Inhibitory activity of Kaki calyx extracts and their triterpene compounds on recombinant hepatitis C virus NS3/4A protease

Nobuko KAKIUCHI, Tairiku KUROIWA, Kazuki TUCHIYA, Toshiyuki ATSUMI, Isao OHTSUKA

Abstract

We tested hexane, methanol and 50% aqueous acetone extracts of kaki calyx for their inhibitory activity on recombinant hepatitis C virus NS3/4A protease. The 50 % aqueous acetone extract had the highest inhibitory activity, and was fractioned by Sephadex LH20 column chromatography. The 70 % methanol fraction of the column chromatography was further fractioned by silica gel column/thin layer chromatography, and triterpenes, betulinic acid, oleanolic acid, and ursolic acid, were isolated. They were identified by analyses using HPLC equipped with a polycyclic aromatic hydrocarbons (PAH) column and 1H-NMR spectroscopy. The triterpenes were thought to be the main inhibitory compounds in the 50 % aqueous acetone extract of kaki calyx, but there may be inhibitory compounds other than the triterpenes.

Key words : HCV, NS3/4A protease, inhibitor, Kaki calix, triterpene

Introduction

Hepatitis C virus (HCV) is a major cause of acute and chronic human hepatitis. Approximately, 3 % of the population (180 million people worldwide) is infected with HCV [1]. Despite of successful anti-HCV therapies developed recent years, many patients with HCV in the world are anticipated alternative treatments that are easier to access and low in cost are needed. Traditional medicines from various areas in the world are hopeful candidate for developing novel HCV drugs[2]. We reported the anti-HCV protease activity of extracts from the peel and seeds of a tropical fruit in Peru, and included tannic conpounds [3]. Furthermore, methanol extracts of 13 crude drugs were tested for inhibitory activity on the enzyme reaction, and 6 of them at a concentration of 100 μg/ml inhibited the reaction more than 90%. Five of these effective extracts contained as much as 800-1500 μg/ml phenolics, but the extract of kaki (persimmon) calyx contained less than 400 μg/ml[4]. Here, we focus on inhibitory principle of kaki calyx other than phenolics.

Materials and methods

Chemicals

Oleanolic acid and betulinic acid were purchased from Aldrich (USA and UK), and ursolic acid from Tocris (UK). The protease substrate having a 13-amino acid sequence of HCV NS5A- NS5B junction (DDIVPCSMSYTWT) and a peptide, P41, consist of the sequence of 20 N-terminal amino acids of HCV NS4A were purchased from BioSynthesis Inc., (USA) or GL Biochem (China). The purities of these peptides were greater than 85 %. Analytical (1.05715.0001) and preparative (1.13895.0001) silica gel 60 F254 thin layer plates were obtained from Merck Millipore (USA).

Herbal material and extract producing

Kaki calyxes (Lot 023415001) were obtained from Uchida Wakanyaku Ltd. (Japan), and were powdered after breaking into small pieces. Fifty grams of the crude drug powder was suspended in 200 ml of hexane and extracted under sonication for 30 min. The hexane layer was removed, and the remaining solid was dried. Two hundred milliliters of 50 % acetone aqueous

School of Pharmaceutical Sciences, Kyushu University of Health and Welfare;
1714-1 Yoshino-cho, Nobeoka city, Miyazaki, 882-8508, Japan.

solution or methanol was added to the solid and sonicated for 30min, and the liquid layer was removed. The extraction with 50 % acetone aqueous solution or methanol was repeated 3 times. After filtration, the extracts were evaporated to remove solvent and weighed.

Fractionation of the 50 % acetone aqueous solution extract of Kaki calyx

The 50 % acetone aqueous extract of Kaki calyx was dissolved in 50 % methanol, and was applied to a column of Sephadex LH20 swelled in 50 % methanol. The column was eluted with methanol-water mixtures in a stepwise manner of 50 %, 60 %, 70% and 100 % methanol, and then eluted with 50 % aqueous acetone solution. Each fraction was evaporated to remove solvent and weighed.

Recombinant HCV protease (ΔNS3) preparation and enzyme reaction.

Expression of a plasmid containing amino acid sequence of HCV polypeptide from 1027 to 1218 and six histidine residues in series at N-terminal adjoining region to the HCV peptide in Escherichia coli JM109 was performed according to the method reported previously [5]. Purification of the expressed peptide using Ni-NTA Quick Start Kit (QIAGEN, Germany) was carried out according to the manufacture' s protocol. The recombinant enzyme together with the HCV NS4 peptide P41 hovering amino acid 1673-1692, which was found to participate in proteolytic reaction, were reacted with a synthetic peptide (DDIVPC / SMSYTWT) corresponding the cleavage site of HCV NS5A/5B [6]. HPLC analysis for enzyme reaction was performed using the Shimadzu liquid chromatography system [4]. Inhibition (%) was calculated based on the HPLC peak area of the remaining substrate of the reaction (Rn), that of positive control (PC), the reaction carried out without sample, and the negative control (NC), the reaction without enzyme and sample, as the following: Inhibition (%) = 100 x (Rn-PC)/(NC-PC)

HPLC analysis of triterpenes

The HPLC analysis was performed on the Shimadzu liquid chromatography system (LC-10ADvp Pumping Unit, DGU-14AM degasser, SPD-10A UV-VIS ultraviolet detector, CTO-10ACcolumn oven, Chromatopac C-R8A data processor, and SLC-10A System Controller) equipped with a Zorbax Eclipse PAH column 250-4.6 (5 μm) (Agilent Technologies). The column was eluted with an acetonitrile-water mixture of 85 % acetonitrile at 30 ° C, and the UV signal at 215 nm was monitored.

Results and Discussion

Inhibitory activity of extracts and fractions of kaki calyx

Hexane, methanol, and 50 % aqueous acetone extracts of kaki calyx were tested for their inhibitory activity on recombinant HCV NS3/4A protease. As shown in Fig 1, the 50 % aqueous acetone extract was found to have the highest activity as well as the highest yield. The extract was fractionated by Sephadex LH20 column chromatography. The fraction eluted with 50 % methanol exhibited less inhibitory activity than the starting extract, and higher activity was observed in fractions eluted with higher methanol contents solutions. The 70 % methanol fraction had the highest inhibitory activity. The fractions from Sephadex LH20 column chromatography were analyzed by TLC developed with a chloroform methanol (9:1) mixture (Fig 2). The 70 % methanol fraction, was found to contain substance(s) with higher mobility.

Further purification of the 70 % methanol fraction of the 50 % aqueous acetone extract of Kaki calyx

The 70 % methanol fraction was further purified by silica gel column chromatography followed by preparative thin layer chromatography. Finally, fractions from the preparative thin layer chromatography, zone 1, 2 and 3 were obtained. Zhang et al reported that best resolution of oleanolic acid and ursolic acid was achieved using PAH (polycyclic aromatic hydrocarbons) polymeric C18 bonded phase column[7].

In addition to oleanolic acid and ursolic acid, betulinic acid was well separated by HPLC with a Zorbax Eclipse PAH column (Fig 3 a). It was found that betulinic acid was enriched in zone 2, as were oleanolic acid and ursolic acid in zone 3 (Fig 3 b, c) .

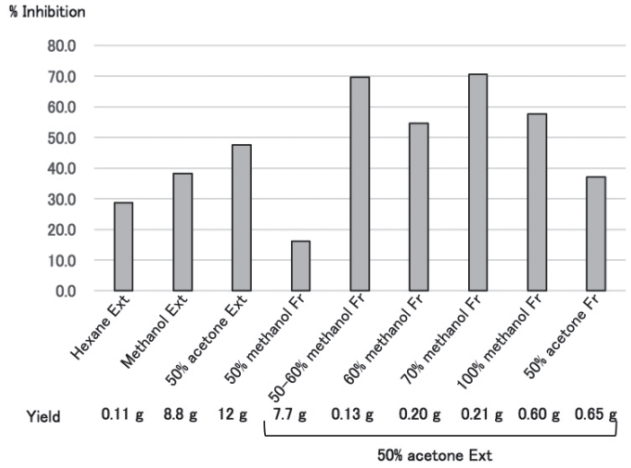

Fig 1 Inhibitory activity and yield of extracts and fractions from Sephadex LH20 column chromatography

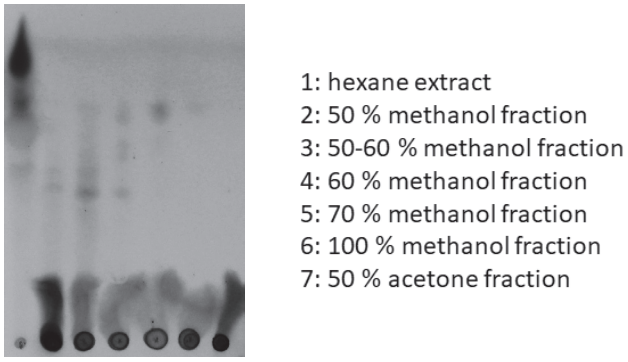

Fig 2 TLC of hexane extract and fractions from Sephadex LH20 column chromatography

The contents of zone 2 and zone 3 were analyzed by 1H-NMR spectroscopy. The 1H-NMR signals of the main compound in zone 2 was identical with those of betulinic acid, while main compounds of zone 3 werethought to be an equivalent mixture of oleanolis acid and ursolic acid. The inhibitory activity of the pure betulinic acid, oleanolic acid and ursolic acid, was tested (Fig 4). They all inhibited the activity more than 50 % at a concentration of 100 μg /ml. The IC_{50} of oleanolic acid and ursolic acid were ~20 μg /ml, and betulinic acid ~60 μg /ml. The IC_{50} of oleanolic acid and ursolic acid for HCV protease were similar to that reported by Ma et al reported, 26 and 16 μg /ml, respectively[8) 9)]. The triterpenes contained in the 50 % aqueous acetone extract of kaki calyx,betulinic acid, oleanolic acid and ursolic acid, were thought to be the main contributors of the inhibitory activity of the recombinant hepatitis C virus NS3/4A protease in the fractions eluted with greater than 70 % methanol. Ursolic acid and acetyl ursolic isolated from the stems of *Cynomorium songaricum* [9)], and oleanolic acid and its synthesized derivatives [8)] were reported to inhibit HCV protease. We found that betulinic acid also had inhibitory activity. Previously, betulinic acid was reported to be a potent inhibitor of eukaryotic topoisomerase I [10)], and selective inhibitor of human melanoma that functions by induction of apoptosis [11)]. The triterpenes were found to be the main inhibitory ingredients in the 50 % aqueous acetone extract of kaki calyx on the enzyme, but there may be inhibitory compounds other than the triterpenes.

References

1 Mohd Hanafiah, K., Groeger, J., Flaxman, A. D., et al :Global epidemiology of hepatitis C virus infection: New estimates of age-specific antibody to HCV seroprevalence. Hepatology 57:1333-1342, 2013

2 Bartenberger, R. :Candidate targets for hepatitis C virus-specific antiviral therapy. Review, Intervirology 40:378-9, 1997.

3 Kakiuchi N., Kanashima T., Myoda T., et al. :Inhibitory activity of extracts of seeds and peel of Peruvian crude drug CamuCamu and their fractions on recombinant HCV protease. In press. Abstract of Annual Meeting of Japanese Pharmaceutical Society, 27PB-pm245, 2015.

4 Kakiuchi N., Suenaga S,. Wakita S, et al. :Inhibitory activity of tannin-containing crude drugs on recombinant HCV protease. J Kyusyu Univ Health Welfare, 17: 65-69, 2016.

5 Vishnuvardhan, D., Kakiuchi, N., Urvil, P. T., et al. :Expression of highly active recombinant NS3 protease domain of hepatitis C virus in E. coli. FEBS Letters, 402:209-212, 1997.

6 Kakiuchi, N., Hijikata, M., Komoda, et al. :Cleavage activity of hepatitis C virus serine protease. J. Biochem. 122:749-755, 1997.

7 Zhang Y., Xue KP., Zhao EY, et al. :Determination of oleanolic acid and ursolic acid in Chinese medicinal plants using HPLC with PAH polymeric C18. Pharmacog. Mag. Oct-Dec 9 (Suppl 1):S19-S24, 2013.

8 Ma CM, Wu XH., Hattori M., et al. : HCV Protease Inhibitory, Cytotoxic and Apoptosis-Inducing Effects of Oleanolic Acid Derivatives. J Pharm Pharmaceut Sci. 12: 243 - 248, 2009.

9 Ma CM., Wei Y., Wang ZG., et al. :Triterpenes from Cynomorium songaricium—analysis of HCV protease inhibitory activity, quantification, and content change under the influence of heating. J Nat Med. 63:9–14, 2009.

10 Chowdhury AR., Mandel S., Mittra S, et al., Betulinic acid, a potent inhibitor of eukaryotic

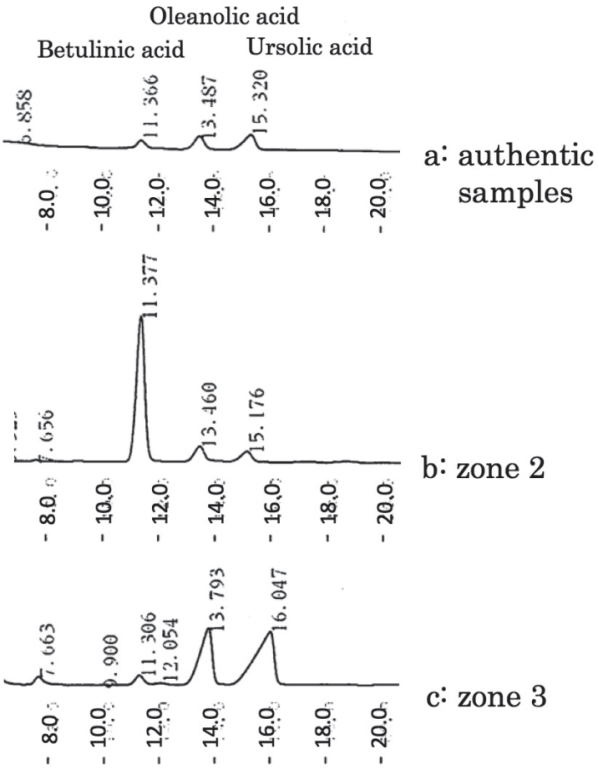

Fig 3 HPLC with a Zorbax Eclipse PAH column of authentictriterpenes (a) zone 2 (a) , and zone 3 (C)

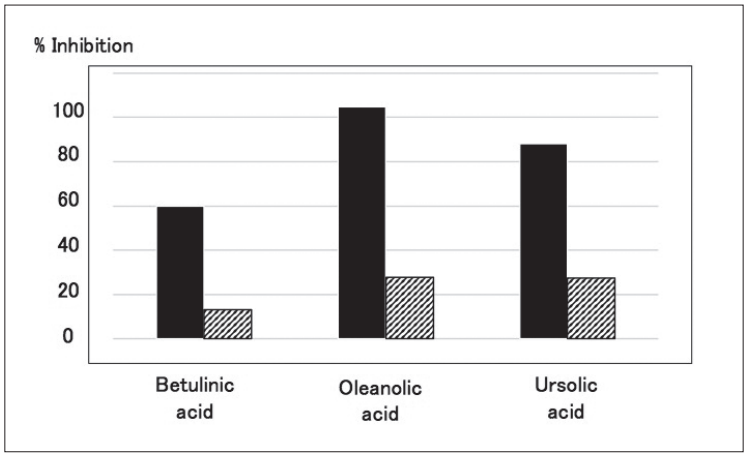

Fig 4 Inhibitory activity of pure triterpenes. Open columns and dashed columns indicate inhibitory activity at a concentration of 100 mg/ml and 10 mg/ml, respectively.

topoisomerase I: identification of the inhibitory step, the major functional group responsible and development of more potent derivatives
Med Sci Monit; 8: BR254-260, 2002.

11 Pisha E., Chai H., Lee IS., et al. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. Nat Med. 1:1046-51, 1995.

九州保健福祉大学研究紀要　*19*：*67～72, 2018*

柿蒂エキスと含有トリテルペン成分の組み替えHCV NS3/4Aタンパク質分解酵素阻害活性

垣内　信子，黒岩　大陸，土谷　和紀，渥美　聡孝，大塚　　功

九州保健福祉大学薬学部生薬学講座　〒882-8508　宮崎県延岡市吉野町1714-1

要旨

柿蒂のヘキサン、メタノール、50 % アセトン抽出物の、組み替え hepatitis C virus (HCV)の NS3/4A 蛋白質分解酵素に対する阻害活性を検討した。50 % アセトン抽出物が最も阻害活性が強く、この抽出物をSephadex LH20カラムで分画を行なった。得た各画分のうち阻害活性の強かった 70 %メタノール画分を、さらにシリカゲルカラム／薄層クロマトグラフィーで分画し、トリテルペンのベルリン酸、オレアノール酸、ウルソール酸を分離した。これらのトリテルペンは polycyclic aromatic hydrocarbons (PAH) HPLC H-NMRスペクトルで同定した。これらトリテルペンは柿蒂の 50 %アセトン抽出物の主たる阻害物質と考えられる。

Key words：HCV, NS3/4A タンパク分解酵素 , 阻害物質 , 柿蒂 , トリテルペン