REPUBLIC OF SINGAPORE
THE PATENT ACT (CHAPTER 221)
CERTIFICATE ISSUED UNDER SECTION 35

I HEREBY CERTIFY that under the provisions of the Patent Act, a patent has been granted in respect of an invention having the following particulars:

TITLE	:	APPLICATION OF AVOCADO EXTRACT, AVOCADENOL B, AND (2R,4R)-1,2,4-TRIHYDROXY HEPTADEC-16-YNE, AND HEALTH FOOD COMPRISING AVOCADO EXTRACT
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DATE OF GRANT	:	14 May 2020

DATED THIS 14 th DAY OF MAY 2020

Daren Tang Heng Shim
Registrar of Patents
Singapore

Application of Avocado Extract, Avocadenol B, and (2R,4R)-1,2,4-Trihydroxy Heptadec-16-yne, and Health Food Comprising Avocado Extract

CROSS-REFERENCES TO RELATED APPLICATIONS

The present application claims priority to PCT Patent Application No. WO2017063150A1 filed on October 14, 2015, incorporated herein by reference in its entirely.

BACKGROUND OF THE INVENTION

The Prevention of Flaviviridae family viral infections is one of the most important issues in public health in many countries. These viruses are mostly found in arthropods, and they mainly infect mammals. Their genetic material is single-stranded RNA, which is approximately 9.6 kb to 12.3 kb in length and has a viral envelope structure.

Flaviviridae family includes a variety of viruses such as Dengue virus, Yellow fever virus, West Nile virus, Japanese encephalitis virus, Hepatitis C virus and Bovine viral diarrhea virus, and so on. These viruses can cause encephalitis, encephalomyelitis, hemorrhagic diseases, or other systemic infections in infected individuals.

Belonging to the genus Flaviviridae Genus, Dengue virus (DENV) mainly consists of three structural proteins, namely capsid protein C, membrane

protein M, and envelope protein E and 7 nonstructural proteins (NS). It has been known that some nonstructural proteins play an important role in the mechanism of Dengue viral infection and are also closely related to the symptoms caused by its infection, such as Dengue fever, Dengue shock syndrome and Dengue hemorrhagic fever.

Furthermore, Dengue viruses can be divided into four serotypes according to their antigenicity, namely DENV-1, DENV-2, DENV-3 and DENV-4, in which all are pathogenic. Dengue virus is mainly transmitted to humans through mosquitoes. Dengue viral infection diseases mainly occur in tropical and subtropical countries with Aedes aegypyi and Aedes albopictus. However, with the increased frequency of travel between countries, Dengue fever has begun to spread to other countries since the 1980s, and has gradually become a global public health problem.

However, there is currently no specific medicament for the treatment of Dengue fever, and vaccine development may be the best way to control dengue viral infections. In recent years, many researches have attempted to develop vaccines that can simultaneously immunize all four serotypes of Dengue viruses, but they still encountered many difficulties in clinical practice, such as the inability to produce vaccines with long-term immune effects. Therefore, “how to effectively prevent Dengue viral infections” is a very important public health issue.

SUMMARY

An embodiment of the present invention provides A use of avocado (Persea americana) extract in the manufacture of a health food for the prevention of Flaviviridae family viral infection.

An embodiment of the present invention provides A use of avocado (Persea americana) extract in the manufacture of a food additive for the prevention of Flaviviridae family viral infection.

A further embodiment of the present invention provides a use of avocadenol B in the manufacture of a medicament for the treatment or prevention of a viral infection of Flaviviridae family viruses.

A further embodiment of the present invention provides a use of a (2R,4R)-1,2,4-trihydroxyheptadec-16-yne in the manufacture of a medicament for the treatment or prevention of a Flaviviridae family viral infection.

A further embodiment of the present invention provides a health food for the inhibition of virus replication or viral inflammation of Flaviviridae family, including an effective amount of avocado (Persea americana) extract as an active ingredient and a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A to 1C, according to some embodiments of the present invention,

illustrate the amounts of Dengue viral protein is detected by Western blotting in Dengue virus-infected Huh-7 cells after treated with different concentration of avocado extract, avoB, and THHY.

Figures 2A to 2C, according to some embodiments of the present invention, illustrate a histogram of the relative amounts of Dengue viral RNA is detected by real-time quantitative reverse transcription polymerase chain reaction in Dengue virus-infected Huh-7 cells after treated with different concentration of avocado extract, avoB, and THHY. (*: $p<0.05$; **: $p<0.01$).

Figures 3A to 3B, according to some embodiments of the present invention, illustrate a histogram of the relative amounts of Dengue viral RNA is detected by real-time quantitative reverse transcription polymerase chain reaction in Huh-7 cells infected by Dengue virus serotypes DENV-1, DENV-2, DENV-3, and DENV-4, after treated with different concentration of avocado extract, avoB, and THHY. (*: $p<0.05$; **: $p<0.01$).

Figures 4A to 4B, 5A to 5B, and 6A to 6B, according to some embodiments of the present invention, illustrate a histogram of the relative amounts of IFN-α2 and IFN-α17 RNAs are detected by real-time quantitative reverse transcription polymerase chain reaction in Dengue virus-infected Huh-7 cells after treated with different concentration of avocado extract, avoB, and THHY.

Figures 7A to 7D and 8 to 9, according to some embodiments of the present invention, illustrate a histogram of relative amounts of OAS-2, OAS-3, and

PKR RNAs are detected by real-time quantitative reverse transcription polymerase chain reaction in Dengue virus-infected Huh-7 cells after treated with different concentration of avocado extract, avoB, and THHY.

Figures 10A to 10C, 11A to 11C, and 12A to 12C, according to some embodiments of the present invention, illustrate a histogram of relative amounts of TNF-α、IL-1β and IL-6 RNAs are detected by real-time quantitative reverse transcription polymerase chain reaction in Dengue virus-infected Huh-7 cells after treated with different concentration of avocado extract, avoB, and THHY (*: $p<0.05$; **: $p<0.01$).

Figures 13A to 13C are line graphs of the viability of Huh-7 cells under treatment with different concentration of avocado extract, avoB, and THHY in accordance with some embodiments of the present invention.

Figure 14A is a histogram of relative amounts of Japanese encephalitis viral RNA in Japanese encephalitis virus-infected BHK cells after treated with different concentration of avocado extract (*: $p<0.05$; **: $p<0.01$).

Figure 14B is a histogram of relative amounts of Japanese encephalitis viral RNA in Japanese encephalitis virus-infected BHK cells after treated with different concentration of avoB (*: $p<0.05$; **: $p<0.01$).

Figure 15A is a histogram of the relative amounts of Hepatitis C viral RNA in hepatitis C virus-infected BHK cells after treated with different

concentration of avocado extract (*: $p<0.05$; **: $p<0.01$).

Figure 15B is a histogram of relative amounts of Hepatitis C viral RNA in hepatitis C virus-infected BHK cells after treated with different concentration of THHY (*: $p<0.05$; **: $p<0.01$).

Figure 15C is a histogram of relative amounts of Hepatitis C viral RNA a in hepatitis C virus-infected BHK cells after treated with different concentration of avoB (*: $p<0.05$; **: $p<0.01$).

DESCRIPTION

The following discloses many different implementation methods or examples to implement different features of the present invention. The following describes specific elements and their examples to illustrate the present invention. Of course, these are only examples and should not limit the scope of the present invention.

The inventors of the present invention have found that the extract of avocado (Persea americana) has an effect of inhibiting dengue virus replication activity and a viral inflammation, and also has the ability to induce interferon (IFN) production by cells infected with dengue virus. In particular, the avocado extract contains avocadenol B or (2R,4R)-1,2,4-trihydroxyheptadec-16-yne as the main active ingredient.

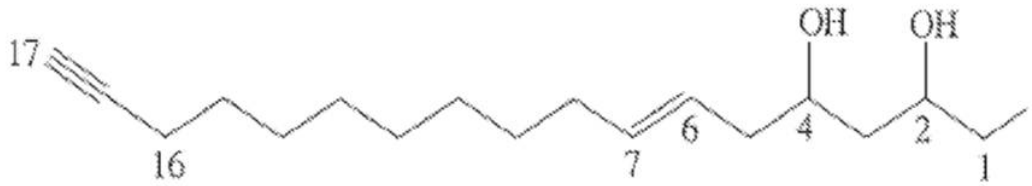
The "avocado extract" used in the present invention refers to an extract extracted from a plant of Persea americana. Avocados are native to Central America and Mexico. They belong to Lauraceae of Angiosperms family. They contain a variety of vitamins, minerals, and beneficial phytochemicals. They are rich in biological activity and anti-oxidant functions without cholesterol. Avocado is listed as the most nutritious fruit in Guinness World Records. It should be noted that the avocados used in the present invention may be derived from any origin or modified variety.

The avocado extract disclosed in the present invention is selected from avocado fruits, dried by slices, and extracted with an organic solvent. The drying temperature is 20°C to 80°C, 40°C to 60°C, for example, 50°C. The above organic solvent is a C1 to C12 alcohol, for example, methanol, ethanol, propanol, isopropanol, butanol, 2-butanol, pentanol, hexanol, heptanol, octanol, nonanol, decanol, undecanol, dodecanol, or a combination thereof, but not limited thereto. The organic solvent is also an aromatic hydrocarbon such as, but not limited to, benzene, toluene or xylene. In one embodiment, methanol is used as the extraction solution, and the concentration of methanol is 80% to 100%, for example, 99.5%.

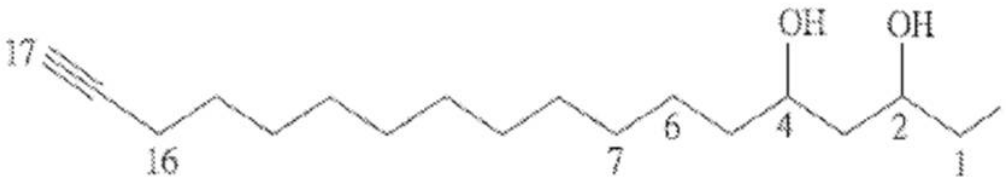
The extraction temperature and time can be determined according to the conditions of the solvent used and the like, and is not particularly limited. The extraction temperature is 5°C to 50°C or 10°C to 30°C, for example, 25°C. Further, the above extraction step can be repeated times to obtain a higher purity extract, for example, the extraction can be repeated 3 times.

Then, the avocado extract obtained in the above extraction step can be further purified to further enhance its purity. The purification step could be column chromatography, thin layer chromatography, gas chromatography, high performance liquid chromatography, ion exchange chromatography or combinations thereof. For example, column chromatography packed with silica can be used.

Through the above-mentioned extraction and purification steps, nuclear magnetic resonance (NMR) analysis and alignment avocadenol B (as shown in the following formula (I) and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne (as shown in formula (II) below) are obtained.



Formula (I)



Formula (II)

Avocadenol B, chemical formula $C_{17}H_{30}O_3$, full name (2R,4R,6E)-1,2,4-trihydroxyheptadec-6-en-16-yne. Studies have confirmed that avocadenol B has anti-mycobacterial activity (*Y.-C.Lu et al. Secondary metabolites from the unripe of Persea americana and their antimycobacterial activities. Food Chemistry 135 (2012) 2904-0929*).

The chemical formula of (2R,4R)-1,2,4-trihydroxyheptadec-16-yne is $C_{17}H_{32}O_3$. No study has pointed out related biological activity.

The inventors performed a cell test of the above avocado extract and avocadenol B and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne produced by purification of the avocado extract. It has been found that avocado extract, avocadenol B, and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne have the efficacy of inhibiting Flaviviridae family virus.

It should be noted that there is no current literature indicating the relationship of the prevention or treatment avocado extract, avocadenol B and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne and Flaviviridae family viruses.

In view of the above "Yellow Fever Virus" viruses disclosed in the present invention include Dengue virus, Yellow fever virus, West Nile virus, Japanese encephalitis, Virus or Hepatitis C virus, etc. It includes all viruses belonging to Flaviviridae family.

In some embodiments, the avocado extract, avocadenol B, and

(2R,4R)-1,2,4-trihydroxyheptadec-16-yne inhibit the protein and RNA production of dengue virus, i.e., inhibit viral replication activity. In some embodiments, the avocado extract, avocadenol B, and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne inhibit the dengue virus-induced inflammation. Also, in some embodiments, the avocado extract, avocadenol B, and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne can induce interferon (IFN) production by cells infected with dengue virus.

Therefore, the present invention also provides a use of the above avocado extract, avocadenol B or (2R,4R)-1,2,4-trihydroxyheptadec-16-yne for preparing of health food or food additives in the prevention of infection by yellow fever virus. Furthermore, the present invention also provides a use of avocado extract, avocadenol B, or (2R,4R)-1,2,4-trihydroxyheptadec-16-yne in the manufacture of a medicament in treatment or prevention of yellow fever virus (Dengue virus). In addition, it can also be used in the manufacture of dietary supplements, nutritional products or medical foods.

In an embodiment, the above health food, food additive, or medicament for preventing an infection Yellow fever virus can further include a pharmaceutically acceptable carrier or salt. The pharmaceutically acceptable carrier or salt could account for 0.5 to 99 wt%, preferably 5 to 95 wt% of the health food, food additive or medicament.

The above pharmaceutically acceptable carriers include additives, excipients, preservatives, flavoring agents and the like that are generally used in the

manufacture of foods or medicaments. For example, starch, corn starch, lactose, dextrin, cyclodextrin, methylcellulose, carboxymethylcellulose, sodium carboxymethylcellulose, gelatin, gum, agar, guar gum, pectin, acacia, tragacanth, carrageenan, or similar additives. Further, pharmaceutically acceptable carriers can also be solvents, dispersion mediums, coatings, antibacterial or antifungal agents, and the like.

Further, pharmaceutically acceptable salts could be inorganic cations, for example, alkali metal salts such as sodium, potassium or ammonium salts; alkaline earth metal salts such as magnesium, calcium salts; salts containing divalent or tetravalent cations such as zinc, aluminum or zirconium salts. In addition, pharmaceutically acceptable salts could also be organic salts such as dicyclohexylamine salts, methyl-D-glucosamine, amino acid salts such as arginine, lysine, histamineor glutamine and so on.

The aforementioned medicaments could be appropriately designed according to the route of administration, such as, tablets, capsules, film-coated tablets, powders, granules, syrups, suspensions, emulsions, injections, suppositories, or patches, etc. The administration route could be, for example, oral administration, subcutaneous injection, intraperitoneal injection, intravenous injection, intramuscular injection, anal administration, inhalatory administration, or local administration. The dosage of the medicament can be appropriately formulated according to the conditions such as patient's weight, age, symptoms of the affected area, physiological conditions, and administration route according to the conditions of the physician or persons in

charge.

In addition, the "effective amount" used in the present invention refers to a dose that has the ability to inhibit viral activity, kill the virus, reduce the number of viruses, or completely eliminate the virus. This effective amount is usually administered to the patient based on the patient's body surface area, the patient's weight, and the condition of the patient. As known to those skilled in the art, the effective dose will also vary with the following conditions, including the route of administration of the medicament, the dosage form of the medicament, or combination with other treatments.

In summary, the inventors of the present invention have found that the avocado extract has the efficacy of inhibiting Flaviviridae family virus. In particular, the avocado extract inhibits dengue virus replication activity and viral inflammation, and also induces dengue virus-infected cells to produce interferon (IFN) against viruses. Further, the inventors also found that the avocado extract contains avocadenol B or (2R,4R)-1,2,4-trihydroxyheptadec-16-yne as a main component of the activity of dengue virus.

The following further describes the present invention in detail by using examples and comparative examples, but it is not intended to limit the content of the present disclosure.

Example

Extraction and purification of avocado

According to the methods mentioned in *Y.-C. Lu et al. Secondary metabolites from the unripe of Persea americana and their anti-mycobacterial activities. Food Chemistry 135 (2012) 2904-0929*, the immature avocado fruit (about 11.9kg) was sliced and placed in an oven at 50°C to obtain a dried avocado sample (about 2.3kg, 19.3% of the original weight). The dried avocado samples were extracted with methanol with the concentration greater than 99.5% at room temperature and the extraction steps were repeated three times. Then, an ethyl acetate (EtOAc) aqueous solution (EtOAc: H_2O=1:1) was added to separate the methanol extract into an EtOAc-soluble fraction and a water-soluble fraction (H_2O-soluble fraction). About 280g of the ethyl acetate-soluble fraction and about 283 g of the water-soluble fraction were obtained, wherein the ethyl acetate-soluble fraction was the avocado extract used in the subsequent experiments.

Subsequently, a portion of the ethyl acetate-soluble fraction (about 100g) was added into a silica gel-packed column (70-230, Merck) for chromatography and purification. After a concentration gradient elution with n-hexane-EtOAc, 12 fractions (A-1 to A-12) were obtained. Next, 10.5g of fraction A-12 was recrystallized with n-hexane to obtain crystals (A-12-C) and mother liquor (A-12-M).

Next, 10 g of A-12-M was chromatographed on a silica gel-packed column (230-400 mesh, Merck) and eluted with a concentration gradient of n-hexane-ethyl acetate to obtain 7 fractions (A-12-M-1 to A-12-M-7).

Further, 7.3 g of A-12-M-4 was chromatographed on a RP-C18 column (spherical C18 100A reversed-phase silica gel (RP-18), 20-40 μM, Silicycle) and eluted with acetone-water (1:1). After elution, 25.2 mg of avocadenol B and 113 mg of (2R,4R)-1,2,4-trihydroxyhexadec-16-yne were obtained. For convenience, avocadenol B and (2R,4R)-1,2,4- trihydroxyheptadec-16-yne are abbreviated as avoB and THHY, respectively, in the following description.

Detection of viral proteins produced in Huh-7 cells

Human hepatoma cell line Huh-7 cells were infected with dengue virus strain 16681 (DENV-2 serotype), and Huh-7 cells were cultured in 24-well plates. The density of Huh-7 cells was $5x10^4$ cells/well. The MOI (multiplicity of infection) of the virus was 0.2. The virus-infected Huh-7 cells were then treated with different concentration of avocado extract, avoB and THHY, and cultured for 3 days. It should be noted that the experimental conditions for dengue virus-infected Huh-7 cells in the following experiments were the same unless otherwise specified. Next, the above cells were dissolved in a RIPA lysis buffer, and the cell lysate was centrifuged to collect the total proteins of Huh-7 cells.

Next, Western blotting was performed to detect dengue virus protein production in Huh-7 cells. The viral protein NS2B was used as a target and detected using a rabbit polyclonal anti-NS2B antibody (GeneTex, CA, USA). GAPDH, stable expression in cells, was used as an internal control. Signal detection was carried out using the ECL detection kit (PerkinElmer, CT).

Western blotting results in Figures 1A to 1C show the viral protein content in dengue virus-infected Huh-7 cells treated with different concentration of avocado extract, avoB, and THHY, respectively, while 0.1% DMSO serves as the control. Figures 1A to 1C demonstrate that when the treating concentration of avocado extract, avoB, or THHY increased, the production of dengue virus protein in Huh-7 cells decreased. Avocado extract, avoB, and THHY all inhibited dengue virus protein production, and the inhibitory effects were significant and concentration-dependent. This indicates that the avocado extract, avoB and THHY have the effects of inhibiting protein production of dengue virus.

Detection of viral RNA produced in Huh-7 cells

In addition, the inventors also detected the amounts of viral RNA produced in dengue virus-infected Huh-7 cells to further confirm the aforementioned protein in test results. Dengue virus strain 16681 (DENV-2 serotype) or four other serotypes of dengue virus strains DENV-1, DENV-2, DENV-3, and DENV-4 were used to infect human hepatoma cell line Huh-7 cells (DENV-1 to DENV-4 virus strains were obtained from the Centers for Disease Control, R.O.C. (Taiwan); DENV-1, No. 8700828A; DENV-2, No. 454009A; DENV-3, No. 8700829A; DENV-4, No. S9201818). The total cellular RNA of Huh-7 cells was purified using the Trizol reagent (Invitrogen, Carlsbad, CA).

Next, the amount of viral RNA in dengue virus-infected Huh-7 cells was detected by real-time quantitative reverse transcription polymerase chain reaction. This PCR reaction was performed under a reaction volume of 10 μl,

wherein the reaction solution contained 200 ng of cDNA, 5 μl of Power SYBER Green PCR Master and 0.4 μM of a primer pair. The PCR reaction condition was set to: 95°C for 10 minutes → [95°C for 15 seconds → 60°C for 1 minute] for 40 cycles → 95°C for 15 seconds → 60°C for 1 minute → 95°C for 15 seconds. In this experiment, the primer pairs were specifically recognizable for the viral protein NS2 of dengue virus, and are specified by the sequence identification numbers (SEQ ID NOs) 1 and 2. The internal control GAPDH was also detected in host cells in this experiment, using the primer pairs specified by SEQ ID NOs 3 and 4.

Figures 2A to 2C show real-time quantitative reverse transcription polymerase chain reaction detection of relative viral RNA levels of in Huh-7 cells infected with 16681 dengue virus at various treating concentration of avocado extract, avoB, and THHY, respectively, where the control in these histogram is 0.1% DMSO. The data in these figures were all quantified by GAPDH normalization.

Figures 2A to 2C show that the dengue virus RNA was significantly reduced in infected Huh-7 cells when the cells were treated with increasing concentration of avocado extract, avoB, and THHY (t-test, $p<0.05$; $p< 0.01$). In addition, the effective concentration 50 (EC_{50}) of avocado extract, avoB, and THHY for inhibiting the production of dengue virus RNA was calculated by interpolation to be 36±3.4 μg/ml, 7.6±1.3 μM, and 2.9±2.6 μM, respectively.

In addition, Figures 3A and 3B show the real-time quantitative reverse

transcription polymerase chain reaction detection of relative RNA levels of Dengue virus in Huh-7 cells infected with four different serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) when the cells were treated with different concentration of avocado extract, avoB, and THHY. A histogram exhibited the relative viral RNA levels in dengue virus-infected Huh-7 cells, all the data was quantified by GAPDH normalization.

As shown in Figure. 3A, dengue virus RNA was significantly reduced in infected Huh-7 cells when the treating concentration of avocado extract increased (t-test, $p<0.05$; $p<0.01$). The results were similar for the four serotypes of dengue virus (DENV-1 to DENV-4). In other words, avocado extract can effectively inhibit the RNA synthesis of dengue virus of DENV-1, DENV-2, DENV-3 and DENV-4. Calculated by interpolation, the EC_{50} of avocado extract in inhibiting the viral RNA production of DENV-1, DENV-2, DENV-3, and DENV-4 were 65±5.1μg/ml、42±6.1μg/ml、33±4.8μg/ml and 74±3.4μg/ml, respectively.

Furthermore, as shown in Figure 3B, when the treating concentration of avoB and THHY increased, the dengue virus RNA in infected Huh-7 cells was significantly reduced (t-test, $p<0.05$; $p<0.01$). The results were similar for the four serotypes of dengue virus (DENV-1 to DENV-4). As demonstrated by the results, avoB and THHY effectively inhibited the RNA synthesis of dengue viruses of DENV-1to DENV-4. Calculated by interpolation, the EC_{50} of avoB in inhibiting the viral RNA production of DENV-1, DENV-2, DENV-3, and DENV-4 were 14.4±2.1 μM, 8.4±1.8 μM, and 13.4±2.2 μM and 15.2±4.1 μM,

respectively, while the EC_{50} of THHY in inhibiting the viral RNA production of DENV-1, DENV-2, DENV-3, and DENV-4 were 16.3±3.4μM、3.4±1.1μM、13.7±4.1μM, and 14.7±2.3μM, respectively.

Detection of interferon produced in Huh-7 cells

Interferon (IFN) is a cytokine secreted by a cell infected with virus. The interferon promotes the uninfected cells to synthesize antiviral proteins to prevent the spread of infection by interaction with relevant receptors on surrounding uninfected cells. Therefore, it has antiviral effect. However, many viruses have the ability against interferons. For example, dengue viruses can disrupt the signal transduction pathway of interferon production in the host cell. Based on the above, the inventors performed follow-up experiments to investigate whether avocado extract, avoB, and THHY would affect the interferon signal transduction pathway in dengue virus-infected cells.

The experimental method was also performed with the aforementioned real-time quantitative reverse transcription polymerase chain reaction, and the target of detection was changed to the RNA of the interferon gene in the host cell. Here, primer pairs with recognition specificity for the interferon genes (IFN-α2 and IFN-α17) were used. In particular, primer pairs shown in SEQ ID NOs: 5 and 6 were used to detect RNA of IFN-α2, and primer pairs shown in SEQ ID NOs. 7 and 8 were used to detect RNA of IFN-α17.

Figures 4A to 4B, 5A to 5B, and 6A to 6B showed the histogram of the relative amounts of RNA of IFN-α2 and IFN-α17 in Huh-7 cells infected with

16681 dengue virus under treatment of different concentration of avocado extract, avoB, and THHY. The amounts were detected by real-time quantitative reverse transcription polymerase chain reaction. The control groups shown in figures were treated with 0.1% DMSO. Furthermore, the data in the figures were all quantified by GAPDH normalization.

As shown in Figures 4A to 4B, 5A to 5B, and 6A to 6B, when the concentration of avocado extract, avoB, and THHY in Huh-7 cells were increased, the amounts of IFN-α2 and IFN-α17 RNAs produced in Huh-7 cells were increased significantly. From the above results, avocado extract, avoB and THHY all induced dengue virus-infected cells to produce interferon against dengue virus.

In addition, the inventors also conducted relevant tests on the downstream signal transduction molecules of interferon, 2'-5' oligoadenylate synthase-1 (OAS-1), OAS-2, OAS-3, and PKR. The effect of interferon signal transduction pathway in infected cells to avocado extract, avoB, and THHY were further confirmed. Furthermore, the RNA of OAS-1 was detected using the primer pairs shown in SEQ ID NOs. 9 and 10; the RNA of OAS-2 was detected using the primer pairs shown in SEQ ID NOs. 11 and 12; the RNA of OAS-3 was detected using the primer pairs shown in SEQ ID NOs. 13 and 14; and the RNA of PKR was detected using the primer pairs shown in SEQ ID NOs. 15 and 16.

Figures 7A to 7D showed the histogram of the relative amounts of RNA of

OAS-1, OAS-2, OAS-3, and PKR in Huh-7 cells infected with 16681 dengue virus under treatment of different concentration of avocado extract, avoB, and THHY. The amounts were detected by real-time quantitative reverse transcription polymerase chain reaction. The control groups shown in Figures were treated with 0.1% DMSO. Furthermore, the data in the figures were all quantified by GAPDH normalization. Similarly, Figures 8 to 9 showed the histograms of relative RNA amounts of OAS-1, OAS-2 and OAS-3 in Huh-7 cells infected with the 16681 dengue virus.

As shown in Figures 7A to 7D and 8 to 9, when the concentration of avocado extract, avoB, and THHY in Huh-7 cells were increased, the amounts of OAS-1, OAS-2, OAS-3, and PKR RNAs produced in Huh-7 cells were increased significantly. Therefore, avocado extract, avoB, and THHY could not only induce interferon production in cells infected with dengue virus, but also promote the synthesis of downstream signal transduction molecules of interferons. Therefore, the mechanism of avocado extract, avoB, and THHY in suppressing dengue virus is indeed closely related to interferons. Furthermore, it could be further deduced that the mechanisms of avocado extract, avoB, and THHY in suppressing dengue virus could be achieved by restoring or enhancing the production of antiviral interferon.

Examination of virus-induced inflammatory responses in Huh-7 cells

Following experiments were performed to determine the effect of avocado extract, avoB, and THHY on virus-induced inflammatory responses.

Experiments were performed with real-time quantitative reverse transcription polymerase chain reaction as described above. The targets of detection were inflammatory reaction associated factors or cytokine RNA in the host cell. Primer sets with specific recognition for tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1 beta), and cytokine-6 (IL-6) were used. In detail, RNA of TNF-α was detected using the primer sets shown in SEQ ID NOs. 17 and 18; RNA of IL-1β was detected using the primer sets shown in SEQ ID NOs: 19 and 20; and RNA of IL-6 was detected using the primer sets shown in SEQ ID Nos. 21 and 22 were used.

Figures 10A to10C, Figures 11A to 11C, and Figures 12A-12C showed the results of real-time quantitative reverse transcription polymerase chain reaction analysis of the relative RNA levels of TNF-α, IL-1β and IL-6 in 16681-dengue-virus-infected Huh-7 cells treated with various concentrations of avocado extract, avoB, and THHY, respectively. The results were shown as histograms. The control in the figures was 0.1% DMSO. The data in the figures was normalized of GAPDH.

As shown in Figures 10A to 10C, 11A to 11C, and 12A to 12C, the RNA production of TNF-α, IL-1β, and IL-6 in Huh-7 cells was significantly reduced as the concentration of avocado extract, avoB, and THHY in Huh-7 cells increased (t-test, $p<0.05$; $p<0.01$). The result indicated that avocado extract, avoB, and THHY could effectively inhibit the inflammatory responses caused by dengue virus infection. In addition, by using the interpolation method, the EC_{50} of avocado extract inhibiting RNA production of TNF-α, IL-1β, and IL-6

could be estimated of 56.7±2.1 μg/ml, 67.4±4.9 μg/ml, and 80.6±1.1 μg/ml respectively. The EC_{50} of avoB inhibiting TNF-α, IL-1β, and IL-6 RNA production were 11.7±3.4 μM, 8.7±3.1 μM, and 22.4±5.7 μM, respectively. The EC_{50} of THHY in inhibiting TNF-α, IL-1β, and IL-6 RNA production were 7.8±2.7 μM, 4.1±1.4 μM, and 48.6±4.2 μM, respectively.

Cytotoxicity analysis

The following experiments were performed to determine the cell viability of avocado extract, avoB, and THHY concentration on Huh-7 cells.

Huh-7 cells were treated with various concentrations of avocado extract, avoB, and THHY. After a 3-day incubation, the cell viability of avocado extract, avoB, and THHY on Huh-7 cells (cytotoxicity) were assayed by MTS assay kit, CellTiter 96 Aqueous One Solution Cell Proliferation assay system Promega, WI, USA. The experiment was performed according to the manufacturer's instructions. The absorption at 490 nm was measured with a 550 BioRad plate-reader (Bio-Rad, Hertfordshire, UK).

After the experiment results were converted, the relative survival rates of Huh-7 cells at different concentration of the avocado extract, avoB and THHY were obtained. The results were shown in Figures 13A to 13C, and the control group in the figures was treated with 0.1% DMSO. The data in the figures was the results obtained from the three repetitive experiments. It should be noted that the CC50 (cytotoxic concentration 50) of the avocado extract, avoB and THHY for Huh-7 cells were 960±5.8 μg/ml, 103±6.2 μM and 142±4.7 μM,

respectively.

In the above experiments, the highest concentration of avocado extract, avoB, and THHY that treated Huh-7 cells were not more than 80 μg/ml, 20 μM and 20 μM, respectively. The highest concentration was much lower than CC50 of the avocado extract, avoB and THHY. In light of this, the above concentration of the avocado extract, avoB and THHY that treated Huh-7 cells treated were not toxic to Huh-7 cells.

In summary, the avocado extract, the avocadenol B and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne could effectively inhibit the generation of RNA and protein of the dengue virus, and have effects for inhibiting the replication activity of the dengue virus. In further, the avocado extract, the avocadenol B and (2R,4R)-1,2,4-trihydroxyheptadec-16-yne could induce the cells infected with the dengue virus to produce interferons against the virus. In addition, they also effectively inhibited the inflammatory response induced by the dengue virus.

It is worth noting that the mechanisms of the avocado extract, avoB and THHY for inhibiting the dengue virus were likely to be achieved by recovering or enhancing the interferon against the viruses in the cells infected with the virus.

Inhibition test of Japanese encephalitis virus

The experiments selected the BHK cells which were infected with Japanese

encephalitis virus. After the cells were treated with different concentration of the avocado extract (50 to 200 μg/ml) and AvoB (1 to 20 μM), the RNA of Japanese encephalitis virus (normalized by GAPDH) was quantified. The RNA content of the BHK cells treated with 0.1% DMSO (also infected with Japanese encephalitis virus) was used as a control group. The results were shown in Figures 14A and 14B, respectively. It demonstrated that the avocado extract and avoB of the present invention all suppressed the replication activity of Japanese encephalitis virus.

Hepatitis C virus inhibition test

The experiments selected the Huh-7 cells infected with the hepatitis C virus. After the cells were treated with different concentration of the avocado extract (20 to 50 μg/ml), THHY (1 to 20 μM) and avoB (1 to 20 μM), the RNA of the hepatitis C virus (normalized by GAPDH) was quantified. The RNA content of the Huh-7 cells treated with 0.1% DMSO (also infected with the hepatitis C virus) was used as a control group. The results were shown in Figures 15A, 15B and 15C, respectively. It demonstrated that the avocado extract, THHY and avoB of the present invention all suppressed the replication activity of the hepatitis C virus.

Although the present invention has been disclosed in several preferred embodiments as described above, it is not intended to limit the present invention. Any person having ordinary skill in the art can make arbitrary modifications without departing from the spirit and scope of the present invention. Therefore, the scope of protection of the present invention shall be

subject to the definition of the scope of the claims.

SEQUENCE

<110> KAOHSIUNG MEDICAL UNIVERSITY

<120> Application of avocado extract, avocadenol B, and (2r,4r)-1,2,4-trihydroxy heptadec-16-yne, and health food comprising avocado extract

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WHAT IS CLAIMED IS:

1. A use of avocado fruit (Persea americana) extract in manufacture of a health food against Flaviviridae family virus infection, wherein the extract comprises avocadenol B or (2R,4R)-1,2,4-trihydroxyhepta-16-yne.

2. The use according to claim 1, wherein the Flaviviridae family virus comprises: Dengue virus, Yellow fever virus, West Nile virus, Japanese encephalitis virus or Hepatitis C virus.

3. The use of claim 1 or 2, wherein the avocado extract has ability to inhibit production of proteins and RNAs of dengue virus.

4. The use of claim 1 or 2, wherein the avocado extract has ability to inhibit dengue virus-induced inflammatory reactions.

5. The use of claim 1 or 2, wherein the avocado extract has ability to induce production of interferon by cells infected with Dengue virus.

6. A use of avocado fruit (Persea americana) extract in manufacture of a food additive against a Flaviviridae family virus infection, wherein the extract comprises avocadenol B or (2R,4R)-1,2,4-trihydroxyhepta-16-yne.

7. The use according to claim 6, wherein the Flaviviridae family virus comprises: Dengue virus, Yellow fever virus, West Nile virus, Japanese

encephalitis virus or Hepatitis C virus.

8. A use of avocadenol B in manufacture of a medicament for the treatment or prevention of infection by Flaviviridae family virus.

9. A use of (2R,4R)-1,2,4-trihydroxyhepta-16-yne for preparation in manufacture of a medicament for treatment or prevention of infection by Flaviviridae family virus.

10. A health food that inhibits virus replication activity or viral inflammatory response of Flaviviridae family virus, comprising:

    an effective amount of avocado fruit (Persea americana) extract as an active ingredient, wherein the extract comprises avocadenol B or (2R,4R)-1,2,4-trihydroxyhepta-16-yne, and

    a pharmaceutically acceptable carrier or salts.

11. The health food according to claim 10, wherein the Flaviviridae family virus comprises: Dengue virus, Yellow fever virus, West Nile virus, Japanese encephalitis virus or Hepatitis C virus.

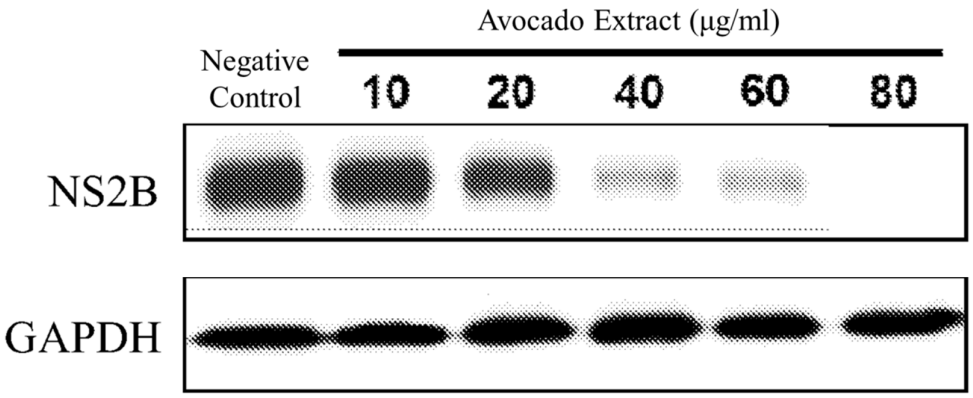


Figure 1A

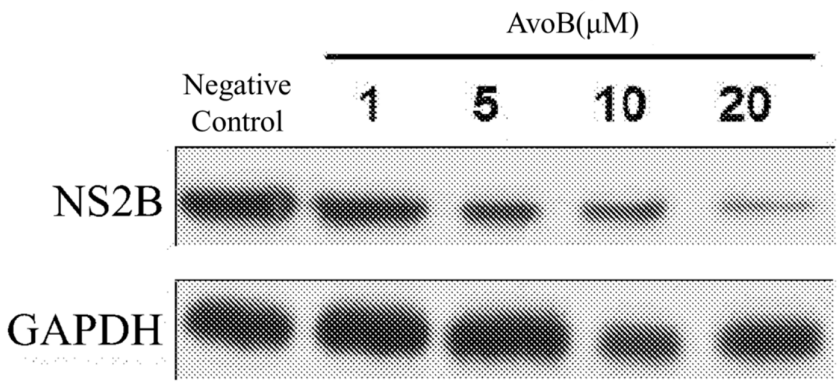


Figure 1B

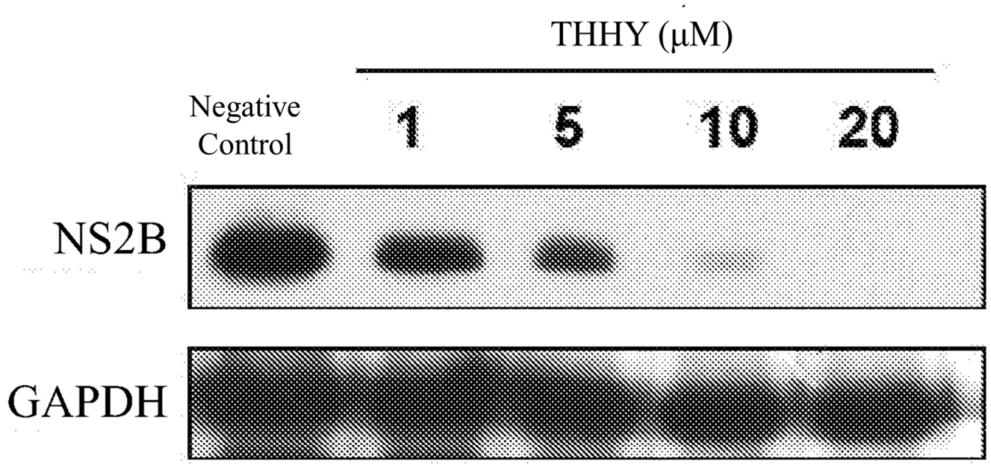


Figure 1C

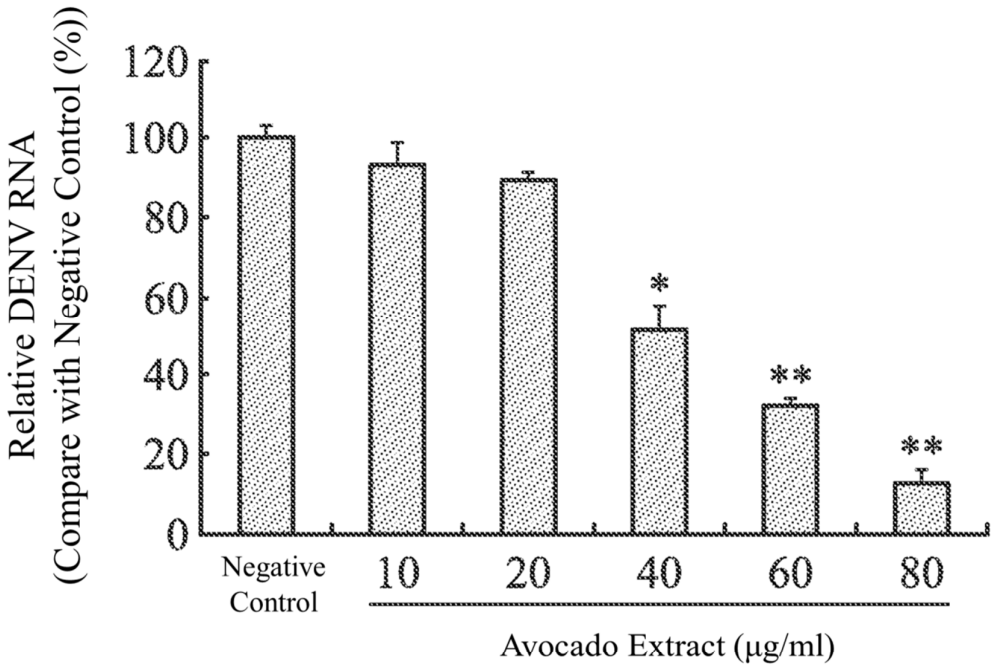


Figure 2A

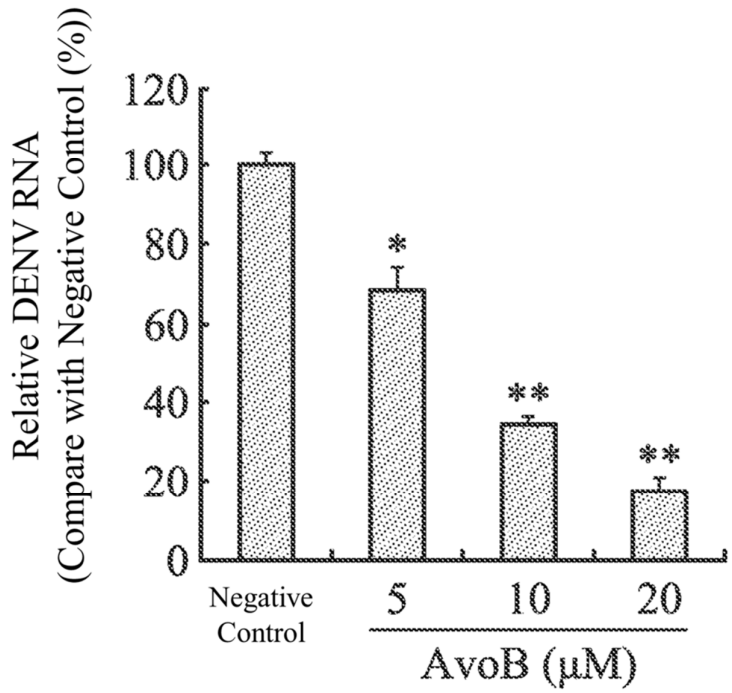
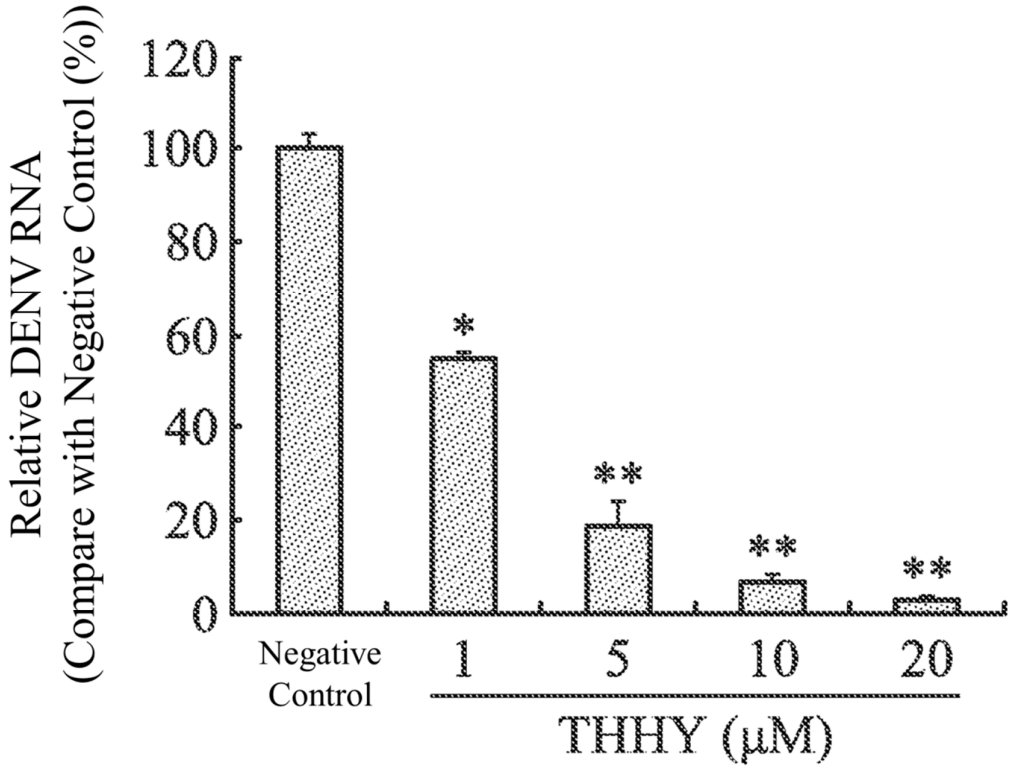


Figure 2B


Relative DENV RNA
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100
80
60
40
20
0
*
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**
**
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5
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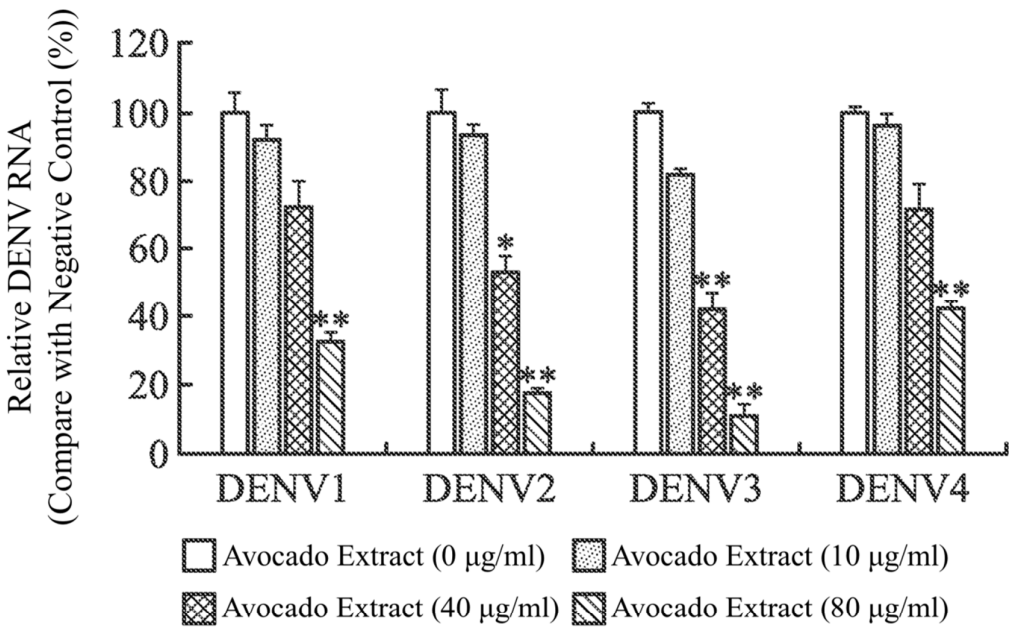
20
THHY (μM)

Figure 2C



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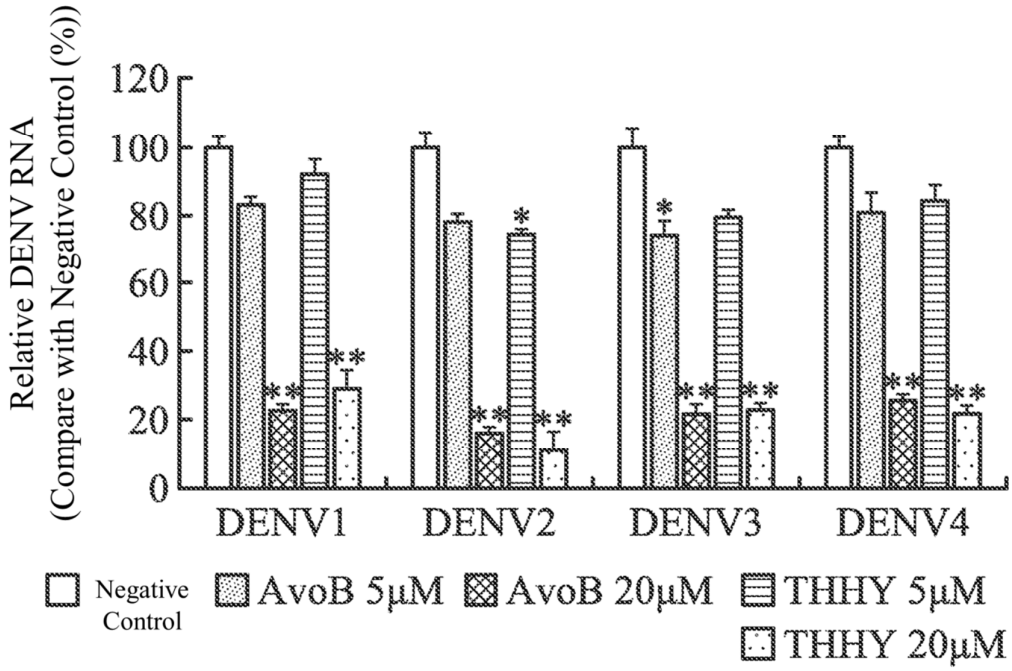
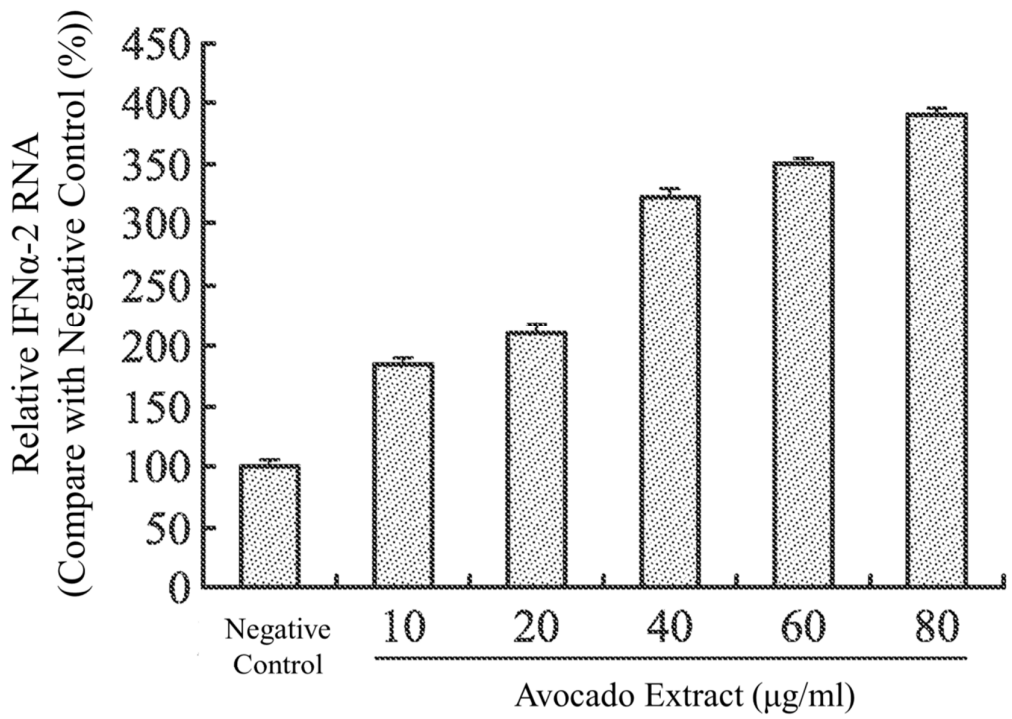


Figure 3B



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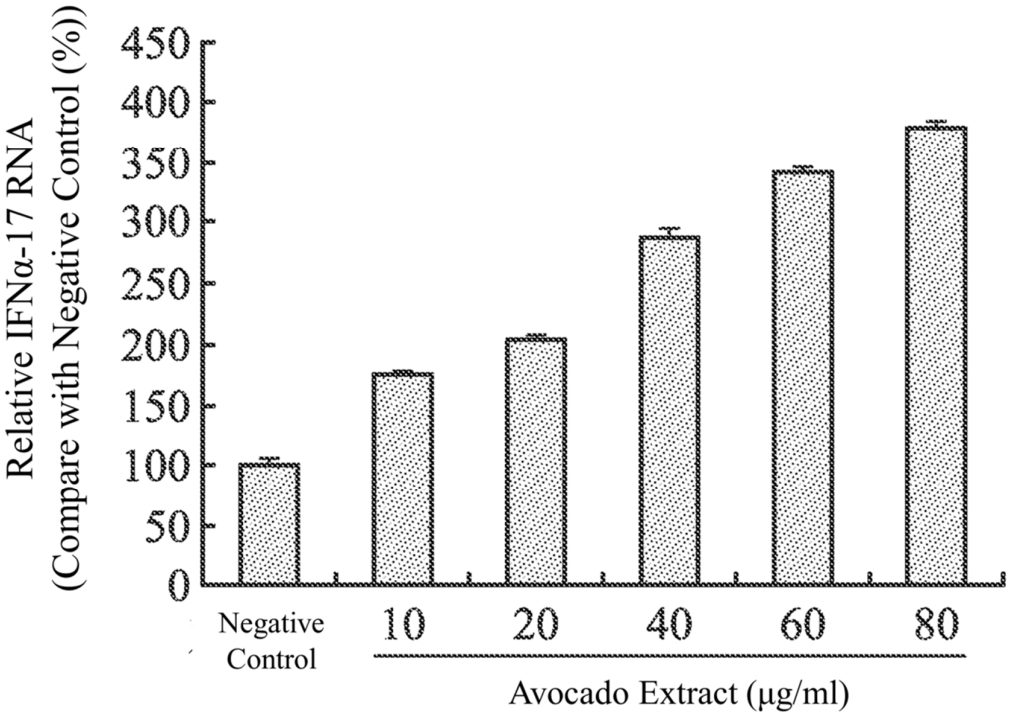
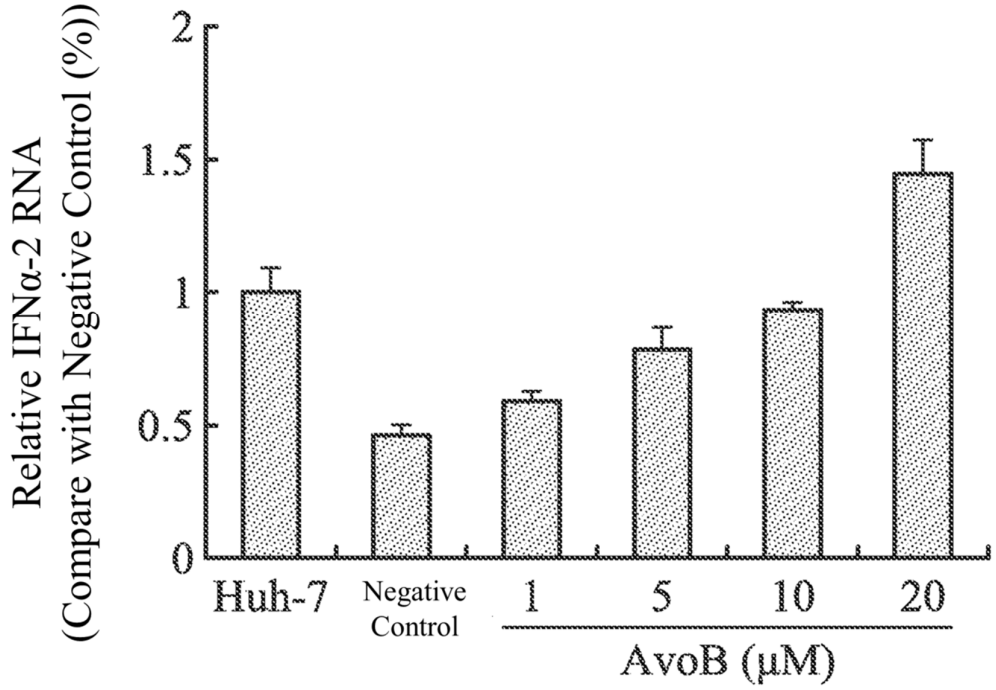


Figure 4B



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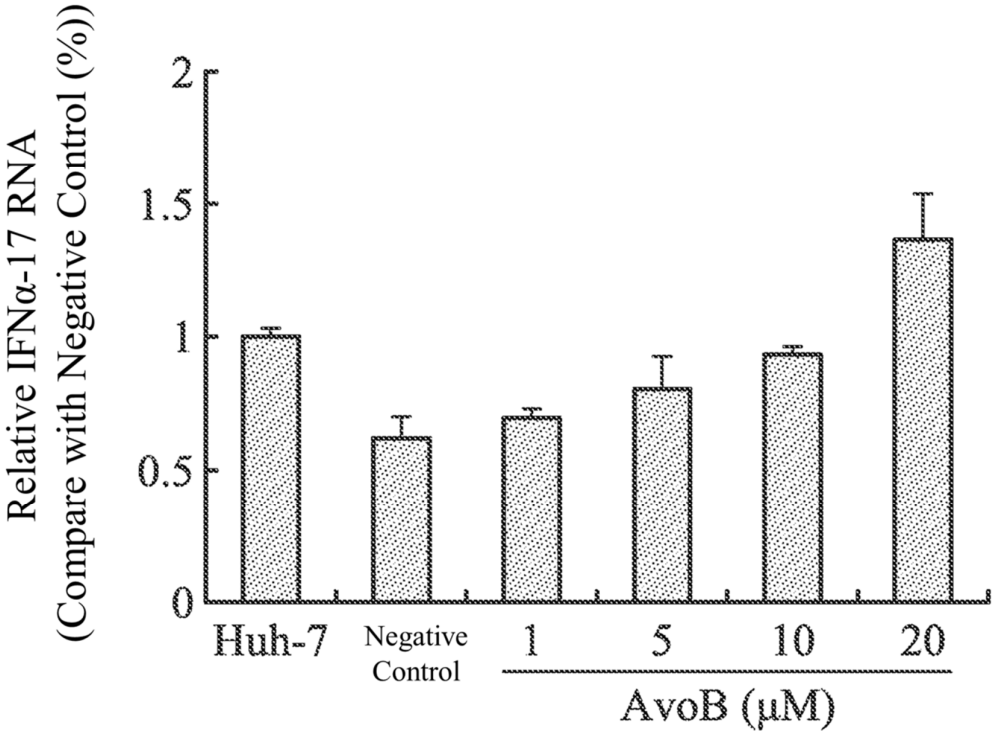
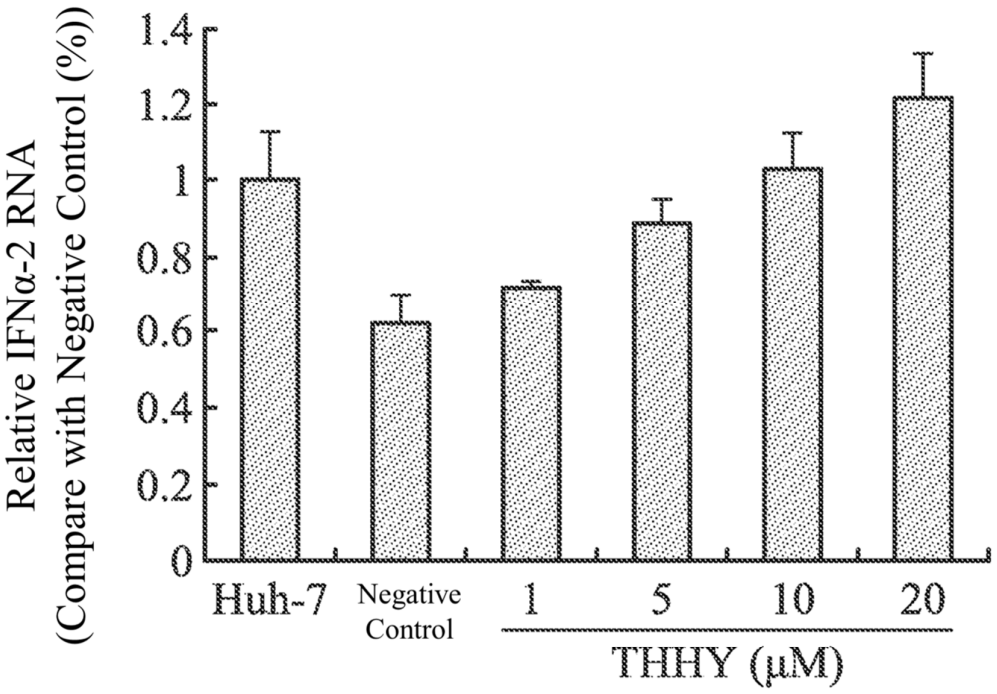


Figure 5B



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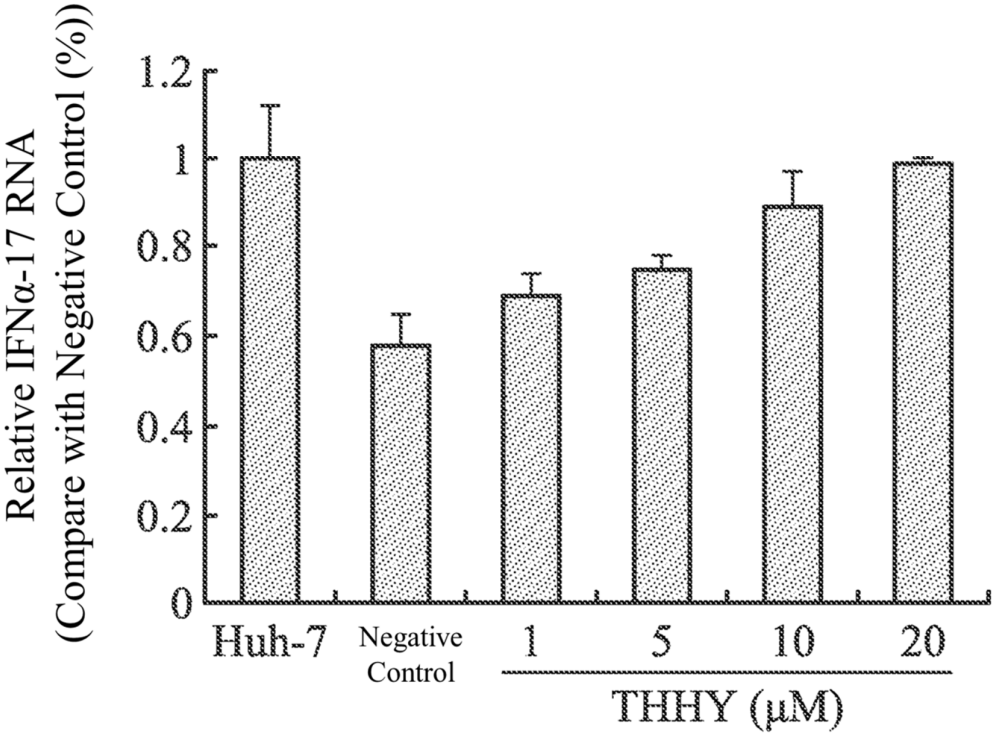

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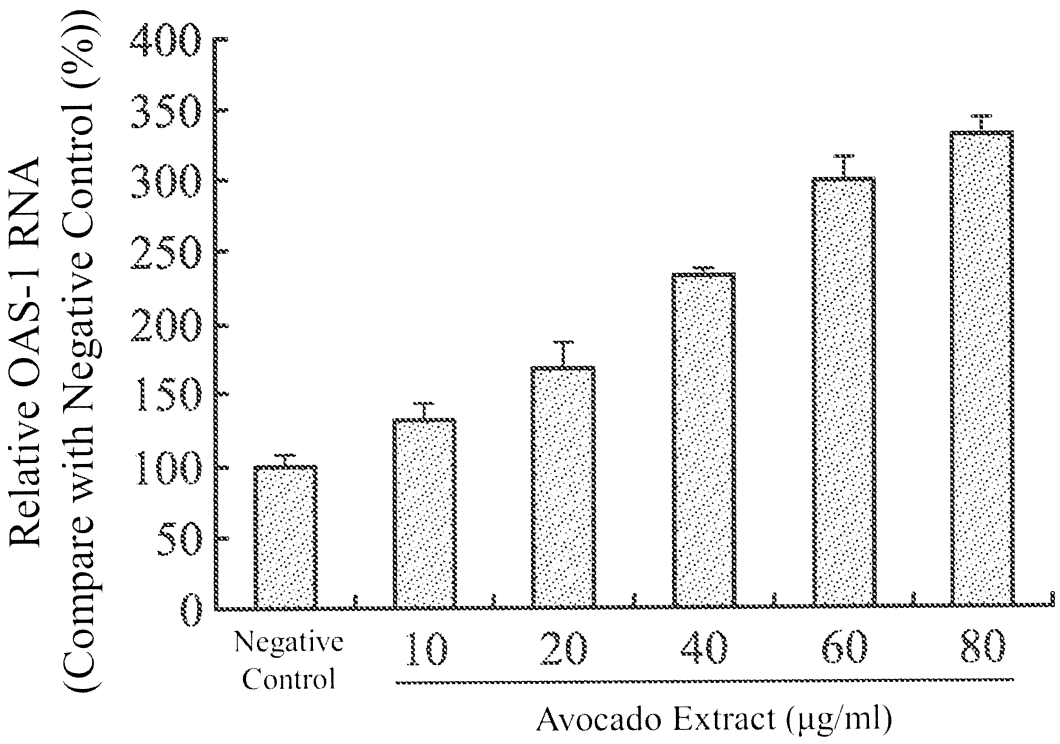

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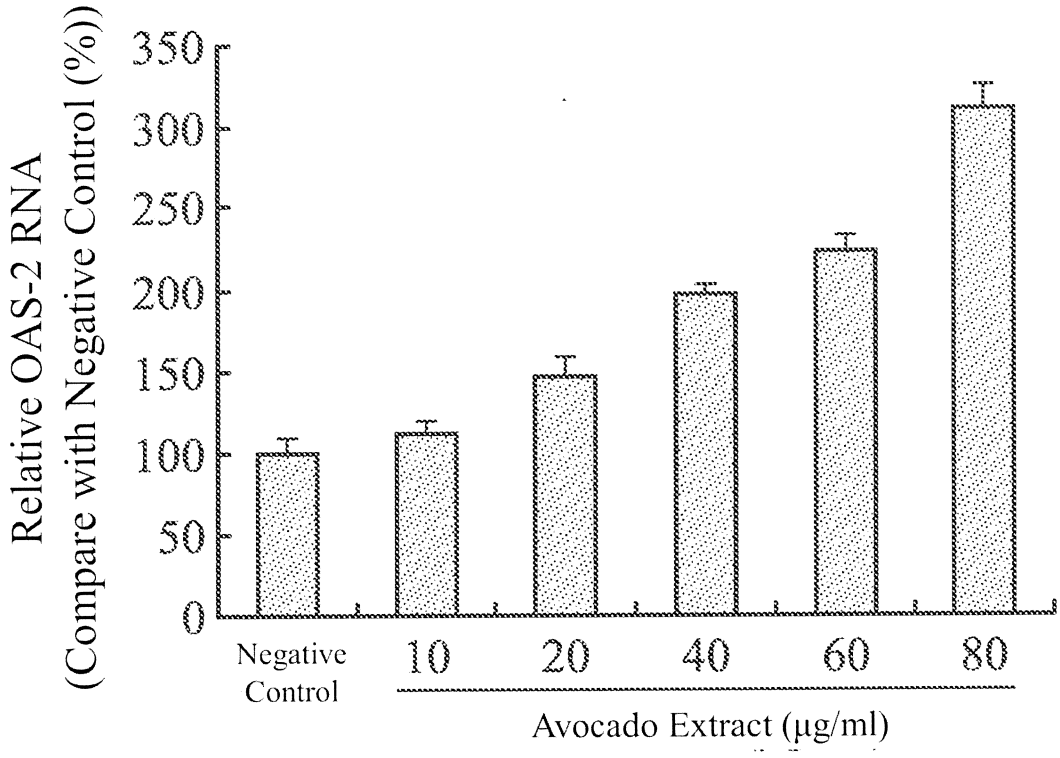


Figure 7B

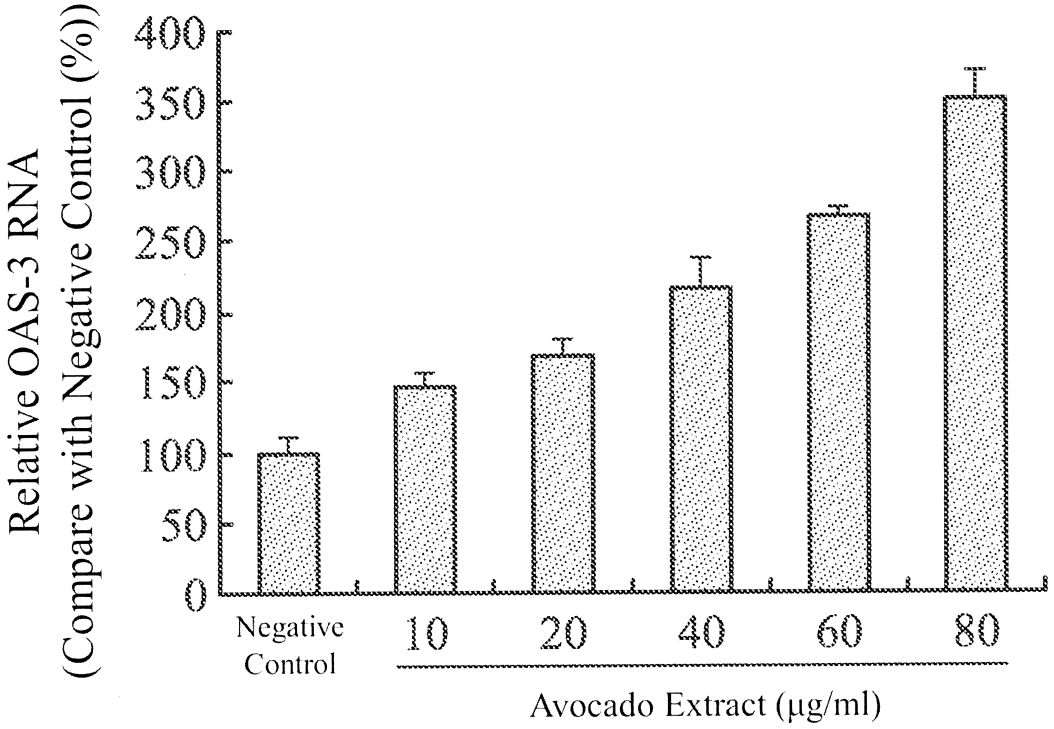


Figure 7C

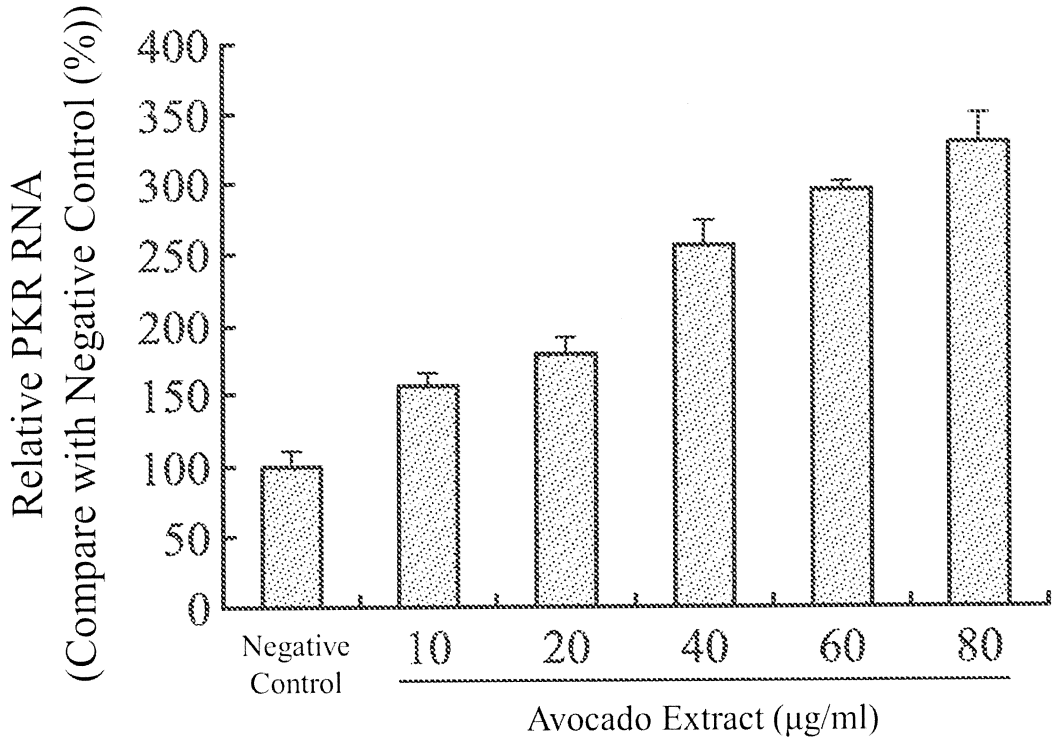
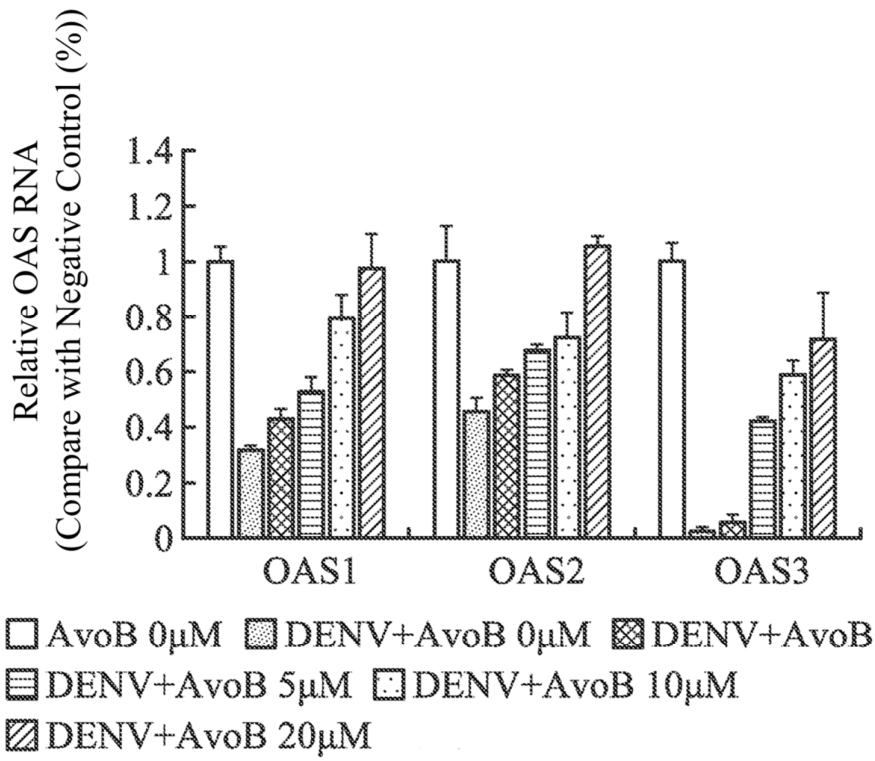


Figure 7D



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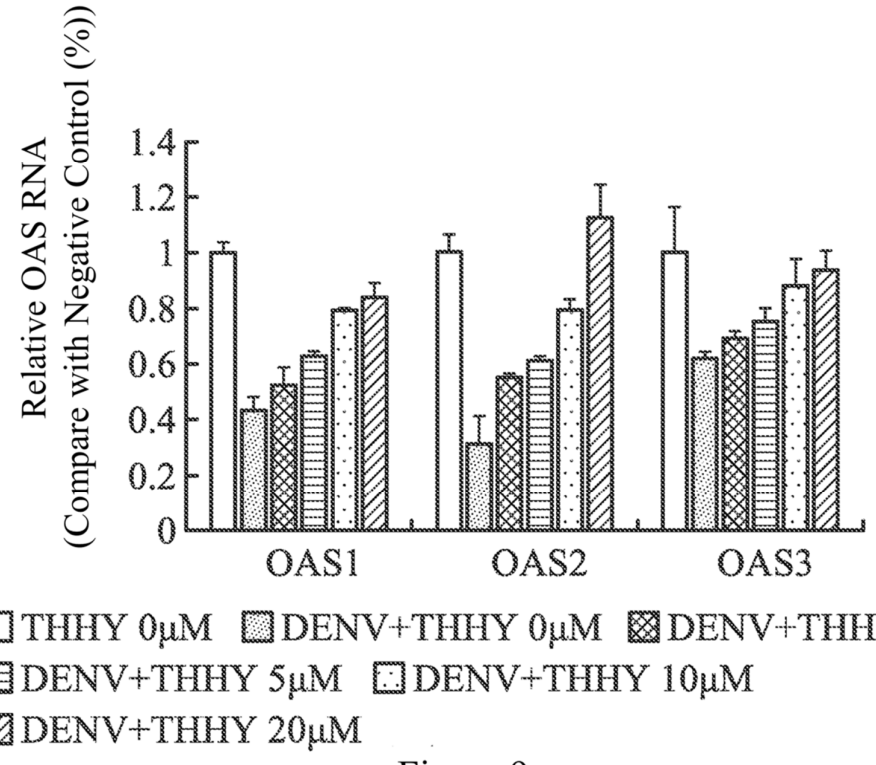

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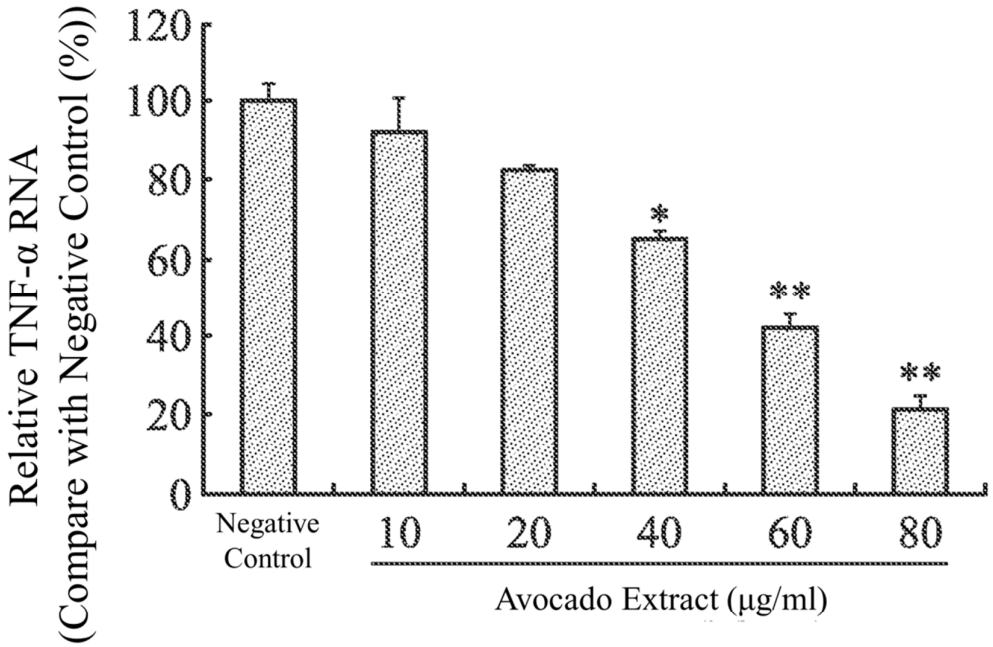

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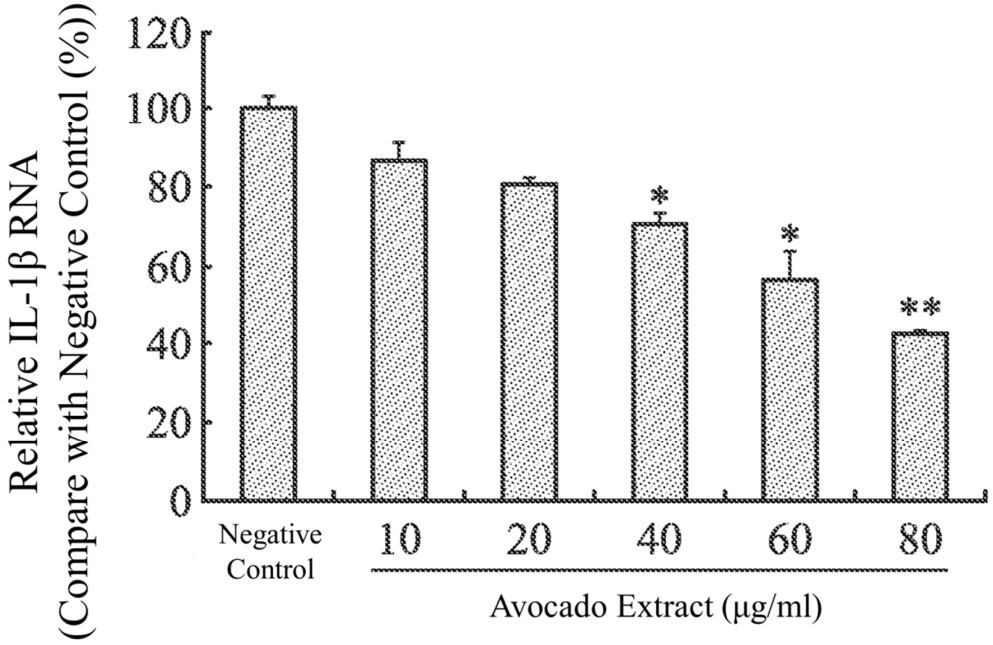

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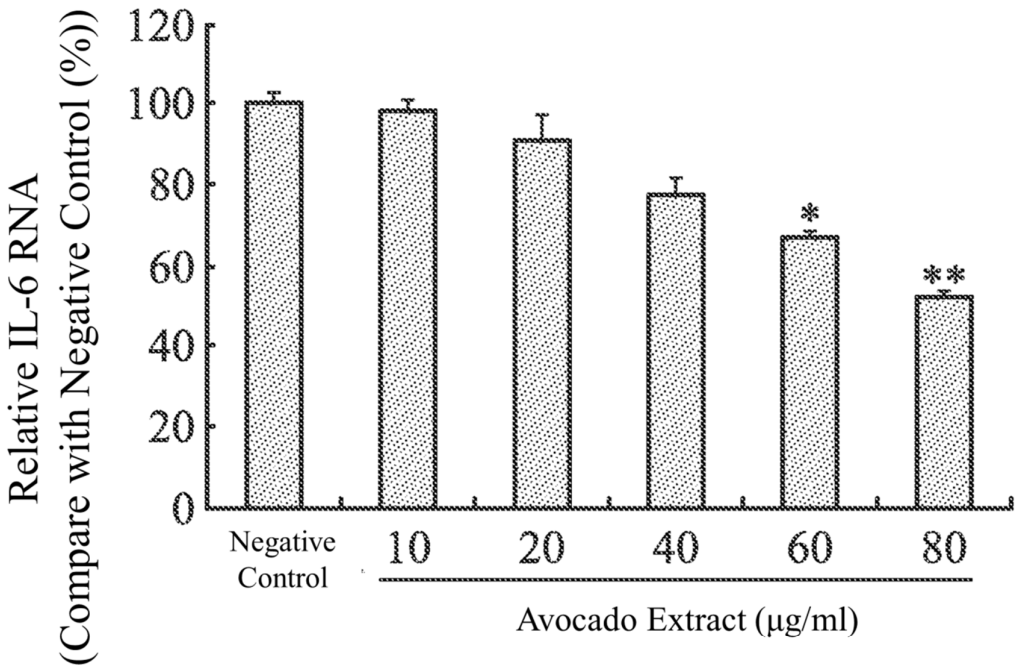

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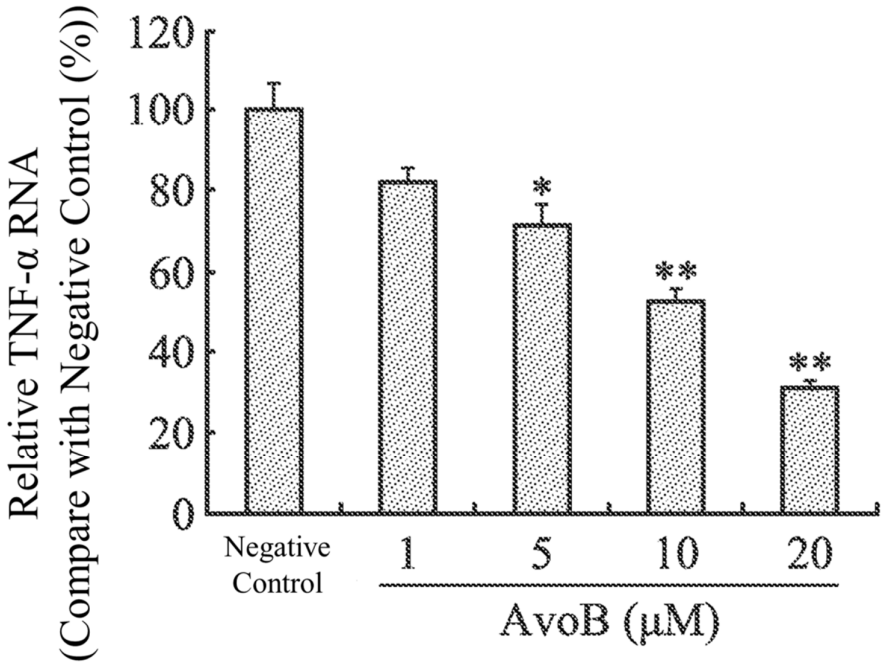

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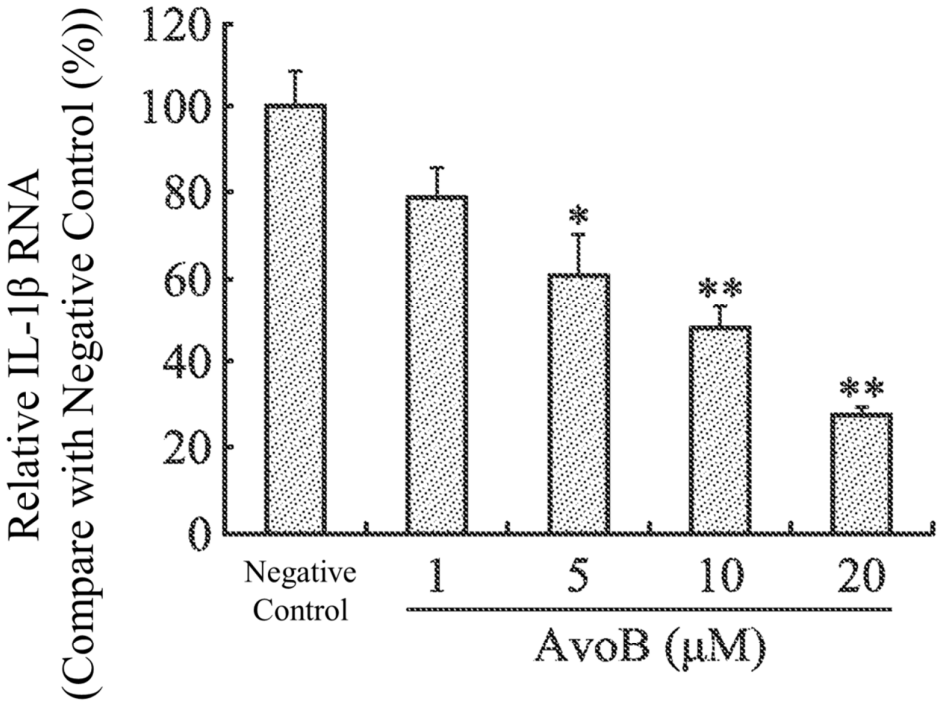


Figure 11B

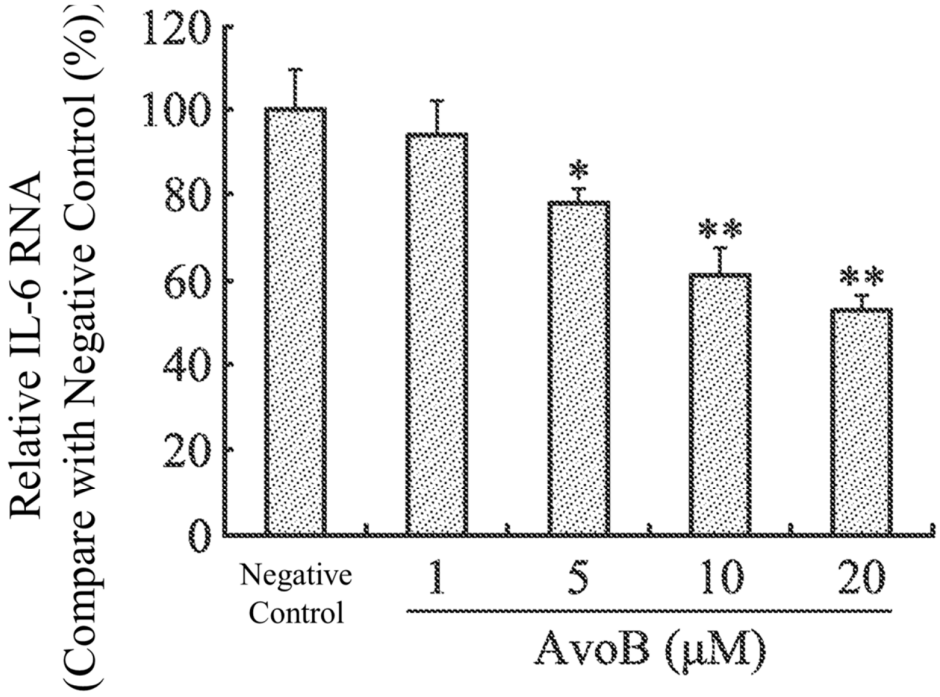
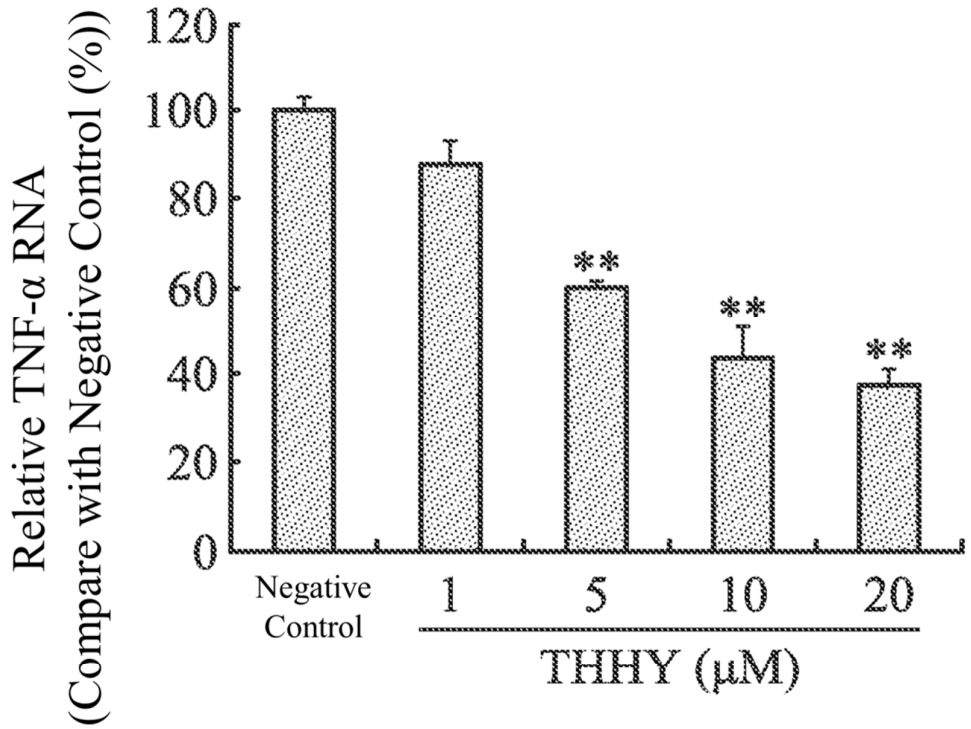


Figure 11C



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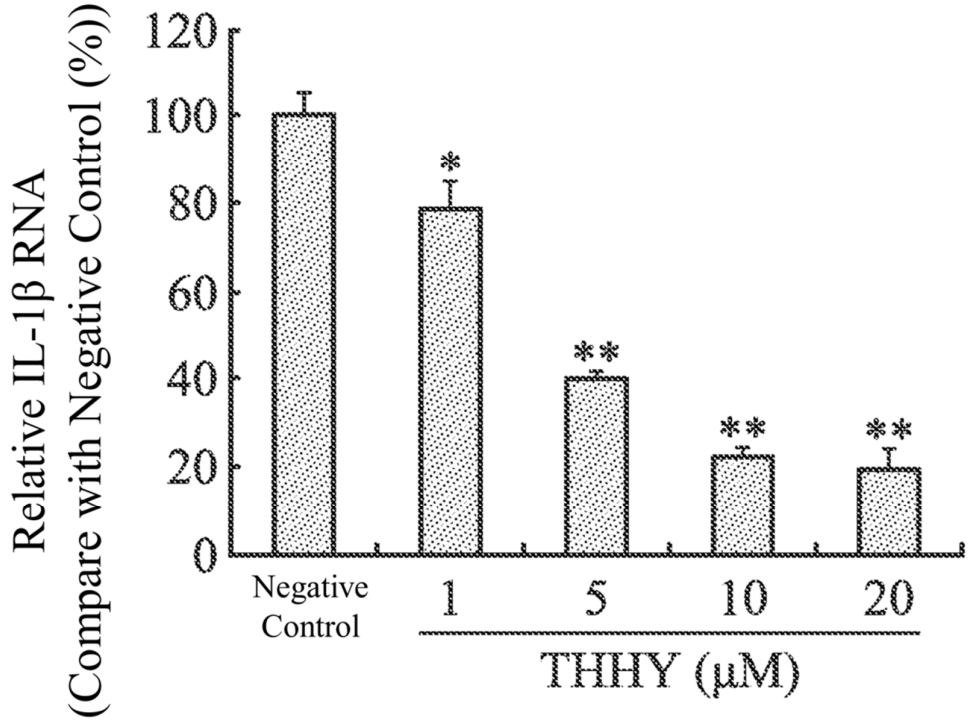

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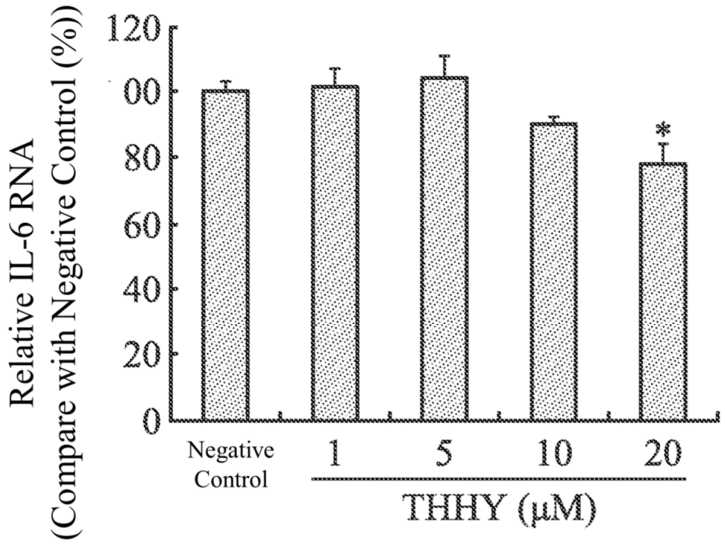

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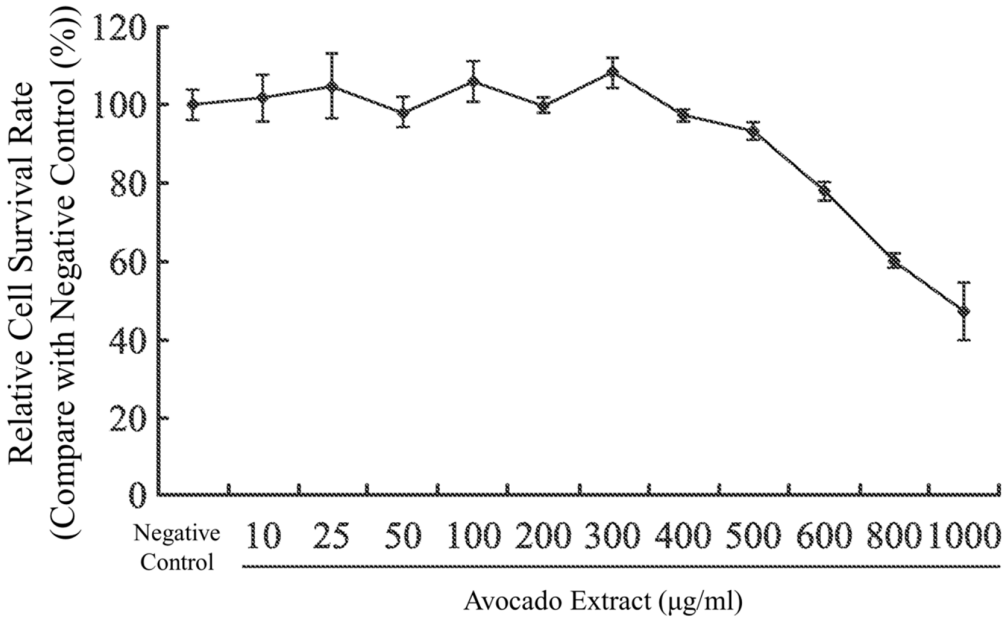

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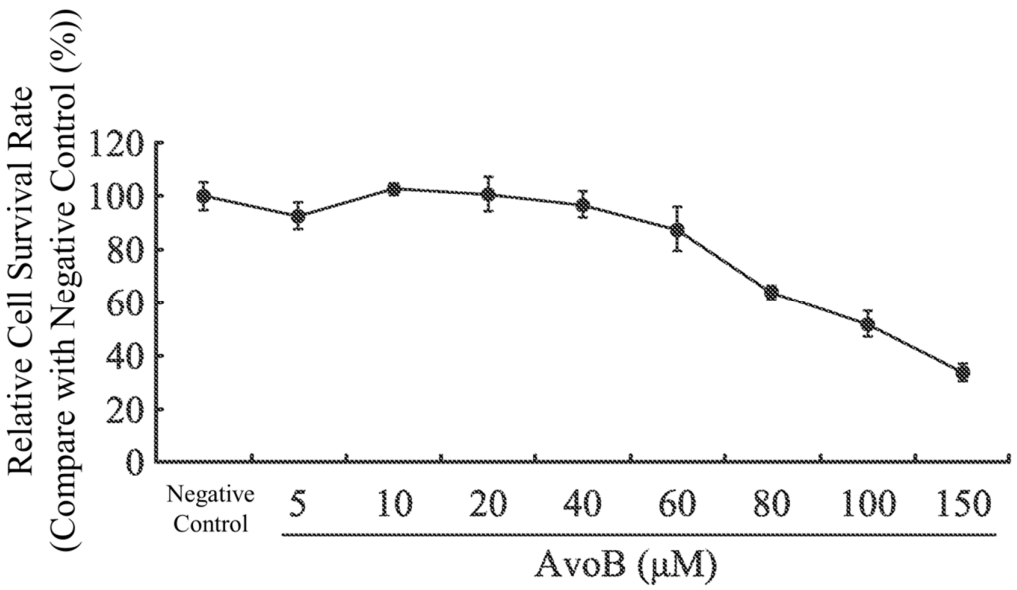

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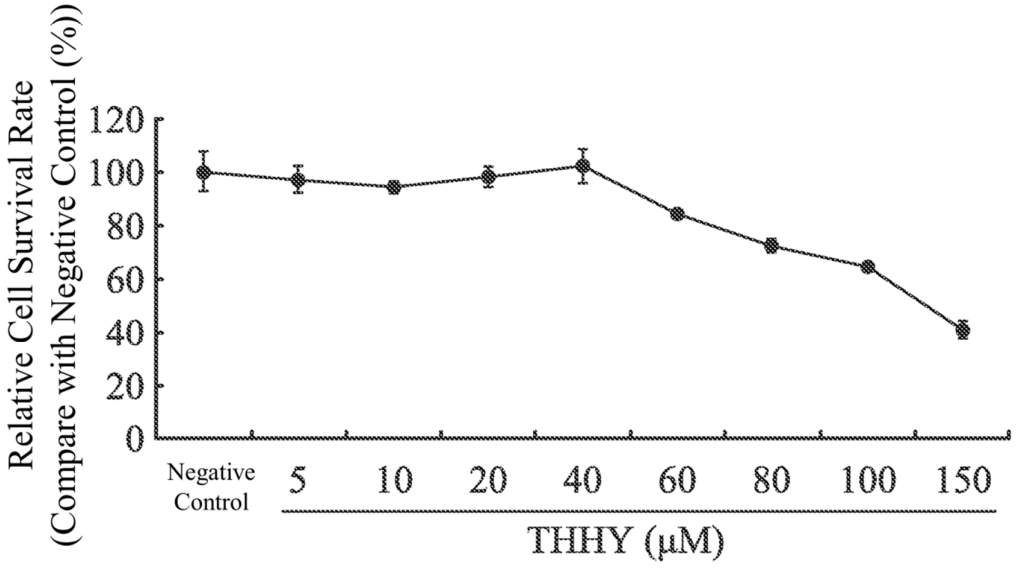

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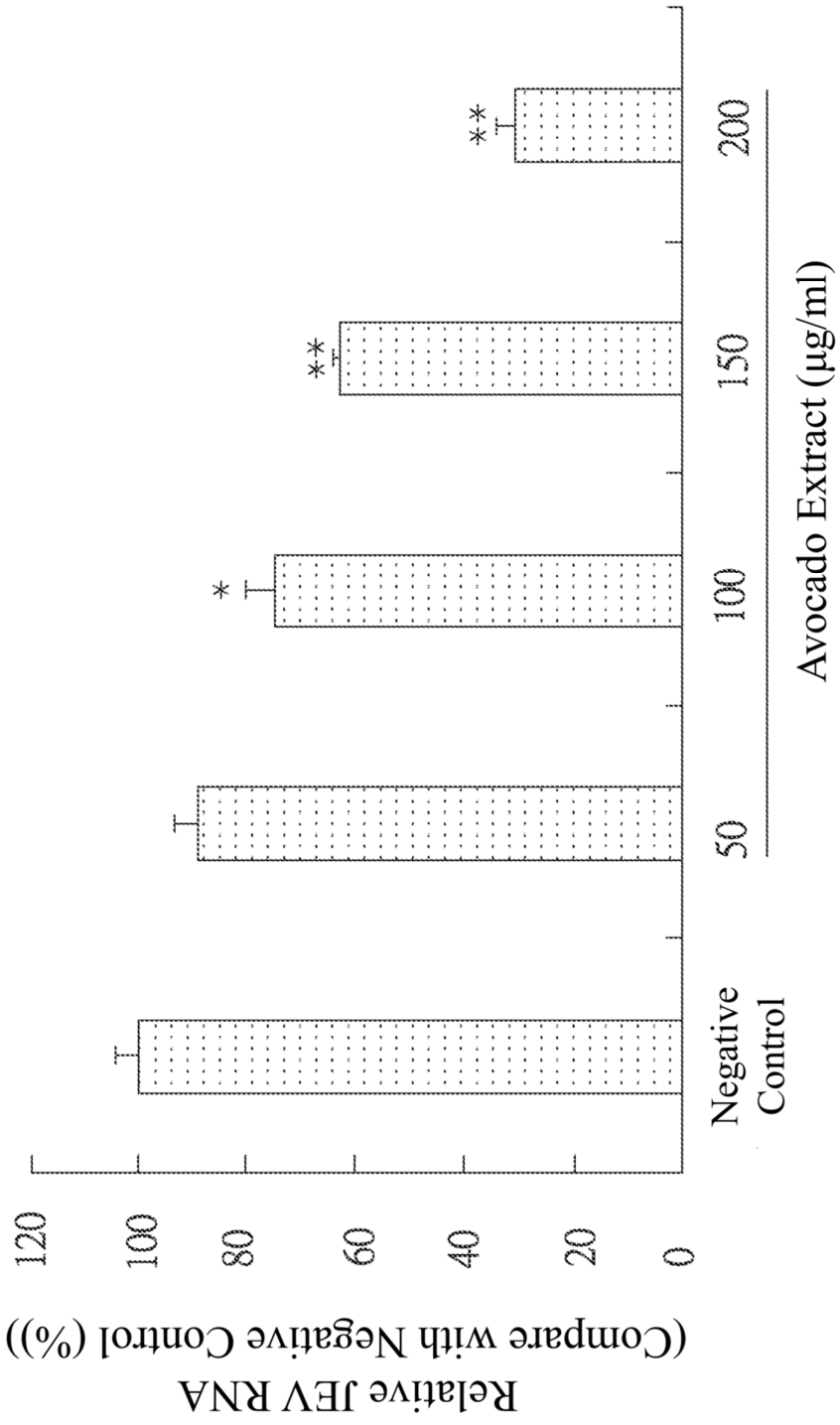
Relative JEV RNA
(Compare with Negative Control (%))
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100
80
60
40
20
0
Negative Control
50
100
150
200
Avocado Extract (μg/ml)
*
**
**

Figure 14A

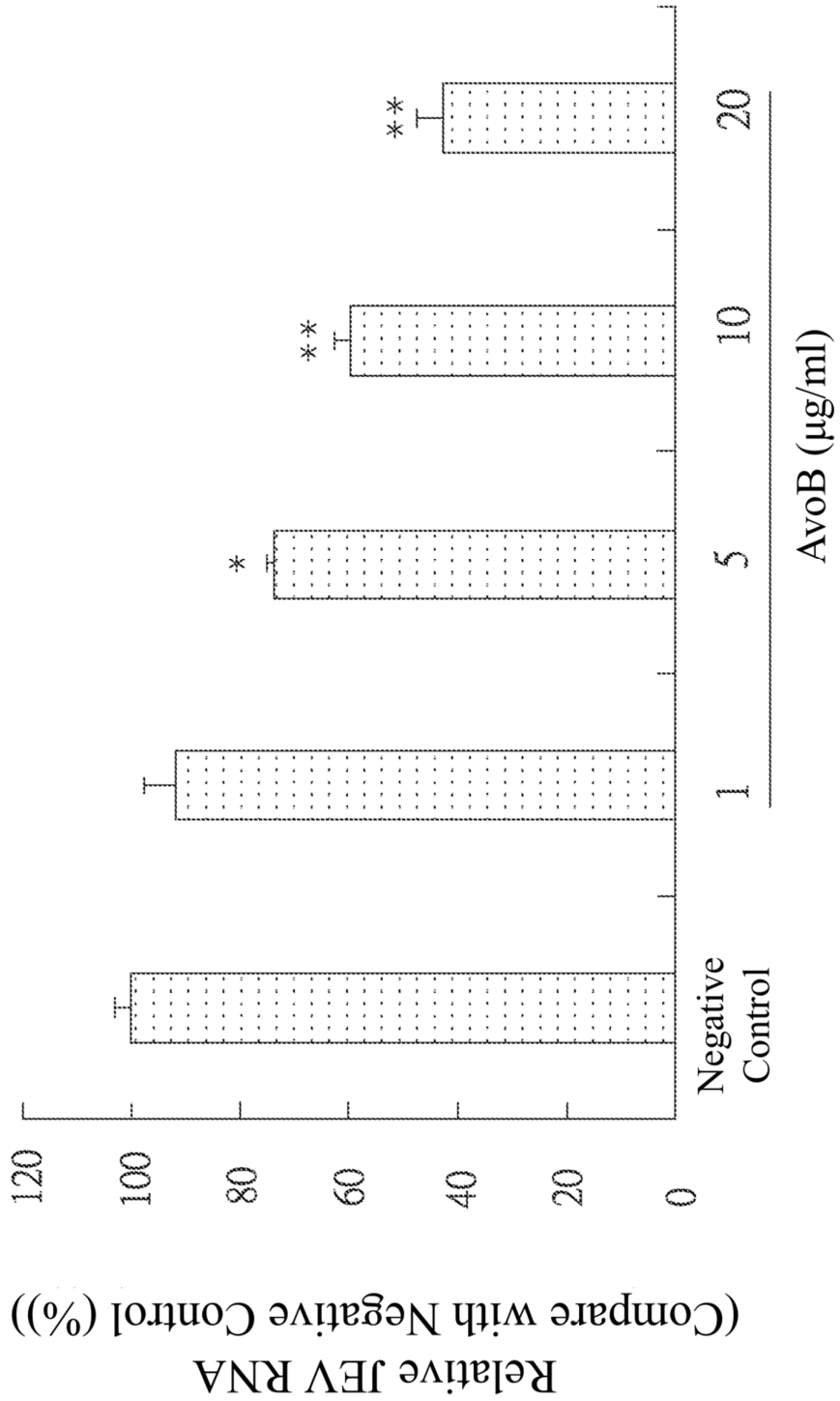
Relative JEV RNA
(Compare with Negative Control (%))
120
100
80
60
40
20
0
Negative Control
1
5
10
20
AvoB (μg/ml)
*
**
**

Figure 14B

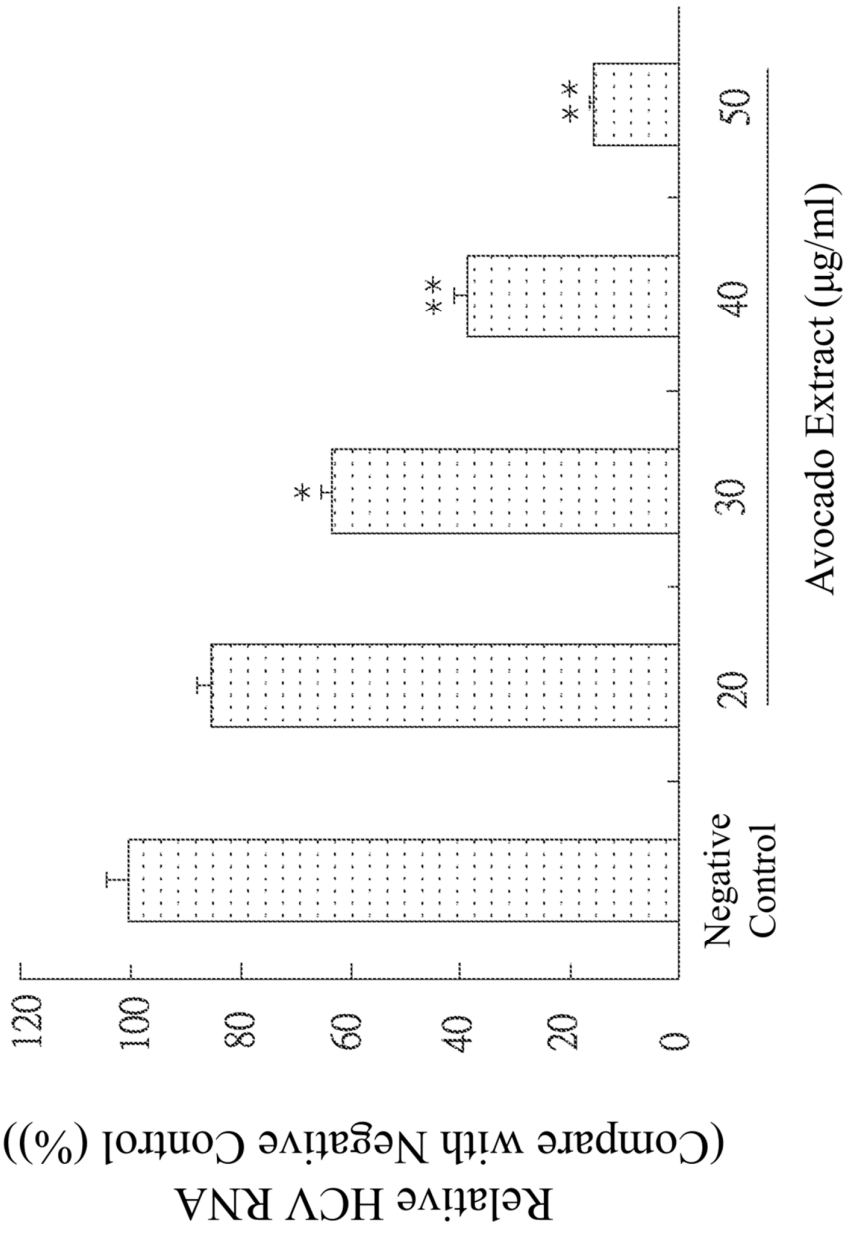
Relative HCV RNA
(Compare with Negative Control (%))
120
100
80
60
40
20
0
Negative Control
20
30
40
50
Avocado Extract (μg/ml)
*
**
**

Figure 15A

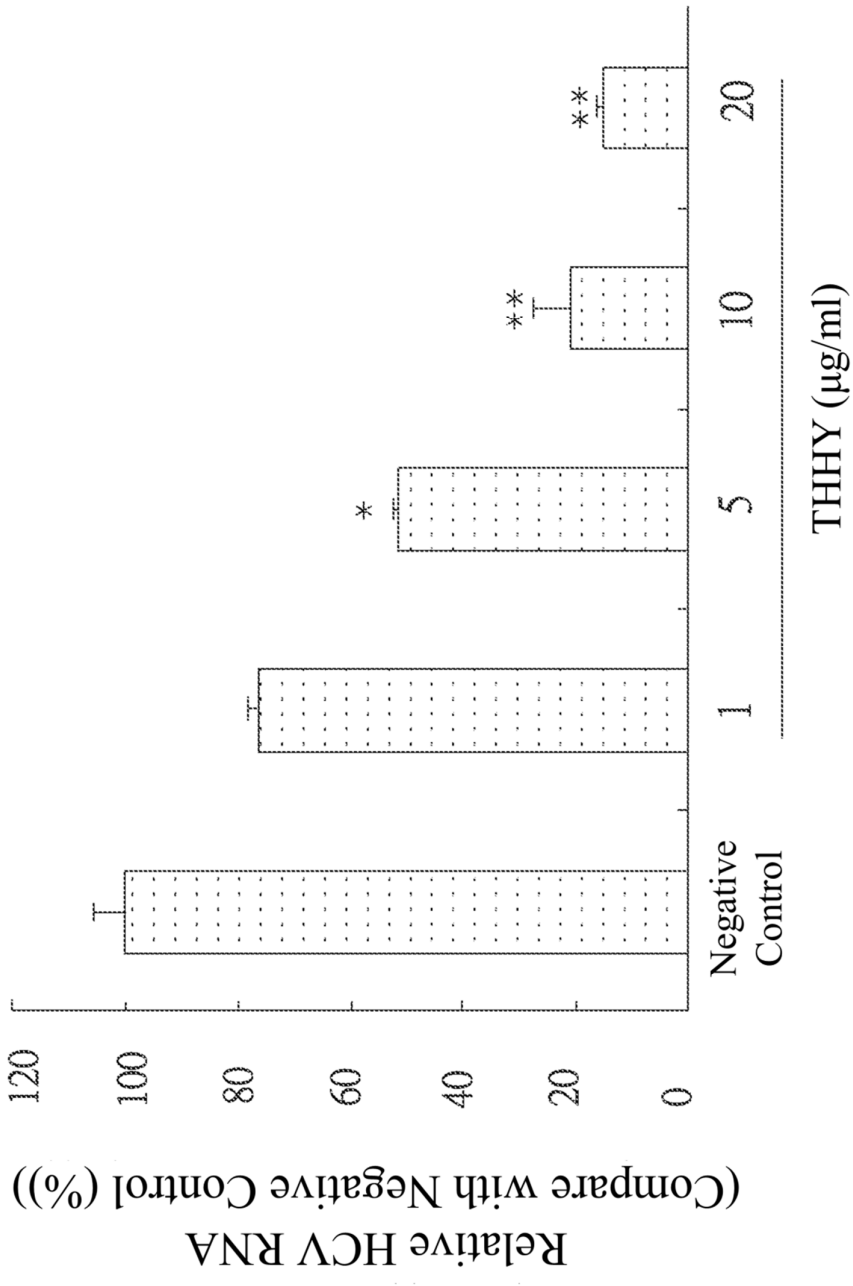
Relative HCV RNA
(Compare with Negative Control (%))
120
100
80
60
40
20
0
Negative Control
1
5
10
20
THHY (μg/ml)
*
**
**

Figure 15B

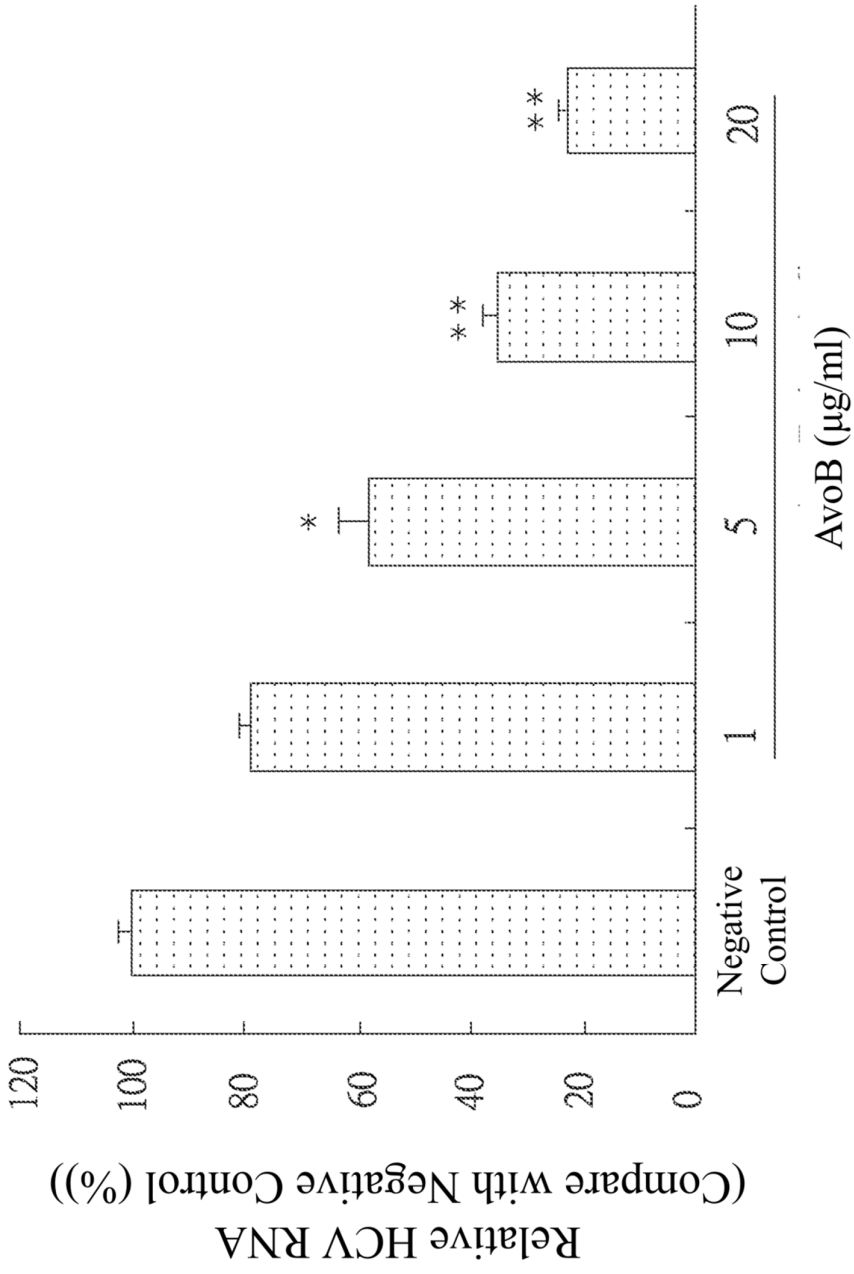
Relative HCV RNA
(Compare with Negative Control (%))
120
100
80
60
40
20
0
Negative Control
1
5
10
20
AvoB (μg/ml)
*
**
**

Figure 15C