

ANTIHEPATITIS C VIRUS ACTIVITY OF INDONESIAN MAHOGANY (*TOONA SURENI*)

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## ABSTRACT

**Objective:** *Toona sureni* (Indonesian mahogany) is a member of Meliaceae family and locally known as suren. Previous study reported that *T. sureni* leaves extract exhibited antiviral activity with 50% inhibitory concentration (IC<sub>50</sub>) value of 13.9 ± 1.6 µg/ml against hepatitis C virus (HCV) J6/JFH1. Cytotoxicity analysis of *T. sureni* leaves extract did not reveal any cytotoxicity effect; therefore, further study was taken to investigate the active substances from the extract.

**Methods:** Bioassay-guided isolation of anti-HCV was conducted using Huh-7.5 cells infected with HCV J6/JFH1 in the presence of extracts, fractions, or compounds from the plant.

**Results:** Ethyl acetate fraction (Fr E) exhibited high anti-HCV activity with IC<sub>50</sub> value of 1.7 µg/ml. Further, separation of Fr E by open column chromatography resulted in nine sub-fractions (sub-Fr E1-E9). Sub-Fr E3 and E4 have IC<sub>50</sub> value of 29.90 µg/ml and 7.68 µg/ml, respectively. Polyphenols compounds have been isolated from sub-Fr E3 and E4. The structures have been determined to be ethyl gallate (1), methyl gallate (2), catechin (3), gallic acid (4), and quercetin 3-O-rhamnoside (5). Among the isolated compounds, gallic acid showed to possess strong anti-HCV activity with IC<sub>50</sub> value of 15.9 µg/ml.

**Conclusion:** *T. sureni* and its isolated compound, gallic acid, may be good candidates to develop for alternative and/or complementary agents of anti-HCV infection.

**Keywords:** *Toona sureni*, Leaves extract, Polyphenols, Antihepatitis C virus, Gallic acid.

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## INTRODUCTION

Hepatitis C virus (HCV) is a major cause of liver disease and potential cause of substantial morbidity and mortality worldwide. Global prevention and control of HCV were met with difficulties due to the complexity and uncertainty related to the geographic distribution of HCV infection and chronic hepatitis C, determination of its associated risk factors, and evaluation of cofactors that accelerate its progression. No vaccine is available to prevent HCV infection, nor does immune globulin provide protection [1,2].

Hepatitis C is an RNA virus that mutates very rapidly, until now, there were seven genotypes (1-7) with more than 70 subtypes. HCV genome encodes structural protein (Core, E1, and E2) and non-structural protein (NS2, NS3, NS4A, NS4B, NS5A, and NS5B). Glycoproteins E1 and E2 were responsible for virus binding to the cell surface meanwhile the function of non-structural protein was RNA replication and virus particle construction [3-5]. The current standard interferon-free treatment regimens with a combination of direct-acting antiviral agents (DAAs) targeting the viral NS3 protease, NS5A multifunction protein, and NS5B RNA-dependent RNA polymerase result sustained virological responses >90%. However, the emergence of DAA-resistant HCV strains, limited access to the DAAs due to their high cost is also an important issue. Many therapies for HCV infection have been developed, but the

therapeutic efficacy still needs to be improved [6]. In future, anti-HCV research should aim at the development of therapies for non-responder patient population and treatment regimens with short duration of treatment [7].

Many plants species are being tested for anti-HCV to find a possible cure for HCV. Some of them have shown significant inhibition in entry, replication, and assembly steps of the viral life cycle [8]. Moreover, some compounds have been isolated and tested for anti-HCV. Previous study reported that chalepin and pseudane IX which isolated from *Ruta angustifolia* leaves showed strong anti-HCV activities with 50% inhibitory concentration (IC<sub>50</sub>) value of 1.7 ± 0.5 and 1.4 ± 0.2 µg/ml, respectively, without apparent cytotoxicity [9]. A major catabolite of chlorophyll A, which was isolated from *Morinda citrifolia* and identified as pheophorbide A, was reported to possess anti-HCV activity with IC<sub>50</sub> value of 0.3 µg/ml. It was reported also that pyropheophorbide A is an anti-HCV compound with IC<sub>50</sub> value of 0.2 µg/ml [10]. Adianti *et al.* suggested that glycoumarin, glycerin, glycerol, and liquiritigenin isolated from *Glycyrrhiza uralensis*, as well as isoliquiritigenin, licochalcone A, and glabridin, would be good candidates for seed compounds to develop antivirals against HCV [11]. Quercetin and gallic acid isolated from *Kalanchoe pinnata* inhibited HCV production in a dose-dependent manner with IC<sub>50</sub> value of 1.5 and 6.1 µg/ml, respectively, without exhibiting cytotoxicity [12].

Evaluation of certain Indonesia medicinal plants explored from East Java revealed that *Toona sureni* is one of the good candidates for the development of anti-HCV drugs. *T. sureni* (Indonesian mahogany) is a member of Meliaceae family and locally known as suren. The study reported that ethanol extract of *T. sureni* leaves exhibited antiviral activity with  $IC_{50}$  value of  $13.9 \pm 1.6$   $\mu\text{g/ml}$  against HCV J6/JFH1. Time of addition experiment revealed that the extract inhibited both at the entry and postentry steps. *T. sureni* leaves extract was not toxic (cytotoxicity concentration  $[CC_{50}] > 500$   $\mu\text{g/ml}$ ,  $SI > 35.9$ ); therefore, further study was taken to investigate the active substances from *T. sureni* leaves extract [13].

Another *Toona* species has been known for other activity. *T. sinensis* has activity for antimicrobial, anticancer, anti-inflammation, radical scavenging, and antioxidant in the liver. *T. ciliata* and *T. pubescens* have been used traditionally as a treatment for diarrhea, as cardiostimulant, astringent, and antioxidant [8,14-18]. However, Jain et al. reported in his research that the antioxidant activity level can affect reduction of chronic hepatitis and cirrhosis [19].

## METHODS

### Plant material

*T. sureni* leaves were collected from Pujon, East Java, Indonesia. Authentication and identification of plant were carried out at the Purwodadi Botanical Garden, East Java.

### Extraction and isolation

Leaves of *T. sureni* were dried at room temperature and pulverized. Dried leaves powder (250 g) were extracted using 80% ethanol by maceration method. The ethanol extract was filtered and concentrated using rotary evaporator. The crude ethanol extract (25.6 g) was suspended in water and partitioned between chloroform, ethyl acetate, and butanol, successively to obtain chloroform, ethyl acetate, butanol, and water fraction, respectively. Ethyl acetate fraction (8.63 g) was further separated on silica gel column using chloroform-methanol 5% gradient system resulted in nine sub-fractions (Sub-Fr E1-E9). Sub-Fr E3 was subjected to open column chromatography using silica gel and chloroform-ethyl acetate (1:1) to yield ethyl gallate (1). Sub-Fr E4 was further subjected to silica gel column (chloroform/methanol 90:1-50:50) resulted in nine sub-fractions (Sub-Fr E4.1-E4.9). Sub-Fr E4.1 further subjected to silica gel column (hexane/ethyl acetate 40:60; 20:80, ethyl acetate, and methanol) to yield ethyl gallate (1). Sub-Fr E4.2 was subjected to semi-preparative high-performance liquid chromatography (HPLC) system (C-18 column, 5  $\mu\text{m}$ ,  $10 \times 250$  mm, flow rate 2.5 ml/min) using acetonitrile-water (20-80%) to yield methyl gallate (2). Sub-Fr E4.3 was also subjected to semi-preparative HPLC using the same condition (flow rate 1.5 ml/min) to obtain gallic acid (3) and catechin (4). Sub-Fr E4.4 was also subjected to semi-preparative HPLC using the same condition (flow rate 1.5 ml/min) to yield quercetin 3-O-rhamnoside (5). Structure elucidation based on nuclear magnetic resonance (NMR) data and references data. The  $^1\text{H}$  NMR spectra were measured with JEOL ECA 500 spectrometer (500 MHz), while HPLC was performed on Shimadzu system.

### Cells and viruses

Huh-7.5 cells were cultivated in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum, non-essential amino acids, penicillin, and streptomycin. Cells were grown at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator.

### Analysis of anti-HCV activities

*T. sureni* extract and fractions were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions at a concentration of 100 mg/ml. The stock solutions were stored at  $-20^\circ\text{C}$  until they were used. Analysis of anti-HCV method was described previously [9,13]. Huh-7.5 cells were seeded in 24-well plates (cell density  $1.9 \times 10^5$  cells/well). A fixed amount of J6/JFH1 with multiplication of infection of 0.5 focus-forming units/cell was mixed with serial dilutions of the extracts (100, 30, 10, 1, and 0.1  $\mu\text{g/ml}$ ) and inoculated to the cells. After 2 h, the cells

were washed with medium to remove the residual virus and further incubated in the medium containing the same concentrations of the test samples as those during virus inoculation.

Culture supernatants were obtained at 2-day postinfection and titrated for virus infectivity. Virus and cells treated with medium containing 0.1% DMSO served as a control. The inhibition percentage of virus infectivity by the samples was calculated by comparing to the control using SPSS probit analysis, and the  $IC_{50}$  value was determined.

### Cytotoxicity assay

The cytotoxicity of the samples was assessed by WST-1 assay. Huh-7.5 cells were seeded in 96-well plates and treated with serial dilution of the samples or control (0.1% DMSO) for 48 h. The condition of cells was observed under microscope to detect the toxic effect on cell morphology. After 48 h, the medium was removed from 96-well plates and then added with 10% of WST-1 reagent (10  $\mu\text{l/well}$ ) and incubated for 4 h at  $37^\circ\text{C}$ . The absorbance of samples was measured at 560 nm and 750 nm. Cell viability percentage and 50%  $CC_{50}$  were calculated.

## RESULTS AND DISCUSSION

Bioactivity-guided isolation of anti-HCV compounds from *T. sureni* was conducted using Huh-7.5 cells infected with HCV J6/JFH1 in the presence of extract, fractions, or compounds from the plant. To isolate an active substance(s) responsible for the anti-HCV activity, the crude ethanol extract was partitioned into chloroform (Fr C), ethyl acetate (Fr E), butanol (Fr B), and water fractions (Fr W), and their anti-HCV activities were evaluated. The result showed that the Fr C and Fr E had strong anti-HCV activities with  $IC_{50}$  values of 1.6 and 1.7  $\mu\text{g/ml}$ , respectively (Table 1).

Due to the anti-HCV activities of Fr E, further separation of Fr E was done by open column chromatography and resulted in nine sub-fractions (Sub-Fr E1-E9). These fractions were evaluated for anti-HCV and cytotoxic activities. The result obtained revealed that sub-Fr E3 and E4 have strong anti-HCV activities with  $IC_{50}$  value of 29.9  $\mu\text{g/ml}$  and 7.7  $\mu\text{g/ml}$ , respectively (Table 2).

Sub-Fr E3 and E4 were subjected to further purification which yielded some polyphenols compounds that have been determined to be ethyl gallate (1), methyl gallate (2), catechin (3), gallic acid (4), and quercetin 3-O-rhamnoside (5) (Fig. 1). Chemical structures of isolated compounds were elucidated based on NMR spectroscopy and reference

**Table 1:  $IC_{50}$  values of a crude ethanol extract and its fractions from *T. sureni***

Samples	$IC_{50}$ , $\mu\text{g/ml}$
Ethanol extract	13.9
Chloroform fraction (Fr C)	1.6
Ethyl acetate fraction (Fr E)	1.7
Butanol fraction (Fr B)	15.4
Water fraction (Fr W)	50.0

*T. sureni*: *Toona sureni*,  $IC_{50}$ : 50% inhibitory concentration

**Table 2:  $IC_{50}$  values of Fr E fractions from *T. Sureni***

Samples	$IC_{50}$ , $\mu\text{g/ml}$
E1	NT
E2	NT
E3	$29.9 \pm 0.7$
E4	$7.7 \pm 0.2$
E5	$27.0 \pm 4.2$
E6	$7.1 \pm 0.3$
E7	$16.8 \pm 0.3$
E8	$7.4 \pm 0.2$
E9	$16.6 \pm 0.1$

\*NT: Not tested. *T. sureni*: *Toona sureni*,  $IC_{50}$ : 50% inhibitory concentration

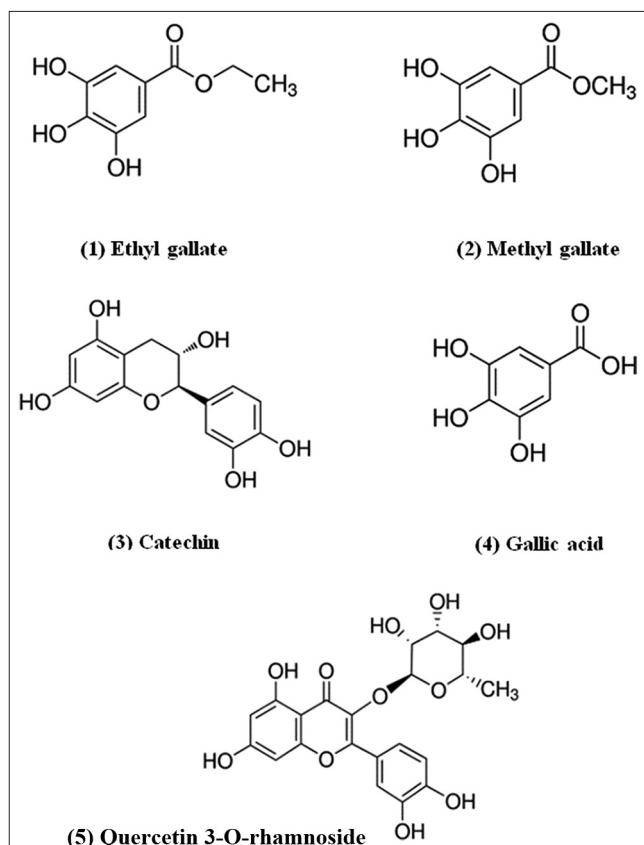


Fig. 1: Chemical structure of isolated compounds from sub-Fr E3 and E4 of *Toona sureni*

Table 3: IC<sub>50</sub> values of isolates from sub-Fr E3 and E4 *T. Sureni*

Samples	IC <sub>50</sub> , µg/mL	CC <sub>50</sub> , µg/mL
Ethyl gallate	52.5±3.5	>100
Methyl gallate	>100	>100
Gallic acid	15.9±0.2	>100
Catechin	52.0±4.9	>100
Quercetin 3-O-rhamnoside	>100	>100

*T. sureni*: *Toona sureni*, IC<sub>50</sub>: 50% inhibitory concentration, CC<sub>50</sub>: Cytotoxicity concentration

data. Anti-HCV activities of those compounds were then evaluated. The results showed that gallic acid mediates a strong anti-HCV activity with IC<sub>50</sub> value of 15.90 µg/ml, while ethyl gallate and catechin revealed moderate activities with IC<sub>50</sub> value of 52.5 and 52.0 µg/ml, respectively. On the other hand, the presence of methyl moiety in the methyl gallate resulting inactive compound against HCV, suggesting that moiety of OH in gallic acid may play an important role for its anti-HCV activities (Table 3).

Gallic acid is also a common natural constituent of a variety of plants. In the present study, gallic acid was identified as a compound with stronger anti-HCV activity than the other isolated compounds. According to Hsu *et al.* reported that gallic acid inhibits HCV in the entry step. This compound inactivates cell-free viral particle and blocks its attachment to the host cells. However, it has a little effect on postbinding of viral entry and fusion stage [20]. Gallic acid is also known to have antioxidant and hepatoprotective activities [21,22]. Most phenolic compounds have radical-scavenging activity. The activity depends on the number and position of hydroxyl group substituent in the molecules as which called on the antioxidant activity of flavonoids is known to be associated with the number of free hydroxyl groups [23]. Jain *et al.* also reported in his research that the antioxidant activity level can affect reduction

of chronic hepatitis and cirrhosis [19]. It is conceivable, therefore, that the anti-HCV mechanism of gallic acid involves its antioxidant activities. Other researchers reported that polyphenols like gallic acid were responsible for the anti-HCV activity in plants [24]. Recent study showed that gallic acid decreases the HCV expression through its antioxidant activities. Gallic acid reduced the level of reactive oxygen species generated by strong oxidizing agent, such as H<sub>2</sub>O<sub>2</sub>, which could inhibit viral replication. This compound attenuates the translation rate of viral proteins or decreases the stability of viral protein [25]. Moreover, gallic acid was reported to inhibit several other viruses such as influenza virus, foodborne viral surrogates, murine norovirus-1, and feline calicivirus-F9 [26,27]. The antiviral activities of gallic acid might be caused by the hydrophobic interaction between the functional group (hydroxyl) and virion components, bind to free radicals and exert an antioxidant effect [25].

## CONCLUSION

Plant of *T. sureni* is a potential source for anti-HCV agent. Ethyl acetate fraction (Fr E) of this plant is active as anti-HCV against J6/JFH1 with IC<sub>50</sub> value of 1.7 µg/ml. Ethyl gallate (1), methyl gallate (2), catechin (3), gallic acid (4), and quercetin 3-O-rhamnoside (5) were isolated from Fr E. Among the isolated compounds; gallic acid showed to possess strong anti-HCV activity with IC<sub>50</sub> value of 15.9 µg/ml. These results suggested that *T. sureni* and its isolated compound, gallic acid, may be good candidates to develop for alternative and/or complementary agents of anti-HCV infection.

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## AUTHOR CONTRIBUTION

AFH, LT, AW, HF and NK contributed to phytochemistry work. TSW, EA, AAP, MA and CAU contributed to anti-HCV work. MIL, S and HH as principle investigators, planned and coordinated the study. All the authors read and approved the final manuscript.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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