incubator at 37 °C and 5% CO₂. Every 15-20 minutes (for 1 hour) the mixture is shaken gently, after 1 hour added MEM medium (with nutrient FBS 2% 5 mL) then incubated in CO₂ incubator at 37 °C and 5% CO₂ until cytopathic effect (observed for 7 days after being infected). The supernatant was harvested by slowly transferring, then centrifuged 4000 rpm at 4 °C for 10 min, then the viral supernatant (stock virus) was stored at -80 °C.

2.3.8. Focus Formation Unit Reduction Assay (FFURA). The antiviral activity of the extract was determined by measuring the result of the reduction of virus amount through Foci Forming Unit (FFU). The supernatant of the vero cell is placed in 24 microplate holes, during the viral transmission process in cells added CMC 1.5% and 2% FBS into the MEM medium. Reduced virus was observed using standard peroxidation based on discoloration for 4 days after transmission. Furthermore, the amount of DENV-2 was calculated using a stereomicroscope and the viral titer was calculated through FFU.

2.3.9. Enzyme Linked Immunosorbent Assay (ELISA reader). The ELISA plate was coated by an antigen with optimum dilution (1: 1000) using a NaHCO₃ buffer (pH 9.6), incubated at room temperature overnight at 4 °C or 2 h at 37 °C. The platter is then washed with PBS 0.05% 3 times then add blockers, BSA 200 μL. The plates were incubated at room temperature for 2 hours and washed again with PBS 3 times. At incubation, positive and negative control serum was diluted in 1% PBS, and 100 μL was included in the washed plate. The plates were incubated at 37 °C for 2 hours and washed again with PBS 3 times. Subsequently, 100 μL of tetramethylbenzidine (TMB) substrate was added, then incubated in dark space for 10 min and the cessation of reaction was carried out with 100 μL of 0.1 M sulfuric acid (H₂SO₄) solution. Inspection results are read on ELISA reader with wavelength 450 nm. For comparison, an ELISA kit was obtained from the Virology Laboratory of the Elizabeth Macarthur Agriculture Institute (EMAI), Woodbridge Rd. Menangle, NSW, Australia.
3. Results and discussion

Infectious diseases caused by dengue virus (Dengue Fever) is a dangerous disease that strikes in almost all tropical regions and subtropics. Until now there has been no drug or special therapy used for the treatment of the disease. For that we need to find an alternative drug that is safe and cheap, so that people can easily. This can help reduce the number of people with Dengue Fever (DHF).

One of the dengue virus that generally attacks some areas is dengue virus type 2 (DENV-2). DENV-2 anti-viral assay was performed on ethyl acetate extract and methanol. The antiviral test is followed by cytotoxicity test against Vero cells in order to provide more accurate information about the activity of the extract against DENV-2.

Before performing these anti-viral tests, screening begins by testing the toxicity of the extract on Brine shrimp *Artemia salina*. It was done as a preliminary test to determine whether the extract has high toxicity to *A. salina*. Previous studies have suggested that high toxicity of compounds/extracts against *A. salina* is positively correlated as antitumor/antivirus [11]. For example, waltherion C compound has isolated from wood stem *Melochia umbellata* var. Degrabrata K. was very active against *A. salina* (LC50 0.29 µg / mL). Waltherione C has been reported as having anti-HIV activity (EC50 0.3 µg/mL) and toxicity against CEM-TART cells (LC50 3.8 µg/mL) (Jadulco et al., 2014). In our hands, waltherione C significant cytotoxicity against P-388 murine leukemia cells with IC50 values of 0.26 µg/mL [12]. Other compounds Moracin M which is a 2-arylbenzofuran derivative also has a high toxicity to *A. salina* (LC50 = 61.7 µg/mL), is very active against murine leukemia P-388 (IC50 2.4 µg/mL [13,14].

![Figure 1. (a) Waltherione C and (b) Moracin M](image)

Toxicity test results on *A. salina*, cytotoxicity to DENV-2 and Vero Cell of Ethyl acetic, methanol and water extract can be seen in table 1.

| No | Extract     | *A. salina* (µg/mL) | DENV-2 (µg/mL) | Vero Cell (µg/mL) |
|----|-------------|---------------------|----------------|------------------|
| 1. | Ethyl acetic| 101.66              | 2.81           | 12.92            |
| 2. | Methanol    | 30.22               | 1.67           | 15.59            |
| 3. | Water       | 82.90               | 2.61           | 11.30            |

Based on the data in Table 1, it shows that the three extracts toxicity (ethyl acetic, methanol and water) against *A. salina* are positively correlated with their cytotoxicity. While the cytotoxicity of Vero cells is significant to show that the three extracts are very active against DENV-2.
4. Conclusion
The toxicity to brine shrimp *Artemia salina* has a positive correlation with the dengue anti virus. The cytotoxic data of ethyl acetate, methanol, and water extract are showing active on vero cells proved that the three extracts had strong potential against dengue virus type 2 (DENV-2).

Acknowledgments
We thank to Hasanuddin University for financial support and my student undergraduate and Magister Program.

References
[1] WHO, 2002, *Dengue and Dengue Hemorrhagic Fever*, Geneva: World Health Organisation, Fact Sheet No. 17
[2] Kapadia, G. J., Shukla Y. N., Morton, J. F., and Lloyd, A., 1977, New Cyclopentide Alkaloids from *Melochia tomentosa*, *Phytochemistry*, 16(9): 1431-1433
[3] Ananil, K., Hudson, J.B., Souzal, C., Akpaganal K, Tower 3, G.H.N.J.T., Amason, J.T. and Gbeassor, M. 2000. Investigation Of Medicinal Plants Of Togo For Antiviral and Antimicrobial Activities. *Pharmaceutical Biology* 38 (1): 40-45
[4] Saravanakumar, A., Venkateshwaran, K., Vanitha, J., Ganesh, M., Vasudevan, M. and Sivakumar T. 2009. Evaluation of antibacterial activity, phenol and flavonoid contents of *Thespesia populnea* flower extracts. *Pak. Journal Pharmacmy Science* 22 (3): 282-286
[5] Arullappan, S., Muhamad, S., and Zakaria, Z., 2013. Cytotoxic Activity of the Leaf and Stem Extracts of *Hibiscus rosa sinensis* (Malvaceae) against Leukaemic Cell Line (K-562). *Tropical Journal of Pharmaceutical Research* 12 (5): 743-746
[6] Dias, G.C.D., Gressler, V., Hoenzel, S.C.S.M., Silva, U.F., Dakol, I.I., and Morel, A.F., 2007. Constituents of the Roots of *Melochia chamaedrys*, *Phytochemistry*, 68, 668-672
[7] Palaksha, M.N., Ravishankar, K., and Sastry, V. G., 2015. Evaluation of in-vitro Anticancer Activity and Quantitative Estimation of Phenolics and Flavonoids of *Melochia corchorifolia* and *Saccharum officinarum* Leaf Extracts. *European Journal of Biomedical Pharmaceutical Sciences* 2 (4): 1410-1420
[8] Jadalco, C.J., Pond, C.D., Wagoner, R.M.V., Koch, M., Gideon, O.G., Matainaho, T.K., Piskaut, P., and Barrows, L.R., 2014, 4-Quinolone Alkaloids from *Melochia odorata*. *Journal Natural Product* 77 (1): 183-187
[9] Erwin, Noor, A., Soekamto, N.H., and Harlim, T. 2010. 6,6’-dimethoxy-4,4’-dihydroxy-3,2’-Furano-Isolavane, A New Compound From *Melochia umbellata* (Houtt.) Stapf var. Degrarbata K. (Palliisa). *Indonesia Journal Chemical* 10 (2): 222 – 225
[10] Erwin, Noor, A., Soekamto, N.H., Altena, I.V., dan Syah, Y.N., 2014, Waltherione C and cleomicosin from *Melochia umbellata* var. Degrarbata K. (Malvaceae), Biosynthetic and Chemotaxonomic, Significance. *Biochemical Systematics and Ecology* 55: 358-361
[11] Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobson, L.B., Nichols, D.E., and McLaughlin, J.L. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. *Plant Medical* 45: 31-34
[12] Alley, M.C., D.A. Scudiero, A. Monks, M.L. Hursey, M.J. Czerwinski, D.L. Fine, B.J. Abbott, J.G. Mayo, R.H. Shoemaker, M.R. Boyd (1988), *Cancer Res.*, 48, 589-601
[13] Soekamto, N. H., Achmad, S.A., Ghisalberti, E. L., Aimi, N., Hakim, E. H., and Syah, Y. M. 2003 Beberapa Senyawa Fenol dari Tumbuhan *Morus macroura* Miq. *Jurnal Matematika dan Sains* Vol. 8 No. 1
[14] Soekamto, N.H., S. A. Achmad, Ghisalberti, E. L., Hakim, E. H., and Syah, Y. M. (2000), Stilben and arylbenzofuran from the heartwood of *Morus macroura*, Proceeding of International Seminar on the role of Chemistry in indutry and environment, *Andalas University*, Padang Indonesia, 30-31 Agustus, 190