Revista Brasileira de Farmacognosia 29 (2019) 739–743

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original article

Chemical constituents of leaves of *Persea americana* (avocado) and their protective effects against neomycin-induced hair cell damage

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ARTICLE INFO

Article history:
Received 16 May 2019
Accepted 1 August 2019
Available online 4 September 2019

Keywords:
Lauraceae
Flavonol glycoside
Megastigmane glycosides
Lignans

ABSTRACT

Persea americana Mill., Lauraceae, commonly known as the avocado, is native to tropical and subtropical regions, including Brazil. From the leaves of *P. americana*, one previously undescribed flavonol glycoside (**1**) together with ten known flavonoids (**2**–**11**), four megastigmane glycosides (**12**–**15**) and two lignans (**16**–**17**) were isolated**.** Their structures were determined by extensive spectroscopic methods including 1D- and 2D-nuclear magnetic resonance and mass spectrometry data. This is the first investigation that reports megastigmane glycoside and lignan classes within the genus *Persea*. All the isolated compounds have been assessed through the cell survival of larval zebrafish following neomycin-induced damage and the cell viability of a House Ear Institute-Organ of Corti 1 mouse auditory cell line. Among the tested compounds, juglanin (**2**) and (+)-lyoniresinol (**16**) showed significant cell regeneration in neomycin-damaged hair cell without cellular toxicity.

Introduction

Persea americana Mill., Lauraceae, the plant that bears the avocado pear as fruit, is native to tropical and subtropical regions (Di Stefano et al., 2017). The Brazilian production of avocado varieties, including Fortuna, Quintal, Geada, Margarida, and Hass, has been growing and its net exports reached 9.8 million dollars in 2017 (Santos et al., 2014). In addition, there has been an increasing interest in the study of various avocado parts due to their health promoting properties and high nutrient value. Bioactive compounds in the avocado have been shown to have possible health benefits with antiviral, analgesic and anti-inflammatory activities (De Almeida et al., 1998; Adeyemi et al., 2002). In Brazil, the leaves are used to treat several disorders, such as urinary infections, bronchitis, and rheumatism.

Hearing loss is a partial or total inability to hear, affecting one or both ears. In recent years, there has been an increased interest in the protection and regeneration of sensory structures using natural products (Yu et al., 2006; Im et al., 2010; Jo et al., 2019). In a continuing project to identify natural products that modulate auditory hair cell function, we previously investigated the efficacy of avocado oil on sensorineural hearing loss *in vitro* and *in vivo* (Nam et al., 2019). In the present study, we continued the phytochemical study of avocado leaves and assessed the protective effects against neomycin (NM)-induced hair cell damaged zebrafish and the cell viability of a House Ear Institute-Organ of Corti 1 (HEI-OC1) mouse auditory cell line.

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https://doi.org/10.1016/j.bjp.2019.08.004

Materials and methods

General experimental procedures

Chemical shifts are reported in parts per million from TMS. All NMR spectra were recorded on an Agilent 400-MR-NMR spectrometer operated at 400 and 100 MHz for hydrogen and carbon, respectively. Data processing was carried out with the MestReNova ver. 6.0.2 program. Mass spectrometry was performed on an Agilent 6530 QTOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with electrospray ionization (ESI) interface and was analyzed in negative mode. The ESI conditions were set as follows: pressure of nebulizer, 45 psi; drying gas temperature, 325 °C; drying gas flow, 11 l/min; sheath gas temperature, 350 °C; sheath gas flow, 11 l/min; capillary voltage, 4000 V; fragmentor, 175 V; skimmer, 65 V; and OCT 1 RF Vpp 750 V. Preparative HPLC was carried out using an Agilent 1200 HPLC system. Column chromatography was performed on silica gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck) or YMC RP-18 resins (30–50 μm, Fujisilisa Chemical Ltd.). For thin-layer chromatography (TLC), a pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S plates (0.25 mm, Merck) were used. Optical rotations were determined on a Jasco (Tokyo, Japan) DIP-370 automatic polarimeter. The melting point was measured on a Gallen-Kamp (Germany) melting point apparatus.

Plant material

The leaves of *Persea americana* Mill., Lauraceae, were collected in Dak Lak province, Vietnam, in March 2018, and authenticated by Dr. Ninh Khac Ban in Institute of Marine Biochemistry, Vietnamese Academy of Science and Technology, Vietnam. A voucher specimen (PA201803) is deposited at the Herbarium of College of Pharmacy, Yonsei Institute of Pharmaceutical Sciences, Yonsei University, Incheon, Korea.

Extraction and isolation

The dried leaves of *P. americana* (1 kg) were extracted with MeOH (3 × 10 l, 50 °) under sonication for 4 h to yield 210 g extract after evaporation of the solvent. This extract was suspended in H_2O and successively partitioned with hexane, $CHCl_3$ and EtOAc to obtain hexane (PA1, 60 g), $CHCl_3$ (PA2, 4 g), EtOAc (PA3, 30 g), and H_2O (PA4, 120 g) partitions after removal of the solvents *in vacuo.*

PA3 was subjected to a silica gel column and eluted with a gradient of $CHCl_3$: MeOH (20:1 → 2.5:1, v/v) to obtain four sub-fractions, PA3A (5.5 g), PA3B (3.3 g) PA3C (7.3 g), and PA3D (1.2 g). The PA3A fraction was chromatographed on a silica gel column eluted with $CHCl_3$:MeOH:H_2O (5:1:0.1, v/v/v) to give three smaller fractions, PA3A1, PA3A2 and PA3A3. PA3A2 further chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm^2 column, 25% aq. MeCN, and a flow rate of 3 ml/min to yield **3** (2.1 mg), **4** (4.2 mg) and **11** (4.1 mg). PA3A3 was also chromatographed on HPLC using 22% aq. MeCN to afford **13** (7.1 mg) and **17** (8.4 mg). The PA3B fraction was also chromatographed on a silica gel column eluted with $CHCl_3$:MeOH:H_2O (5:1:0.1, v/v/v) to obtain **6** (4.0 mg), **7** (6.4 mg), **8** (6.6 mg) and **10** (3.4 mg). The PA3C fraction was further chromatographed on a silica gel column eluted with $CHCl_3$:MeOH:H_2O (4:1:0.1, v/v/v) to afford **1** (2.1 mg), **2** (2.7 mg) and **9** (2.3 mg).

PA4 was chromatographed on a Diaion HP-20 column eluting with H_2O containing increasing concentrations of MeOH (25, 50 and 75%) to obtain three sub-fractions of PA4A (12 g), PA4B (5 g) and PA4C (4 g). The PA4B was chromatographed on an RP-18 column eluting with MeOH:H_2O (1:1, v/v) to obtain **5** (4.2 mg) and PA4B1. The PA4B1 fraction was chromatographed on HPLC using J'sphere ODS H-80, 250 mm × 20 mm^2 column, 18% aq. MeCN, and a flow rate of 3 ml/min to yield **12** (3.6 mg), **14** (1.5 mg), **15** (2.4 mg) and **16** (3.2 mg).

Spectroscopic data

*Kaempferol 3-O-β-D-fucopyranoside (**1**)*

Yellow amorphous powder. m.p. 220–223 °C; $[\alpha]_D^{20}$: –25.0 (MeOH, *c* = 2.0); UV (MeOH) λ_{max}: 266, 295 nm; IR (KBr) ν_{max}: 3380 (OH), 1685 (conjugated ketone), and 1605 cm^{-1} (phenyl). NMR spectroscopic data, see Table 1; HR-ESI-MS m/z: 431.0982 [M−H]– (calcd for $[C_{21}H_{19}O_{10}]$-, 431.0984).

*Acid hydrolysis of **1***

Compound **1** (2 mg) was dissolved in 1 N HCl (dioxane–H_2O, 1:1, 1 ml) and heated to 80 °C in a water bath for 3 h. The acidic solution was neutralized with silver carbonate and the solvent thoroughly driven out under N_2 gas overnight. After extraction with $CHCl_3$, the aqueous layer was concentrated to dryness using N_2 gas. The residue was dissolved in 0.1 ml of dry pyridine, followed by addition of L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 ml). The reaction mixture was heated at 60 °C for 2 h. Trimethylsilylimidazole solution (0.1 ml) was then added, followed by heating at 60 °C for 1.5 h. The dried product was partitioned with hexane and water (0.1 ml each), and the organic layer was analyzed by gas chromatography (GC): column SPB-1 (0.25 mm × 30 m), detector FID, column temp 210 °C, injector temp 270 °C, detector temp 300 °C, carrier gas He (30 ml/min). Under these conditions, standard sugars gave peaks at t_R (min) 8.85 and 9.35 for D- and L-fucose. Peaks at t_R (min) 8.85 of D-fucose were observed.

Animals

Wild-type adult zebrafish (ZF, *Danio rerio*) was maintained at a S type (1500(W) * 400(D) * 2050(H) mm, Daejeon, Korea) ZF system. Three pairs of ZF were put overnight in a spawning box and eggs were collected at 3 h post-fertilization, then incubated in petri dishes with 0.03% of sea salt solution prepared from sea salts purchased from Sigma-Aldrich Co. (St. Louis, USA). Embryos were maintained in a cycle of 14 h light/10 h dark in an incubator at 28.5 °C ± 5.0, until 6 days post-fertilization (dpf) when the experiments were performed.

Neomycin (NM) induced ototoxicity in ZF model

To induce ototoxicity in ZF, wild-type ZF larvae were placed into 96-well plate and treated with 100 μl of 2 μM neomycin sulfate (MB Cell Co., CA, USA) for 1 h. To evaluate the effect of avocado leave extract compounds on otic hair cell recovery, the ZF in NM solution was first rinsed with 0.03% sea salt solution and then exposed to 1 μM of isolated compounds for 8 h at 28 °C. After the treatment, ZF was rinsed with 0.03% sea salt solution and stained with 0.1% YO-PRO-1, purchased from Thermo Fisher Scientific Inc. (MA, USA) for 30 min, finally were anesthetized with 0.04% tricaine. The otic hair cells were then counted after visualization under a fluorescence microscope (Olympus 1 × 70; Olympus Co., Tokyo, Japan). All images were analyzed by Focus Lite software (Focus Co., Daejeon, Korea).

Cell culture and viability (MTT assay)

The House Ear Institute-Organ of Corti 1 (HEI-OC1) mouse auditory cell line were cultured under permissive conditions (33 °C with 10% CO_2) with high-glucose Dulbeccoś Eagleś medium (DMEM, Sigma–Aldrich Co., St. Louis, USA) containing 10% fetal bovine serum (FBS; Welgene Inc., Gyeongsangbuk-do, Korea) and 50 U/ml

Table 1
NMR spectroscopic data for compound **1**.

Pos.	δ_C [a, b]	δ_H [a, c] (*J* in Hz)	δ_C [d, e]	δ_H [d, f] (*J* in Hz)
2	159.1	–	158.0	–
3	136.2	–	135.3	–
4	179.7	–	179.3	–
5	163.0	–	163.2	–
6	99.9	6.19 (s)	100.2	6.73 (s)
7	166.2	–	166.4	–
8	94.8	6.39 (s)	94.9	6.74 (s)
9	158.5	–	158.0	–
10	105.5	–	105.6	–
1'	122.6	–	122.4	–
2', 6'	132.4	8.08 (d, 8.2)	132.3	8.51 (d, 8.2)
3', 5'	116.1	6.86 (d, 8.2)	116.4	7.20*
4'	161.6	–	162.0	–
1"	105.2	5.03 (d, 7.6)	104.9	6.10 (d, 7.8)
2"	72.8	3.72 (dd, 7.6, 8.8)	73.3	4.71 (dd, 9.0, 7.8)
3"	75.2	3.49 (br d, 8.8)	75.7	4.24 (dd, 9.0, 4.2)
4"	73.0	3.53*	72.7	4.05 (br s)
5"	70.6	3.53*	72.7	3.92 (br q, 6.0)
6"	16.5	1.09 (d, 6.2)	17.4	1.44 (d, 6.0)

[a] Measured in MeOD-d_4, [b] 100 MHz, [c] 400 MHz,, [d] measured in pyridine-d_5, [e] 150 MHz, [f] 600 MHz *overlapped, assignments were done by HSQC, HMBC and COSY experiments.

INF-γ (Peprotech Inc., Seoul, Korea) without antibiotics as previously describe (Kalinec et al., 2016).

The cells were subcultured in a density of 1×10^4 cells/well in 96-well flat bottom plates. The cells were incubated for 24 h and then pre-treated for 1 h with active compounds at a concentration of 1 μM, followed by cotreatment of the 1 μM of compounds with 15 mM NM for 24 h. After treatment at 33 °C, cells were exposed to 0.5 mg/ml of MTT (Duchefa Biochemie, Amsterdam, Netherlands) solution for 4 h. After incubation, the solution was removed and 100 μl of Dimethyl sulfoxide was added to each well to solubilize the formazan crystals. Absorbance was measured (Synergy HT, BioTek Instruments, Inc., VT, USA) at 570 and 630 nm, and average OD in control cells was taken as 100% viability.

Statistical analysis

Data were analyzed using GraphPad Prism (version 5) statistical software package (GraphPad, San Diego, CA, USA). All data are expressed as means ± standard error of the mean (SEM). The statistical significance of the differences between groups was determined using a one-way repeated measures ANOVA and Tukey's post-hoc test. *p* values of <0.05 (*), <0.01 (**) and <0.001 (***) were considered statistically significant.

Results and discussion

Compound **1** was obtained as a yellow amorphous powder and its molecular formula was determined to be $C_{21}H_{20}O_{10}$, by HR-ESI-MS [M−H]− ion at m/z 431.0982 (calcd. for $[C_{21}H_{19}O_{10}]$−, 431.0984). The proton signals corresponding to aromatic rings of flavanol skeleton were observed at δ_H 6.19 (s), 6.39 (s), 6.86 (d, *J* =8.2 Hz), and 8.08 (d, *J* = 8.2 Hz). The β-linkage of the sugar moiety was deduced from the coupling constant (*J* = 7.8 Hz) of the anomeric proton signal at δ_H 5.03. The ^{13}C-NMR and HSQC spectra of **1** revealed 21 carbons signals, including nine quaternary carbons (one carbonyl, six oxygenated), eleven methines (six olefinics, five oxygenated) and one methyl carbon (Table 1). The presence of fucopyranoside was deduced by one methyl proton at δ_H 1.09 (d, *J* = 6.2 Hz), four oxygenated protons at δ_H 3.49, 3.53, 3.53, and 3.72; and the anomeric proton at δ_H 5.03. The sugar moiety was further compared by NMR data in pyridine solvent with those of sugar moiety of batatin I (Escalante-Sánchez and Pereda-Miranda, 2007). The HMBC correlations between H-1" (δ_H 5.03) and C-3 (δ_C 136.2) suggested the presence of *O*-β-fucopyranosyl sugar moiety at C-3 (Fig. 1). Based on the evidence above, compound **1** was determined to be kaempferol 3-*O*-β-D-fucopyranoside.

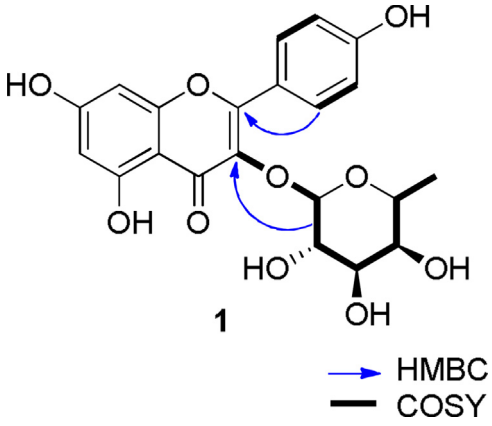
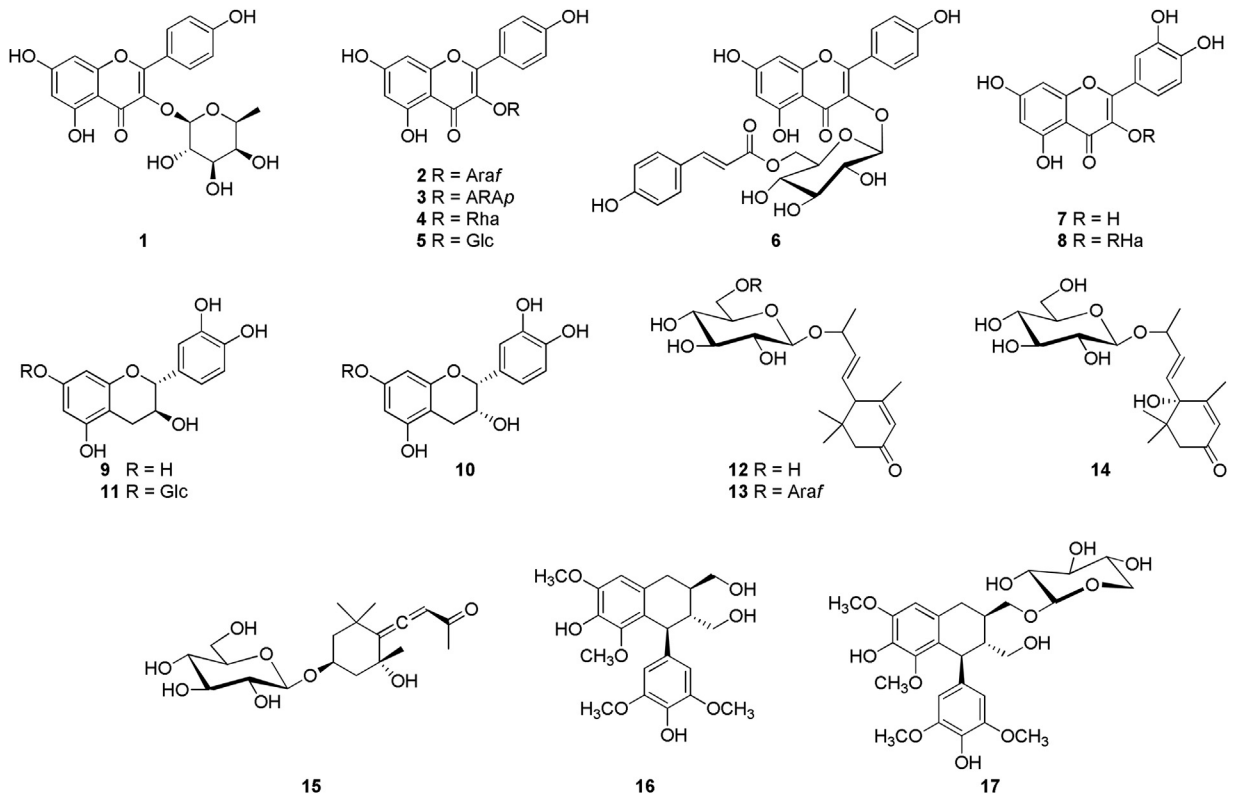


Fig. 1. The key HMBC and COSY correlations of compound **1**.

Comparison of the NMR and MS data with reported values in the literature led to the identification of known compound structures: juglanin and astragaline (**2** and **5**) (Kim et al., 1994), juglalin (**3**) (De Almeida et al., 1998), afzelin and quercitrin (**4** and **8**) (Arot and Williams, 1997), *trans*-tiliroside (**6**) (Lee et al., 2005), quercetin (**7**) (Lin et al., 2009), catechin and epicatechin (**9** and **10**) (Davis et al., 1996), senecin (**11**) (Foo and Karchesy, 1989), (6*R*,9*R*)-3-oxo-α-ionol-9-*O*-β-D-glucopyranoside and (6*S*,9*R*)-roseoside (**12** and **14**) (Kuang et al., 2008), ficumegasoside (**13**) (Van et al., 2011), icariside B_1 (**15**) (Hisamoto et al., 2004), (+)-lyoniresinol (**16**) (Li and Seeram, 2010) and (+)-isolariciresinol 9-*O*-β-D-xylopyranoside (**17**) (Kwon et al., 2010).



In the present study, eleven flavonoids including one new flavonol glycoside (**1**–**11**), four megastigmane glycosides (**12**–**15**) and two lignans (**16**–**17**) were isolated from the leaves of *P. americana*. Previously reported secondary metabolites from *P. americana* are divided into alkanols/aliphatic acetogenins, flavonoids, and phenolics (De Almeida et al., 1998; Abe et al., 2005; Lee et al., 2012; Rodríguez-Sánchez et al., 2013; Di Stefano et al., 2017). Flavonoids, especially the *C*-glycosyl flavones such as oritentin, isoorientin, vitexin, and isovitexin, are the main metabolites reported in the genus *Persea* (Wofford, 1974). However, those compounds were not identified in the present study. Instead, *O*-glycosyl flavanols and *O*-glycosyl flavonols were observed. In addition, there have been no reports about the isolation of megastigmane glycosides and lignans from *P. americana*. Thus, this is the first investigation that reports megastigmane glycoside and lignan components in the *Persea* genus.

Table 2
Number of otic hair cells from zebrafish and cell viability of compounds (1 μM) isolated from *Persea americana* leaves.

Compound	# of otic hair cells	cell viability (%)
NOR	16.63 ± 1.35	100.00 ± 5.00
NM	4.70 ± 1.49	63.10 ± 3.19
1	5.90 ± 1.89*	58.36 ± 1.35
2	6.18 ± 1.73**	95.19 ± 10.19***
3	4.95 ± 1.76	N.D.
4	5.69 ± 1.99	N.D.
5	5.42 ± 2.72	N.D.
6	5.13 ± 1.51	N.D.
7	6.11 ± 1.80*	99.43 ± 9.03***
8	4.84 ± 2.18	N.D.
9	5.89 ± 1.33*	96.55 ± 8.60***
10	4.30 ± 1.70	N.D.
11	5.72 ± 1.89*	58.51 ± 3.57
12	5.94 ± 2.29*	56.53 ± 2.66
13	6.09 ± 1.91*	97.31 ± 10.67***
14	6.09 ± 2.42*	95.10 ± 14.16**
15	5.75 ± 1.66*	63.04 ± 3.91
16	7.09 ± 2.43***	98.79 ± 13.39**
17	6.39 ± 2.52**	58.90 ± 5.26

NM for the neomycin-induced group. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ indicate statistically significant differences compared to the untreated control (NM).

To search for new and protective phytochemicals that recover auditory hair cell function, the biological activity of isolated compounds was evaluated against NM-induced hair cell damaged zebrafish. Otic hair cells under NM exposure significantly decreased the number of the cells, compared to the normal group. However, 1 μM of isolated compounds **1**, **2**, **7**, **9** and **11**–**17** treatment significantly increased the number of otic hair cells (Table 2). All megastigmane glycosides and lignans isolated in the present study exhibited an increased efficacy on otic hair cell recovery after NM-induced cell damage. Especially, juglanin (**2**), (+)-lyoniresinol (**16**), and (+)-isolariciresinol 9-*O*-β-D-xylopyranoside (**17**) demonstrated significant hair cell recovery ($p < 0.01$ and <0.001) compared to the untreated group. Furthermore, compounds increased the numbers of otic hair cell on the ZF model have assessed their cell viability against NM-induced HEI-OC1 cells. In our previous study, we figured out that as the concentration of NM increased, the cell viability of HEI-OC1 cells decreased (Nam et al., 2019). Notably, among the noteworthy compounds, **2**, **7**, **9**, **13**–**14** and **16** did not show cytotoxicity against HEI-OC1 cells. Taken together, these results unraveled a novel therapeutic role of juglanin (**2**) and (+)-lyoniresinol (**16**) for otoprotective phytochemicals and offered an alternative option for the treatment of damaged hair cells.

Conclusions

A phytochemical investigation of *P. americana* leaves resulted in the isolation of previously undescribed kaempferol 3-*O*-β-D-fucopyranoside, together with sixteen known compounds. It should be noted that megastigmane glycosides and lignans were isolated for the first time from the genus *Persea* and these could be potential chemotaxonomic markers of *P. americana*. To identify hearing protective phytochemicals, isolated compounds were evaluated for the protective effects on NM-induced hair cell damaged ZF model and HEI-OC1 cells. These bioactivity results demonstrated that juglanin

(**2**) and (+)-lyoniresinol (**16**) from the avocado leaves protect otic hair cells from NM damage without cellular toxicity.

Authors' contribution

SP: manuscript submission, interpretation of NMR data, writing manuscript. YHN, IR, MSP, KWL, BNH: zebrafish study. JHP, HJK, YO, MO: sample preparations. JSL, DHK, YHP: cell viability test. ISM, SYC, KWJ, THK: manuscript revision. SHK: supervision of the study.

Ethical disclosures

Protection of human and animal subjects.

The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data

The authors declare that no patient data appear in this article.

Right to privacy and informed consent

The authors declare that no patient data appear in this article.

Acknowledgments

This work was supported by the Technology Innovation Program (10080691, Development of an individual approval health functional food and global product; protection of hearing loss and improvement of auditory function for the people with light to moderate hearing loss (26–55 dB of pure tone audiometry threshold using avocado fraction) funded by the Ministry of Trade, Industry & Energy (MOTIE, Korea).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.bjp.2019.08.004.

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