



RESEARCH ARTICLE

REVISED *Acacia mangium*: A promising plant for isolating anti-hepatitis C virus agents

[version 3; peer review: 1 approved, 2 approved with reservations, 1 not approved]

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V3 First published: 08 Dec 2022, 11:1452
<https://doi.org/10.12688/f1000research.124947.1>
 Second version: 28 Feb 2023, 11:1452
<https://doi.org/10.12688/f1000research.124947.2>
 Latest published: 02 Aug 2023, 11:1452
<https://doi.org/10.12688/f1000research.124947.3>

Abstract

Background: Medicinal plants have been demonstrated to possess various pharmacological effects including anti-hepatitis C virus. *Acacia mangium* is one of the *Acacia* genus that contain various metabolites. The metabolites play an important role for antiviral activities. The current study examined the anti-hepatitis C virus (HCV) activities of *Acacia mangium* extracts in solvents with various polarities and further evaluated the mechanism of action of the extracts on the protein virus and combination treatment models.

Methods: Anti-hepatitis C virus activities was conducted with *in vitro* cells culture of Huh 7it both in a single or combination treatment. Its NS3 protein inhibition was evaluated with western blotting assay.

Results: The results revealed the strong anti-HCV activities of the extracts. The 50% inhibition concentrations (IC₅₀s) of the ethanol, n-hexane, dichloromethane and methanol extracts were of 4.6 ± 0.3, 2.9 ± 0.2, 0.2 ± 0.3, and 2.8 ± 0.2 µg/mL, respectively, and no cytotoxic effect was detected. These extracts displayed stronger effects than the positive control ribavirin. The mode of action of the ethanol extract was evaluated at 30 µg/mL, revealing that the inhibitory effect was stronger on the post-entry step than on the entry step. Western blotting revealed that the extracts decreased NS3 protein expression, indicating that virus replication was suppressed. Further evaluation illustrated that combined treatment with the ethanol extract enhanced the anti-viral activity of simeprevir.

Conclusions: These results indicated that *A. mangium* leaves could represent sources of anti-HCV agents.

Open Peer Review

Approval Status    

	1	2	3	4
version 3 (revision) 02 Aug 2023				 view
version 2 (revision) 28 Feb 2023		 view	 view	
version 1 08 Dec 2022	 view			

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Keywords

Acacia mangium, hepatitis C virus, infectious disease, medicinal plant, medicine, health



This article is included in the **Pathogens** gateway.



This article is included in the **Plant Science** gateway.

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Competing interests: No competing interests were disclosed.

Grant information: This research was supported by the internal university grant of Universitas Airlangga with the grant number 407/UN3.14/PT/2020.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Wahyuni TS, Sukma NS, Permanasari AA *et al.* **Acacia mangium: A promising plant for isolating anti-hepatitis C virus agents [version 3; peer review: 1 approved, 2 approved with reservations, 1 not approved]** F1000Research 2023, 11:1452 <https://doi.org/10.12688/f1000research.124947.3>

First published: 08 Dec 2022, 11:1452 <https://doi.org/10.12688/f1000research.124947.1>

REVISED Amendments from Version 2

We have revised following the reviewer suggestions

1. Abstract section, background, line 3: change word "by" to "with".
2. Abstract section, Method, lines 9, 10, 11 of abstract section.
3. Method section: add sentence in cytotoxicity assay section, lines 3-5 of this section.

Any further responses from the reviewers can be found at the end of the article

Introduction

Hepatitis C virus (HCV) infection is an acute or chronic liver disease. HCV infection has a high prevalence globally, and approximately 71 million people are at risk of liver cirrhosis or hepatocellular carcinoma attributable to chronic infection (Lange *et al.*, 2014). To date, no effective hepatitis C vaccine has been developed because HCV is a commonly mutated virus (Pawlotsky *et al.*, 2015; Pawlotsky *Jm et al.*, 2018).

Hepatitis C treatment has evolved with the availability of direct acting antivirals, which have achieved sustained virological response (SVR) rates exceeding 90% (Segarra-Newnham *et al.*, 2020). However, some low-income countries cannot access those therapies because of their costs, and the combination of interferon-alpha and ribavirin (RBV), which produces an SVR rate of 50%, remains in use. Combination treatment has also been reported to have serious side effects and risks of resistance, making this strategy less effective (Swain *et al.*, 2010). Efforts to develop new agents for HCV are necessary. Further issues to overcome include the development of drugs that can inhibit the virus with fewer side effects and affordable prices for all countries. Therefore, it is necessary to develop affordable, safe, and effective HCV therapies.

Traditional herbal medicine has become a popular treatment, and plants are among the primary components of such medicines. Our previous studies reported medicinal plants possessing anti-HCV activities (Adianti *et al.*, 2014; Wahyuni *et al.*, 2013; Wahyuni *et al.*, 2014). Many medicinal plants have also been reported to inhibit HCV by inhibiting various steps of the HCV life cycle (Hussein *et al.*, 2000; Ravikumar *et al.*, 2011; Wahyuni *et al.*, 2016).

Plants in the Fabaceae family are frequently used by traditional healers to treat liver diseases, including HCV infection. The chemical compounds present in the Fabaceae family include saponins, tannins, flavonoids, proteins, stylbenoid, xanthenes, terpenes (triterpenes, diterpenes), phytoalexin, galactonate, lactogenic agents (polyketide), and anthraquinone. Most of those compounds are reported to possess anti-viral, hepatoprotective, and anti-cancer activities (Roy *et al.*, 2016). One genus of the Fabaceae family that has been demonstrated to inhibit HIV is *Acacia*, and the active species include *A. nilotica* (50% inhibitory concentration [IC₅₀] = 40.5 µg/mL) and *A. confusa* (IC₅₀ = 5 µg/mL) (Hussein *et al.*, 2000; Lee *et al.*, 2011).

A. mangium contains alkaloids, flavonoids, polyphenols, glycosides, saponins, steroids, tannins, and terpenoids, and their leaves contain phenolic groups including tannins and flavonoids. *A. mangium* was reported to contain 2,3-cis-3,4,7,8-tetrahydroxyflavanone and teracacidin (Barry *et al.*, 2005). *A. mangium* was reported to possess various bioactivities such as anti-oxidant, antibacterial activities, antifungal activities (Batiha *et al.*, 2022; Mihara *et al.*, 2005; Prayogo *et al.*, 2021). Other species of *Acacia*, *Acacia confusa* and *Acacia nilotica* were reported to inhibit HCV (Lee *et al.*, 2011; Rehman *et al.*, 2011). This current study evaluated the anti-HCV activity of various extracts of *A. mangium* leaves. In addition, the cytotoxicity of the extracts was evaluated. The mode of action was additionally assayed to determine the part(s) of the HCV life cycle inhibited by the extracts. Moreover, its mechanism of action was examined by Western blotting and combination treatment with current anti-HCV drug. This study was used simeprevir for combination treatment which known as NS3 protease inhibitor. It is particularly effective against HCV genotypes 1 and 4, making it a valuable treatment option for patients infected with those genotypes (Rice *et al.*, 2014).

Methods

Materials

A. mangium leaves were obtained from an area in Mojokerto Regency, Indonesia. The plant was verified by an expert botanist from Materia Medica Indonesia, East Java (see the *Underlying data* (Wahyuni, 2022)).

The materials used in the bioassays were as follows: Huh7it-1 cells (Apriyanto *et al.*, 2016); adapted variant of HCV strain (JHF1a) (Yu *et al.*, 2010); Dulbecco's Modified Eagle's Medium (DMEM, GIBCO Invitrogen) supplemented with 10% of fetal bovine serum (FBS, GIBCO Invitrogen), 150 µg/mL kanamycin (Sigma-Aldrich), and non-essential amino

acids (NEAAs, GIBCO Invitrogen); Dulbecco's phosphate-buffered saline (GIBCO Invitrogen); formaldehyde (HCHO, Sigma-Aldrich); trypsin-EDTA (Sigma-Aldrich); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTT, Sigma-Aldrich); bovine serum albumin (BSA, Biowest); Triton X-100 Sigma-Aldrich); 3,3'-diaminobenzidine (DAB, Thermo Fisher Scientific); anti-HCV human antibody and HRP-conjugated goat anti-human Ig antibody (Thermo Fisher Scientific); RIPA buffer; polyacrylamide gel and polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA); β -actin antibody (MBL, Nagoya, Japan); and a chemiluminescence detection system (Bio-Rad; GE Healthcare, UK).

Preparation of *A. mangium* leaf extracts

A. mangium leaves (2 kg) were dried, ground into powder, and further extracted with two kind of extraction processes. First, 200 g of powder was extracted by maceration process with a total of 2 liters of 96% ethanol and another 200 g was successively extracted with 2 liters of n-hexane, dichloromethane, and methanol. Specifically, 10 mg of the extract powder were dissolved in 100 μ L of dimethyl sulfoxide to obtain 100,000 μ g/mL stock solution (Wahyuni *et al.*, 2013).

Cell and virus culture

Huh7it-1 cells were cultured in DMEM supplemented with 10% FBS, 150 μ g/mL kanamycin, and NEAAs in 5% CO₂ at 37°C and maintained for bioassay purposes. Cells were incubated at 37 °C for 2 days. Cells which showed more than 80% confluence were used for further bioactivity assay. The detailed protocol for cell passage is available at <https://dx.doi.org/10.17504/protocols.io.n92ldpbd715b/v1>.

Virus stock was obtained by propagating HCV in Huh7it-1 cells. Culture supernatants at day 3, 5 and 7 after virus infection were collected. Virus titers were calculated by titration assay (Wahyuni, 2013). In brief the virus harvested was diluted on x5, x25, x125, x625, and x3125 then put onto Huh7it-1 cells and incubated for 4 hours, the remaining virus was removed and refed with new medium for further incubation for 2 days. The infected cells were stained with DAB staining reagent and further calculated. The number of viruses represented as titer virus. The high titer virus stock (higher than 1×10^5) was chosen for anti-HCV assay. The stock was stored at -80°C until use (Wahyuni *et al.*, 2018).

Anti-HCV activity

An anti-HCV assay was conducted using HCV-infected Huh7it-1 cells. Various concentrations of the extracts were mixed with virus solution (multiplicity of infection of 0.1) and inoculated into the cells at a final concentration of 0.01, 0.1, 1, 10, 50, or 100 μ g/mL. The cultures were incubated for 48 h with 2 h of virus inoculation and further incubation for 46 hours at 37°C. The viral levels of the supernatants were examined by titration assay. Culture cell supernatant was collected, diluted 10x with medium and inoculated to the Huh7it cells. This was incubated for 2 days and the infected cells were calculated after the immunostaining process. The inhibitory effect of the extracts were calculated compared to the untreated control. The 50% inhibitory activity was conducted by SPSS software version 25 (Wahyuni *et al.*, 2019; Wahyuni *et al.*, 2018). Ribavirin was used as the positive control.

Viral titration and immunostaining

Huh7it-1 cells were incubated with serial dilutions of the supernatant for 48 h. The cells were fixed with formaldehyde, stabilized with triton and subjected to immunostaining with primary antibody (human serum) and secondary antibodies (HRP-conjugated goat anti-human). The detailed immunostaining protocol is available in the *Underlying data* (Permanasari and Wahyuni, 2022a). DAB staining was performed to visualize the infected cells. The percent inhibitory effect was calculated by comparing the reduction of infected cells to the control (Wahyuni *et al.*, 2018).

Mode of action analysis

The mode of action assay was performed to examine whether *A. mangium* extract affected the entry or post-entry step of the HCV life cycle. Three parallel experiments were performed. First, the extract was only added to the cells during viral inoculation. Second, the extract was only added to the cells after viral inoculation. Third, the cells were treated with the extract both during and after inoculation. After 48 h of incubation, all supernatants were collected to examine their viral levels by titration assay. Viral supernatant was diluted with medium and inoculated to the Huh7it cells. Infected cells were calculated to further determine the percentage inhibitory against HCV. Anti-HCV activity was expressed by 50% inhibition concentration (IC₅₀) (Hafid *et al.*, 2017).

Cytotoxicity assay

The MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay was used to measure cytotoxicity. Huh7it-1 cells were incubated with various concentrations of the extracts for 48 h. After incubation, 10% MTT was added to the cultures for 4 h. The remaining reagent was discarded and DMSO was added in each wells to dissolve the formazan formation. Absorbance was measured at 560 and 750 nm to calculate the percentage cell viability relative to the control.

SPSS software version 25 probit analysis was used to calculate the 50% cytotoxic concentration (CC₅₀) (Wahyuni *et al.*, 2018). The protocol of the MTT assay is available at <http://dx.doi.org/10.17504/protocols.io.6qpvr4x5pgmk/v1>.

Immunoblotting assay

Huh7it-1 cells were treated with mixtures of the extracts (10 or 50 µg/mL) and HCV. After incubation for 2 days at 37 °C, the cells were collected, lysed, and protein levels were determined using a BCA assay kit (Thermo Fisher Scientific). Equal amounts of proteins were subjected to SDS–polyacrylamide gel electrophoresis followed by transfer to a polyvinylidene difluoride membrane. Samples were run in transfer buffer at 0.3 A for 30 minutes followed by processing to SDS running buffer at 0.1 A for 25 minutes. The membrane was applied into a blocking buffer of skim milk and reacted to antibodies. The primary antibody was an HCV NS3 mouse monoclonal antibody (clone H23; Abcam, Cambridge, MA, USA), and the secondary antibody was HRP-conjugated goat anti-mouse immunoglobulin. β-actin (MBL, Nagoya, Japan) served as the internal control (Permanasari *et al.*, 2021; Widyawaruyanti *et al.*, 2021). Membranes were incubated at room temperature for 1 hour in each antibody. NS3 protein expression was detected using an enhanced chemiluminescence detection system (GE Healthcare, Buckinghamshire).

Anti-HCV activity of the combination of *A. mangium* extract and simeprevir

Combination treatment was performed by adding equal volumes of *A. mangium* extract and simeprevir (Toronto Research Chemical, Canada). Simeprevir was added at 0.25×, 0.5×, 1×, 2×, and 4×IC₅₀ for monotherapy and combination. All treatments were performed for 48 h incubation. The IC₅₀ of simeprevir when used in combination with *A. mangium* extract and monotherapy were calculated and compared using the SPSS probit assay version 25 (Wahyuni *et al.*, 2020).

Results

In vitro activity of *A. mangium* against HCV

All *A. mangium* leaf extracts strongly inhibited HCV in a dose dependent manner (Figure 1). Inhibition concentrations of 50% of all extracts were calculated by probit analysis. Dichloromethane extract displayed the strongest effects, with an IC₅₀ of 0.2 ± 0.3 µg/mL, whereas the IC₅₀s of the extracts ranged 2.8–4.6 µg/mL (Table 1). While the positive control of ribavirin revealed the IC₅₀ values of 10.4 ± 0.2 µg/mL. All of the extracts were demonstrated to possess stronger activity compared to the positive control of ribavirin. The raw data are available in the *Underlying data* (Permanasari and Wahyuni, 2022b).

Cytotoxic effects of *A. mangium* extracts

The cytotoxicity assay of the extracts observed no toxic effect in the Huh7it. The percentage of cell viability demonstrated that all extracts possessed cell viability higher than 80% in the concentration of 400 µg/mL. However, n-hexane and dichloromethane extract showed a reduction in the percentage of cell viability at the dose of 400 µg/mL (Figure 2). The raw data are available in the *Underlying data* (Permanasari and Wahyuni, 2022c).

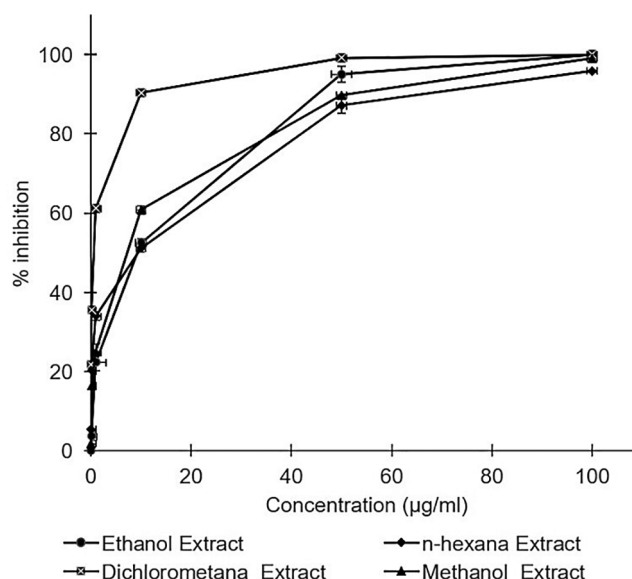
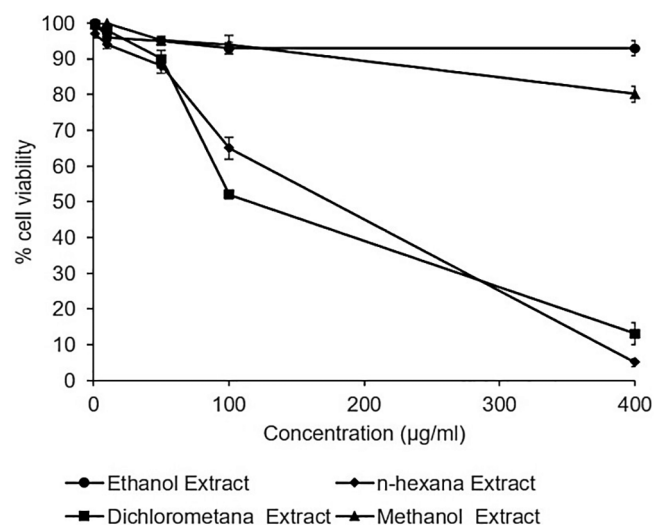


Figure 1. Concentration-dependent inhibition of hepatitis C virus infectivity by the ethanol, n-hexane, dichloromethane, and methanol extracts of *Acacia mangium*. Huh7it-1 cells were cultured and inoculated with mixtures of the virus and each extract at various concentrations. Virus inhibition was calculated relative to the untreated control. The data represent the mean of three independent experiments.

Table 1. The 50% inhibitory concentration (IC₅₀) and 50% cytotoxic concentration (CC₅₀) of *Acacia mangium* leaf extracts.

Extract/fraction	IC ₅₀ (µg/mL)	CC ₅₀ (µg/mL)	Selectivity index (CC ₅₀ /IC ₅₀)
Ethanol 96%	4.6 ± 0.3	>400	>86.9
n-Hexane	2.9 ± 0.2	121.2 ± 1.2	41.8
Dichloromethane	0.2 ± 0.3	125.6 ± 0.5	628
Methanol	2.8 ± 0.2	>400	>142.8
Ribavirin	10.4 ± 0.2	>400	>38.46

**Figure 2.** Percent viability of extracts in Huh7it-1 cells. Cells were treated with various concentrations of each extract of *Acacia mangium*. MTT (3-(4,5- Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent was added after 2 days of incubation, and cell viability was examined using a microplate reader at wavelengths of 450 and 630 nm. The percent cell viability was calculated, and the 50% cytotoxic concentrations were determined by SPSS probit analysis. The data represent the mean of three independent experiments.

Mode of action evaluation

The mode of action assay was performed using three series of experiments. The result illustrated that the inhibitory effect was higher on the post-entry step than on the entry step. The percentage virus inhibition of post entry steps revealed more than 20% differences than the entry step (Figure 3). This result suggested that the extract dominantly affected post-infection processes such as virus replication, virus assembly, and virus release. The raw data are available in the *Underlying data* (Permanasari and Wahyuni, 2022d).

Anti-HCV activity of the combination of *A. mangium* extract and simeprevir

Evaluation of the combination effect between the extract and simeprevir showed there is an enhancement of the effects of the extract to simeprevir, which is known as an HCV NS3 protein inhibitor. The addition of *A. mangium* extract increased the anti-HCV effect of simeprevir, as the IC₅₀ of simeprevir when used in combination with *A. mangium* extract was reduced by 2-fold compared to that of simeprevir alone (Table 2).

Extract of *A. mangium* inhibited NS3 protein expression

To examine the mechanism of action of the ethanol extract, western blotting analysis was performed. The result demonstrated a reduction of the NS3 protein level due to the extract intervention. Immunoblotting revealed that treatment with *A. mangium* extract at 10 or 50 µg/mL decreased the NS3 protein expression by 40% and 95%, respectively, versus the control (Figure 4) (*Underlying data*, Permanasari and Wahyuni, 2022e).

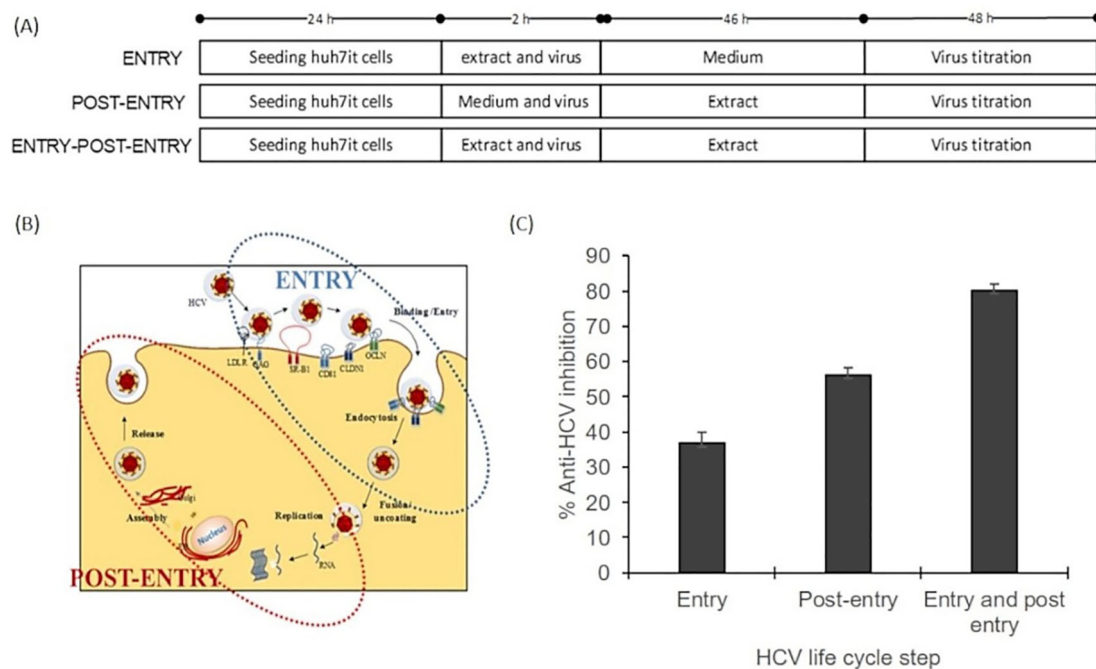


Figure 3. Mode of action analysis of the ethanol extract of *Acacia mangium* illustrated that HCV inhibition occurred dominantly in the post-entry step. (A) Cells were cultured with the ethanol extract of *A. mangium* (30 µg/mL) in three parallel experiments. First, cultured cells were treated with the extract only during inoculation (entry step). Second, cells were treated with the extract only after inoculation (post-entry step). Third, cells were treated with the extract both during and after inoculation. (B) The entry step comprises the processes of viral binding to host receptors, viral entry into the cells and endocytosis, whereas the post-entry comprises translation, replication, assembly, and release. (C) Percent inhibition of the entry step, post-entry step, and both steps. The data represent the mean ± SEM of three independent experiments.

Table 2. Anti-Hepatitis C virus activities of *Acacia mangium* in single and combination with simeprevir.

Sample	50% inhibitory concentration*
<i>Acacia mangium</i> extract alone	4.75 ± 0.07 µg/mL
Simeprevir alone	19.65 ± 0.49 nM (0.0149 ± 0.0005 µg/mL)
Combination simeprevir and <i>Acacia mangium</i> extract	9.4 ± 0.2 nM (0.0069 ± 0.00014 µg/mL)

*Data represent the mean ± SD of three independent experiments.

Discussion

The study found that *A. mangium* possesses potential anti hepatitis C virus activity through some mechanism. The anti-HCV activities of the *A. mangium* leaves extracted using solvents of different polarities, namely 96% ethanol, n-hexane, dichloromethane, and methanol was examined. The data illustrated the strong anti-HCV activities of all extracts. Moreover, no toxic effect was observed according to the CC₅₀ and selectivity index.

Ethanol is commonly used as a solvent in traditional drug development. Therefore, the strong anti-HCV activities of the ethanol extract and the lack of cytotoxicity provide necessary information for developing extracts of *A. mangium* as an alternative or complementary anti-HCV agent. However, we used various solvents with different polarities to further isolate the active anti-HCV compounds. The result demonstrated that the dichloromethane extract of *A. mangium* possessed the strongest inhibitory activity.

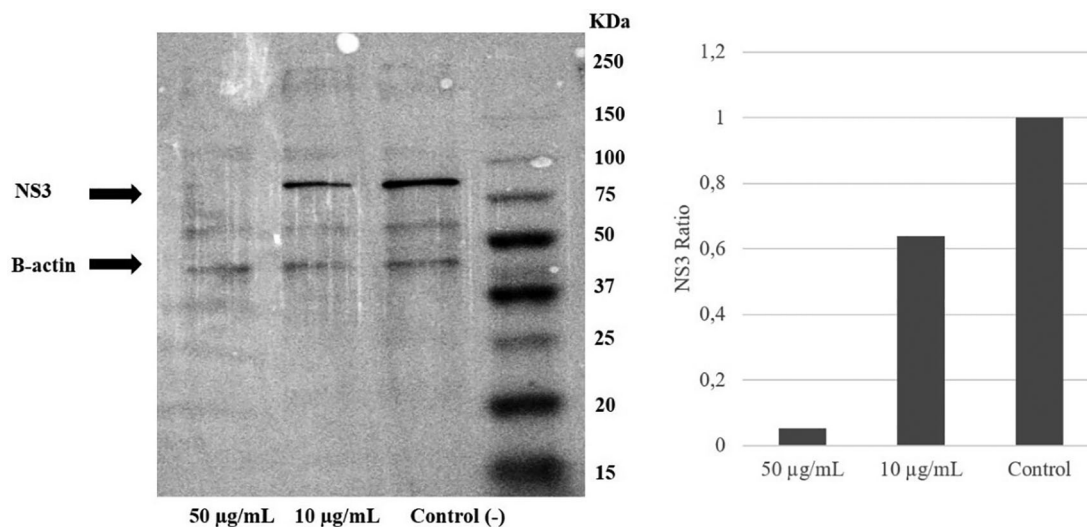


Figure 4. Extract of *Acacia mangium* reduced NS3 protein levels in a concentration-dependent manner. Huh7it-1 cells were treated with a mixture of the extract (final concentration, 10 or 50 µg/mL) and virus. The cells were lysed in RIFA buffer, and an equal amount of proteins were separated by SDS-polyacrylamide gel electrophoresis.

Mode of action analysis was performed as the first screening assay to determine the mechanism of *A. mangium* extract. The results illustrated that the ethanol extract more strongly inhibited HCV in the post-entry stage than in the entry stage. Virus production starts with binding of the virus to the host cell receptor, followed by virus entry and endocytosis. These processes are included in the entry step. The entry of HCV into hepatocytes is mediated by the viral E1 and E2 glycoproteins, which are the surface proteins of viral particles. HCV infection occurs through complex interactions of

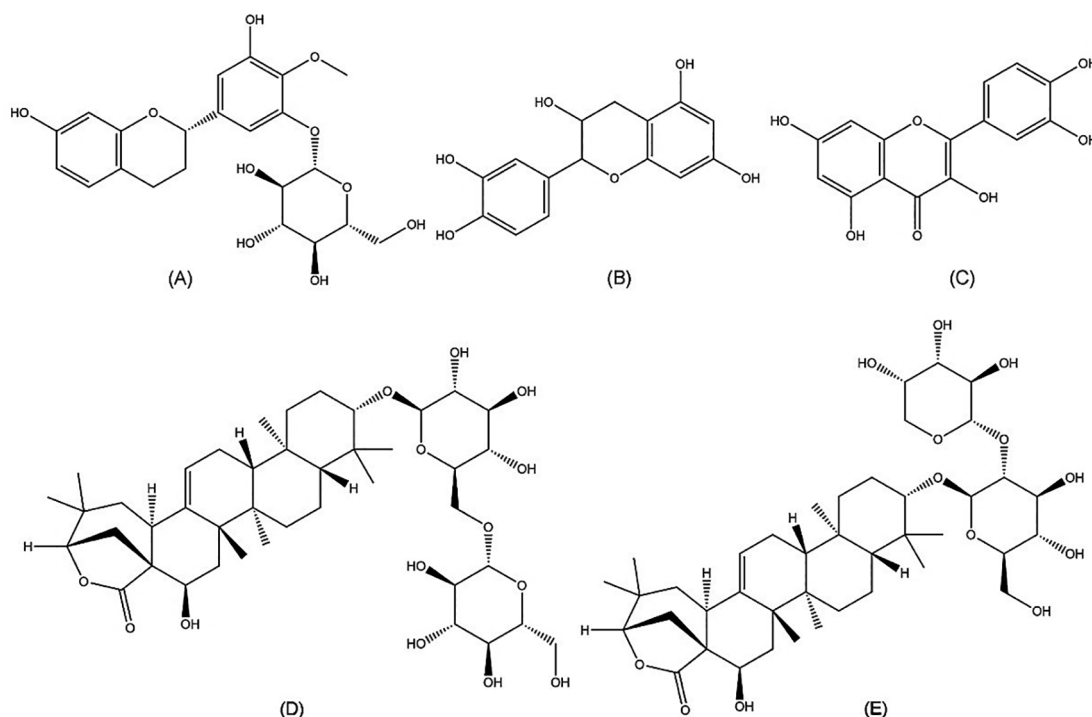


Figure 5. Molecular structures of five bioactive compounds from the *Acacia* genus. (A) Auriculose (<https://pubchem.ncbi.nlm.nih.gov/compound/Auriculose>), (B) epicatechin (<https://pubchem.ncbi.nlm.nih.gov/compound/epicatechin>), (C) quercetin (<https://pubchem.ncbi.nlm.nih.gov/compound/5280343>), (D) proacaciaside I (<https://pubchem.ncbi.nlm.nih.gov/compound/102446075>), and (E) proacaciaside II (<https://pubchem.ncbi.nlm.nih.gov/compound/102446076>).

viral lipoviral particles with cellular factors, including low-density lipoprotein receptors, glycosaminoglycans, scavenger receptor class B type I, tetraspanin (CD81), claudin-1, and occludin (Dubuisson *et al.*, 2008; Moriishi and Matsuura, 2003). Viral particles enter host cells through clathrin-mediated endocytosis, after which they are sent to the endosome. Meanwhile, the post-entry steps include translation, replication, and assembly. NS3–NS5 form a replication complex that produces new viral genomic RNA. Genomic RNA and HCV core proteins accumulate to form a nucleocapsid, which is excreted through the lumen side of the endoplasmic reticulum. After this excretion, the nucleocapsid can interact with very low-density lipoprotein (VLDL), followed by translocation to the Golgi for maturation. Mature HCV–VLDL complexes are released exocytically via the VLDL secretory pathway. Finally, new cells can be infected by released free HCV particles or by cell-to-cell transmission (Fénéant *et al.*, 2014; Lindenbach and Rice, 2013; Zeisel *et al.*, 2015).

To further clarify the mechanism of the anti-HCV effects of *A. mangium* extract, Western blotting was performed to evaluate the effect of the extract on NS3 protein. *A. mangium* extract decreased NS3 protein levels versus the control. NS3 is a non-structural virus protein that plays an important role in replication. It is an attractive target for HCV treatment. Inhibition of NS3 could result in decreased virus production. Moreover, the anti-HCV activity of the extract was evaluated in combination with the NS3 protein inhibitor simeprevir. *A. mangium* extract was demonstrated to enhance the inhibitory activity of simeprevir against HCV. This suggested that the extract potentiated the effect of simeprevir on secondary targets of HCV.

Chemical compounds play an important role in anti-HCV activities. Further isolation of the active compounds from *A. mangium* against HCV is needed. However, it has been reported that *Acacia* species are rich in polyphenols, flavonoids, alkaloids, saponins, and terpenoids. It was reported compounds in the genus *Acacia* include epicatechin, quercetin, proacaciaside I, and proacaciaside II (Figure 5). Those compounds were previously demonstrated to exhibit bioactivities such as anti-bacterial, anti-fungal, and anti-parasitic effects, which could contribute to anti-HCV properties (Chew *et al.*, 2011; Rangra *et al.*, 2019).

Conclusions

A. mangium leaf extracts possess strong anti-HCV activities without toxic effects. The extracts strongly inhibited the post-entry step, decreased NS3 protein levels, and enhanced the anti-HCV activities of simeprevir. These results suggest that *A. mangium* could be used to develop complementary and alternative treatments for HCV.

Data availability

Underlying data

Figshare: Plant Determination of *Acacia mangium*.jpg (It provided the taxonomy information of *Acacia mangium*) <https://doi.org/10.6084/m9.figshare.20973511> (Wahyuni, 2022)

Figshare: Immunostaining HCV protocol. Immunostaining HCV.docx (It provided the detail steps of immunostaining process.) <https://doi.org/10.6084/m9.figshare.20977168> (Permanasari and Wahyuni, 2022a).

Figshare: in vitro activity of *A. mangium* against HCV. raw data figure 1.docx (It demonstrated the figure of cells with infected cells, the tables which showed number of infected cells and percent inhibition of the three replication of experiments.) <https://doi.org/10.6084/m9.figshare.20977714> (Permanasari and Wahyuni, 2022b).

Figshare: Cytotoxic effect of *A. mangium* extracts. cytotoxicity.docx (It provided the percent viability of extract ethanol, n-hexane, dichloromethane, and methanol <https://doi.org/10.6084/m9.figshare.20977939> (Permanasari and Wahyuni, 2022c))

Figshare: Untitled Item. Mode of action.docx (It provided the number of infected cells in three kind of inoculation method, entry, post entry and both.) <https://doi.org/10.6084/m9.figshare.20977933> (Permanasari and Wahyuni, 2022d).

Figshare: NS3 *Acacia mangium* inhibition. NS3 beta actin 4 (1).tif (It provided the raw data of western blotting assay) <https://doi.org/10.6084/m9.figshare.21352095> (Permanasari and Wahyuni, 2022e).

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/) (CC-BY 4.0).

Acknowledgments

We would like to thank Professor Hak Hotta (Konan Women's University), Dr. Takaji Wakita (National Institute for Infectious Diseases, Japan), and Dr. Yohko Shimizu (Kobe University) for providing Huh7it-1 cells.

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Current Peer Review Status:    

Version 3

Reviewer Report 30 November 2023

<https://doi.org/10.5256/f1000research.150283.r222958>

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Moustafa Sarhan

Zoology Department, Faculty of Science, Al-Azhar University, Assiut, Egypt

In the current study, the authors evaluated the anti-HCV activity of various extracts of *Acacia mangium* leaves. They also determined the cytotoxicity of the extracts. The mode of action experiments revealed the reduction of NS3 protein levels during the post-entry step with some effects on the entry step. Overall, the work is good however there are some concerns:

1. The title needs to be improved to be more indicative of the work, like this "Evaluation of anti-hepatitis C virus of leaves extracts of *Acacia mangium*"
2. The graphs in Fig. 1 and Fig. 2 are not so clear. To make them more clear you can use colors for drawing the lines.
3. In the mode of action experiments, why you did not check the effect of the extract on the cells alone for 2 hours and then add the virus?
4. Why was ethanol extract selected to check the mode of action? Methanol extract also has a good effect! Dichloromethane has the highest effect and SI!
5. You may need to use fractionation-guided HPLC to find out the bioactive compound(s) in the leaf extract. You should add to the discussion and conclusion.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Molecular Biology, Antiviral peptides

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 05 Jun 2024

tutik sri wahyuni

Dear Reviewer,

Thank you for your comment and suggestion.

1. Regarding the title, we would like to express that this results provide the promising candidate for anti-HCV of Acacia mangium
2. We have provide the Fig 1 and Fig 2 with high resolution figures. We suggest to download the figure so it can access clearly.
3. In the mode of action we used three pararel assay. Evaluate the effect in during inoculation (sample and virus), post inoculation/post entry and whole step. The treatment of cell with extract only is suitable for evaluate pre entry/ before virus infection that suitable for prevention evaluation assay.
4. We do further analyze for ethanol extract because ethanol extract less toxic and it is a recommendation solvent in product development.
5. We have on going process for further examine the difference profile among the extract and purification to obtain the active compounds.

Thank you in advance

Competing Interests: There is no conflict of interest

Version 2

Reviewer Report 05 June 2023

<https://doi.org/10.5256/f1000research.144860.r172677>

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Borris Rosnay T Galani

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In the submitted manuscript, Wahyuni *et al.* examined the *in vitro* antiviral effects of different organic extracts of *Acacia mangium* leaves on HCV infection in cell culture systems. The extracts' selectivity indices were determined using immunostaining and cytotoxicity tests. Western blot examination of the ethanol extract revealed that it primarily functions as a replication inhibitor, boosting simeprevir effectiveness when used in combination and significantly lowering NS3 viral protease expression compared to untreated controls. This research is original and contributes to our understanding of the biological potential of this plant in the treatment of viral hepatitis.

However, certain flaws were discovered in this version that must be addressed before further consideration:

Introduction:

- Many references in the introduction are quite old, for example, Swain *et al.* 2010; Javed *et al.* 2011. The literature needs to be updated (2018-2023).
- New efforts also result in the approval of direct-acting antivirals, or DAAs, which aid in the recovery from HCV in three months. Sofosbuvir, Daclatasvir, and other DAAs are quite effective. The major restriction here is the emergence of resistance to these new medications. To validate the title of this article, I would recommend that the authors evaluate the antiviral effects of the plant extracts on HCV-resistant strains.
- Please write the species names in italics and correct spelling errors: *Acacia nilotica*, not 'Acacia nilotoca'.
- Simeprevir is an NS3/4A protease inhibitor used to treat HCV-genotypes 1 and 4 patients. Since the authors used this drug as a positive control in their tests, actual data on this compound needs to be presented in the introduction as well as its major limitation.
- Why did the authors confine the study to *in vitro* systems rather than including an *in vivo* examination? summaries of the opportunities provided by *in vitro* systems must be presented here.

Methodology:

- Please also include the length of the maceration of plant extracts.
- Please properly cite Wahyuni, 2013 and include it in the references. But this reference is also old.
- The Huh7it cell systems must be introduced correctly. What makes them superior to other JFH-1 systems?

- Two positive controls (ribavirin and simeprevir) were employed independently in this study with no evidence of their mode of action. To minimize confusion, this must be described explicitly. Also, clarify why Sofosbuvir a very popular replication inhibitor has not been used in this assessment. It would have helped to understand the extent of this effect on the replication step.
- For the suppression of HCV infectivity under the section "Anti-HCV activity," I would propose that the authors specify which viral proteins the primary (human) antibody employed for immunostaining precisely targets, and that they solely refer to prior publications (Permanasari & Wahyuni, 2022).
- I believe that at least one mean comparison test should be used to detect the statistical difference between IC50 values.
- What about the isobologram curves now that combination experiments have been completed? Before concluding the Simeprevir relationship, it would have been great to know whether this combination is synergistic or antagonistic.

Results & Discussion

- According to my interpretation of Table 1, the dichloromethane extract has the lowest IC50 value and the highest selectivity index (SI), and therefore should be used for further analysis. I'm curious why the authors chose to focus on the ethanol extract instead. Please stick to facts.
- Based on this result, I think the phytochemical characterization of the dichloromethane extract by HPLC/UV is necessary.
- Kindly support these findings with *in silico* studies of *Acacia mangium* compounds with the HCV NS3/4A protease.
- The information in Figure 1 and Table 1 is almost the same. I would advise the authors to concentrate solely on Table 1 because it includes IC50, CC50, and SI values, which are more relevant. Because IC50 is a result of a dose-dependent impact, showing it directly explains it and eliminates the need for Fig. 1.
- Fig 5: Honestly, I don't see the importance of these compounds since you have not isolated all these from your plant. In my opinion, you must isolate these compounds and test their effect on replication.
- What about the effects of these extracts on other non-structural proteins including the RdRp which is a key enzyme in the replication step?
- The effect of this extract on NS3/4A activity is also required

Conclusion

- Before concluding that *A. mangium* extracts might be exploited to produce supplementary and alternative therapies for HCV more *in vivo* research is needed to evaluate the anti-HCV potential and assess the toxicity.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmacology, Hepatology and Virology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 28 Jul 2023

tutik sri wahyuni

Dear Reviewer

Thank you for your comments and suggestions.
Herewith our respond to the reviewer comments

Introduction:

Many references in the introduction are quite old, for example, Swain et al. 2010; Javed et al. 2011. The literature needs to be updated (2018-2023).

Answer: We revised some references.

Javed was change to Segarra-Newnham M, See N, Fox-Seaman G. Retreatment of Hepatitis C Infection With Direct-Acting Antivirals. Fed Pract. 2020 Jul;37(7):316-319. PMID: 32908335; PMCID: PMC7473721.

New efforts also result in the approval of direct-acting antivirals, or DAAs, which aid in the recovery from HCV in three months. Sofosbuvir, Daclatasvir, and other DAAs are quite effective. The major restriction here is the emergence of resistance to these new medications. To validate the title of this article, I would recommend that the authors

evaluate the antiviral effects of the plant extracts on HCV-resistant strains.

Answer: Thanks for the recommendation. However, our main concern here is to show the potential of the *Acacia mangium* plant as a complementary drug against HCV. Therefore, we are also conducting combination treatment with simaprevir.

Please write the species names in italics and correct spelling errors: *Acacia nilotica*, not '*Acacia nilotoca*'.

Answer: The spelling and grammar errors have been proofread by a native English speaker and have been revised as per your suggestions.

Simeprevir is an NS3/4A protease inhibitor used to treat HCV-genotypes 1 and 4 patients. Since the authors used this drug as a positive control in their tests, actual data on this compound needs to be presented in the introduction as well as its major limitation.

Strengths of Simeprevir:

High efficacy: Simeprevir has demonstrated high rates of sustained virologic response (SVR) in clinical trials, leading to viral clearance and improved patient outcomes.

Genotype specificity: It is particularly effective against HCV genotypes 1 and 4, making it a valuable treatment option for patients infected with these genotypes.

Once-daily dosing: Simeprevir is typically administered as a once-daily oral medication, which improves convenience and patient adherence to the treatment regimen.

Weaknesses of Simeprevir:

Resistance development: The long-term use of Simeprevir, especially as a monotherapy, can lead to the development of viral resistance, potentially reducing its effectiveness.

Limited efficacy in certain populations: Simeprevir may not be as effective in certain patient populations, such as those with liver cirrhosis or prior treatment failure.

Drug interactions: Simeprevir has the potential for drug interactions, particularly with certain medications metabolized by cytochrome P450 enzymes, which may require dosage adjustments or careful monitoring.

Reference:

Rice CM, et al. (2014). "Simeprevir - a protease inhibitor for the treatment of hepatitis C virus infection." *N Engl J Med*, 370(8): 796-797.

Why did the authors confine the study to in vitro systems rather than including an in vivo examination? summaries of the opportunities provided by in vitro systems must be presented here.

Answer: The study of in vitro systems refers to conducting experiments or investigations in a controlled laboratory environment outside of a living organism. In vitro studies involve using cells, tissues, or organs cultured in a laboratory setting, often in artificial conditions that mimic aspects of the natural environment. And for HCV the in vivo model was very limited

Methodology:

Please also include the length of the maceration of plant extracts.

Answer: The maceration process has been described

Please properly cite Wahyuni, 2013 and include it in the references. But this reference is also old.

Answer: The appropriate method was described very well in Wahyuni 2013. And the method was used the same until now.

The Huh7 cell systems must be introduced correctly. What makes them superior to other JFH-1 systems?

Answer: The Huh7 cell system, is a widely used in vitro cell culture model for studying the hepatitis C virus (HCV). It is derived from the Huh-7 human hepatoma cell line and has been extensively employed for HCV research, including the study of the JFH-1 strain of the virus. Two positive controls (ribavirin and simeprevir) were employed independently in this study with no evidence of their mode of action. To minimize confusion, this must be described explicitly. Also, clarify why Sofosbuvir a very popular replication inhibitor has not been used in this assessment. It would have helped to understand the extent of this effect on the replication step.

Answer: We used ribavirin and simeprevir that still used as anti-hcv drug in some area in developing countries due to limited others DAAs

For the suppression of HCV infectivity under the section "Anti-HCV activity," I would propose that the authors specify which viral proteins the primary (human) antibody employed for immunostaining precisely targets, and that they solely refer to prior publications (Permanasari & Wahyuni, 2022).

Answer: We used the serum of a patient with a positive HCV genotype 2a, which did not have a specific viral protein target.

I believe that at least one mean comparison test should be used to detect the statistical difference between IC_{50} values.

Answer: We complete it with Student's t-test: The t-test is a parametric statistical test that compares the means of two groups. Since we have two sets of IC_{50} values

What about the isobologram curves now that combination experiments have been

completed? Before concluding the Simeprevir relationship, it would have been great to know whether this combination is synergistic or antagonistic.

Answer: We have completed with the diagram which showed synergetic effect by Combination Index (CI) analysis through compusyn software. The Combination Index is a quantitative measure used to evaluate drug interactions in combination therapies. It is calculated based on the dose-response curves of individual drugs and the combination. The CI value assesses whether the observed effect of the combination is synergistic ($CI < 1$), additive ($CI = 1$), or antagonistic ($CI > 1$).

Results & Discussion

According to my interpretation of Table 1, the dichloromethane extract has the lowest IC₅₀ value and the highest selectivity index (SI), and therefore should be used for further analysis. I'm curious why the authors chose to focus on the ethanol extract instead. Please stick to facts.

Answer: The author was more explored in ethanol extract because of the potency in developing extanol extract as herbal drug product.

Based on this result, I think the phytochemical characterization of the dichloromethane extract by HPLC/UV is necessary.

Kindly support these findings with in silico studies of Acacia mangium compounds with the HCV NS3/4A protease.

Answer: We will conduct it on the future experiments

The information in Figure 1 and Table 1 is almost the same. I would advise the authors to concentrate solely on Table 1 because it includes IC₅₀, CC₅₀, and SI values, which are more relevant. Because IC₅₀ is a result of a dose-dependent impact, showing it directly explains it and eliminates the need for Fig. 1.

Answer: The figure 1 we described the doses dependent manner among concentration and percent inhibition.

While in the table represent the IC₅₀, CC₅₀ and SI, so we think it also necessary to include figure 1.

Fig 5: Honestly, I don't see the importance of these compounds since you have not isolated all these from your plant. In my opinion, you must isolate these compounds and test their effect on replication.

Answer: The five compounds were active compounds that showed potential as both antibacterial and antiviral agents since we did not perform the isolation. This information will be very useful for predicting compounds that may play as anti-HCV agents. We agree that more study was needed to elucidate the compounds and determine their activities. What about the effects of these extracts on other non-structural proteins including the

RdRp which is a key enzyme in the replication step?

Answer: We not perform the PCR for RdRp. Our study was detected the NS3 protein level, that also a key component of the hepatitis C virus (HCV) replication. It plays essential roles in viral replication and is considered an attractive target for antiviral drug development. The effect of this extract on NS3/4A activity is also required

We agree with the reviewer suggestion, however some limiting of experiment condition that could not examine that matter

Conclusion

Before concluding that *A. mangium* extracts might be exploited to produce supplementary and alternative therapies for HCV more in vivo research is needed to evaluate the anti-HCV potential and assess the toxicity.

Answer: Yes, sure. However, our concern was to analyze the activity of *Acacia mangium* in inhibiting HCV growth in an in vitro model. The in vivo study will be conducted the the future research.

Competing Interests: There is no competing interests

Reviewer Report 19 May 2023

<https://doi.org/10.5256/f1000research.144860.r170379>

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Courage Sedem Dzah 

¹ Ho Technical University, Ho, Volta Region, Ghana

² Ho Technical University, Ho, Volta Region, Ghana

The authors have studied the antiHCV effect of leaf extracts of *Acacia mangium*. It is a good work as natural bioactives are being exploited for beneficial use. Value addition is important and so, I think this work is very good in that sense. However, there are a few issues to be addressed technically:

Abstract

1. "...*Acacia mangium* is one of the *Acacia* genus that contain with various metabolites..." - Take out the "with" I have underlined.
2. "The current study examined the anti-hepatitis C virus (HCV) activities of *Acacia mangium* extracts in solvents with various polarities and further evaluated the mechanism of action of the extracts on the protein virus and combination treatment models." - Are you

evaluating effects on combination treatment models too or on protein virus? I do not think the models are also dependent variables.

3. "Anti-hepatitis C virus activities was conducted by *in vitro* culture cells..." - Please replace "by" with "using". Because culture cells cannot conduct a test. Also, better to say cell culture than "culture cell".
4. "Further examined its NS3 protein inhibition was evaluated by western blotting assay..." - This whole statement is grammatically incorrect. Take out "examined" and replace "by" with "using".
5. What is ribavirin? Any brief details on it and why was it used as a control?
6. "The mode of action of the ethanol extract was evaluated at 30 µg/mL, revealing that the inhibitory effect was stronger on the post-entry step than on the entry step." - what does entry and post-entry steps mean in this context and why study kinetics at a higher concentration than the 4.6 ug/ml for ethanol extract?
7. Why use ribavirin as a positive control and also use simeprevir in combination treatment? Comparing simeprevir-based treatment with the control will not make sense.
8. "...The current study examined the anti-hepatitis C virus (HCV) activities of *Acacia mangium* extracts in solvents with various polarities..." - Please indicate here early enough that you used the leaves of the plant. This was not mentioned until your conclusion and it should not be so.

Methods

1. Why extract compounds from 200g of leaf powder? What informed this figure?
2. At what temperature was the extraction done?
3. For how long was the maceration done?
4. For your MTT assay, why did you not rinse your cells and take out media and add DMSO before incubating again for colour development for spectral reading? DMSO is added to MTT assays at the end of the experiment to dissolve the formazan crystals that were created during the experiment. If DMSO is not added and the formazan crystals are not dissolved, this would cause significant changes in the absorbance spectrum due to the sodium bicarbonate that would still be there.

Cytotoxic effect result

1. How sure are you that you have removed interference from cell culture media and that your result is not as a result of potential interference?

Other concerns

1. What compounds from the plant leaf specifically are responsible for the results you have gotten?
2. Figure 5 throws light on 5 bioactive compounds. Are the observations as a result of all of these compounds or some of them or just one of them?

3. Does it mean that the results are only due to these five compounds or there may be other compounds beyond these five that may give the results you have?

4. Please, do consider English Language Editing to avoid some errors.

Thank you for the opportunity.

Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: 1. Plant bioactive compounds extraction, purification, identification, characterization, bioactivity assessment, bioavailability and potential industrial applications (e.g. bioactive compound-based nanoparticles, nano-emulsions, etc) 2. Food Science and Engineering. 3. Physical processing and treatment of biomass

I confirm that I have read this submission and believe that I have an appropriate level of expertise to state that I do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 01 Jun 2023

tutik sri wahyuni

Dear Reviewer,
Thank you for your comment and suggestion.

Here with some point of our responses:

1. "...*Acacia mangium* is one of the *Acacia* genus that contain with various metabolites..." -
Take out the "with" I have underlined.

Answer: We have revised the manuscript and remove the word "with"

2. "The current study examined the anti-hepatitis C virus (HCV) activities of *Acacia mangium* extracts in solvents with various polarities and further evaluated the mechanism of action of the extracts on the protein virus and combination treatment models." - Are you evaluating effects on combination treatment models too or on protein virus? I do not think the models are also dependent variables.

Answer: Our study evaluated the effect of extract in the NS3 protein of virus. And it demonstrated the inhibition effect of extract in NS3 protein level of virus. Furthermore, the combination analysis was conducted between extract and simeprevir which showed enhancement effects of extract on anti-HCV activity of simeprevir. Those results suggested that extract has a role in protein inhibition.

However, our study did not examine directly the mixture of extract and simeprevir on protein virus.

3. "Anti-hepatitis C virus activities was conducted by *in vitro* culture cells..." - Please replace "by" with "using". Because culture cells cannot conduct a test. Also, better to say cell culture than "culture cell".

Answer: Thank you for the suggestion. We have revised in the manuscript, changed the word 'by' with 'using' and changed 'culture cells' with 'cell culture'.

4. "Further examined its NS3 protein inhibition was evaluated by western blotting assay..." - This whole statement is grammatically incorrect. Take out "examined" and replace "by" with "using".

Answer: We have revised in the manuscript

5. What is ribavirin? Any brief details on it and why was it used as a control?

Answer: Ribavirin was used as a treatment in hepatitis C virus infection. It has several mechanism, (1) Modulate the host immunity by interfere the T helper 1 and T helper 2, (2) Inhibit the inosine monophosphate dehydrogenase and depletion of guanosine triphosphate which resulting the inhibition of viral replication and protein synthesis, (3) ribavirin also act as mutagen in the viral target.

Although the Direct Acting Antiviral Agents have been used as current treatment of hepatitis C virus, however some developing area still used ribavirin and specific patient condition was need with ribavirin combination treatment.

6. "The mode of action of the ethanol extract was evaluated at 30 µg/mL, revealing that the inhibitory effect was stronger on the post-entry step than on the entry step." - what does entry and post-entry steps mean in this context and why study kinetics at a higher concentration than the 4.6 µg/ml for ethanol extract?

Answer: The Hepatitis C Virus life cycle is mainly divided into two stage; entry step (viral

adsorption and penetration) and post-entry step (uncoating, translation, replication, assembly and release). The time-of-addition study is performed to determine antiviral effect of the plant extract in the HCV life cycle, and the plant extract concentrations which exhibits >90% virus inhibition (30 µg/mL in this experiment) is usually used to analyse.

7. Why use ribavirin as a positive control and also use simeprevir in combination treatment? Comparing simeprevir-based treatment with the control will not make sense.

Answer: The purpose using ribavirin as positive control not for comparing with simeprevir. We use simeprevir as a combination treatment due to the mechanism of simeprevir as NS3 protein inhibitor.

8. "...The current study examined the anti-hepatitis C virus (HCV) activities of *Acacia mangium* extracts in solvents with various polarities..." - Please indicate here early enough that you used the leaves of the plant. This was not mentioned until your conclusion and it should not be so.

Answer: We have revised by added leave in the introduction section. We have mentioned in the material section that leaves were used of *Acacia mangium* extract.

9. Why extract compounds from 200g of leaf powder? What informed this figure?

Answer: Plant material preparation was derived from the powder of dried leaves. A number of 200 g was used for extraction process with ethanol solvent and other 200 g of *A. mangium* powder was extracted with difference polarities solvent, n-hexane, dichloromethane and methanol). 200 g of sample is enough for the research purpose.

10. At what temperature was the extraction done?

Answer: The extraction was done in the room temperature.

11. For how long was the maceration done?

Answer: The maceration was done overnight in three times.

12. For your MTT assay, why did you not rinse your cells and take out media and add DMSO before incubating again for colour development for spectral reading? DMSO is added to MTT assays at the end of the experiment to dissolve the formazan crystals that were created during the experiment. If DMSO is not added and the formazan crystals are not dissolved, this would cause significant changes in the absorbance spectrum due to the sodium bicarbonate that would still be there.

Answer: The MTT assay was conducted as you was mentioned. We have revised in the manuscript.

After 2 days incubation, the medium was discarded, rinse with medium then 150 mikroliter of 10% of MTT reagent was added and incubate for 4 hours. After incubation time removed

the MTT and DMSO was added to dissolve the formazon.

13. How sure are you that you have removed interference from cell culture media and that your result is not as a result of potential interference?

Answer: The cytotoxic assay was done following our previous method and replication was also conducted to ensure the valid result.

Other concerns

14. What compounds from the plant leaf specifically are responsible for the results you have gotten?

Answer: Our study have not deemine the single compounds with responsible to the activities. Following to the references epicatechin, quercetin, proacaciaside I, and proacaciaside II were reported as isolated compounds of Acacia genus that may contribute in their activities.

15. Figure 5 throws light on 5 bioactive compounds. Are the observations as a result of all of these compounds or some of them or just one of them?

Answer: The compounds in Figure 5 were known as isolated compounds of Acacia genus that may give contribution in its activities.

16. Does it mean that the results are only due to these five compounds or there may be other compounds beyond these five that may give the results you have?

Answer: Our studies have not determined the specific compounds with contribute for its anti-HCV activities. There are strill many unidentified compounds from this plants.

17. Please, do consider English Language Editing to avoid some errors.

Answer: We have done the proof read editing and revised some portion.

Thank you

Competing Interests: There is no conflict of interest

Version 1

Reviewer Report 21 February 2023

<https://doi.org/10.5256/f1000research.137197.r162586>

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Current study evaluated the anti-HCV activity of various extracts of *A. mangium*. In addition, the cytotoxicity of the extracts was evaluated. The mode of action was additionally assayed to determine the part(s) of the HCV life cycle inhibited by the extracts. Moreover, its mechanism of action was examined by Western blotting and combination treatment with current anti-HCV drug. The work is important for the development of novel antivirals, and the results are interesting. I recommended it to be indexed after minor revision.

For Abstract: Parts Background and Methods need to be reorganized, and the content does not match the requirements. Part Background only needs to introduce the research background. Part Methods does not need to write the specific operation process.

For Introduction: The research progress on the biological activity of *Acacia mangium* extract should be increased.

Table 1: Extracts from different solvents show different activities because the chemical components in them have changed. Can you detect which components have changed by liquid chromatography? In addition, have you ever tested the biological activity of different batches of extracts from the same solvent?

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Discovery of novel antivirals based on natural products

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 24 Feb 2023

tutik sri wahyuni

Dear Reviewer,

Thank you for the review comments.

Herewith I submitted the revised version of manuscript following the reviewer comments.

We revised the abstract section in the part of introduction and method. Other revision was also mentioning the bioactivities information of *Acacia mangium* and other *Acacia* genus in the introduction section.

Other comments were regarding the HPLC profile of each extracts that have differences polarities. Not much information regarding this data, however some information of *A. mangium* compounds and other *Acacia* genus have been explained in the text.

Comment regarding the examination of difference batch of extract have been done and gave the relatively the same activity.

Best regards,
Tutik Sri wahyuni, PhD.

Competing Interests: No competing interests were disclosed.

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