In vitro and *in silico* Evaluations of Chemical Constituents from the Rhizomes of *Aglaonema simplex* (Blume) Blume as Hyaluronidase Inhibitor

Thongchai Khammee*, Sawittree Rujitanapanich and Sutthiduean Chunhakant

Department of Chemistry, Faculty of Science and Technology, Phranakhon Rajabhat University, Changwattana Road, Bangkhen, Bangkok 10220

Amornmart Jaratrungtawee

Bruker Switzerland AG, 10th Floor, Sathorn City Tower, South Sathorn Road, Thungmahamek, Sathorn, Bangkok 10120

Mayuso Kuno

Department of Chemistry, Faculty of Science, Srinakharinwirot University, Sukhumvit 23, Wattana, Bangkok 10110

Received: August 26, 2019; Accepted: September 17, 2019

Abstract

From rhizomes extract of Wan Khan Mak [*Aglaonema simplex* (Blume) Blume] long-chain ester, butyl stearate (1) and a phytosterol, β-sitosterol (2) were isolated**.** The structures of two isolated compounds were elucidated by using analysis of their spectroscopic data, and compared with the reported value of the known compounds. The isolated compounds were further evaluated for *in vitro* hyaluronidase enzyme inhibition assay. Compound 2 (IC_{50} = 888.5±44.9 μg/mL) exhibited mild hyaluronidase inhibitory activity as compared with the standard 6-*O*-palmitoylascorbic acid (IC_{50} = 186.1±3.9 μg/mL). Moreover, a molecular docking study of the highest hyaluronidase inhibitor was carried out to understand the binding mode of the inhibitors into the binding site of hyaluronidase.

Keywords: *Aglaonema simplex*; rhizome; hyaluronidase inhibitor; molecular docking

1. Introduction

Inflammation is a fundamental mechanism to prevent foreign pathogens from causing injury to the cells or tissues, which act by removing dangerous stimuli and entering the therapeutic process (Chen *et al*., 2017). However, excessive inflammation from inflammatory mediators may be associated with organ or tissue dysfunction. Furthermore, inflammatory processes that occur in the body are related to the occurrence of

*Corresponding author: thongchai.k@pnru.ac.th

DOI: 10.14456/tjst.2020.17

various diseases, such as cardiovascular disease, Alzheimer's disease, Parkinson's disease, diabetes, cancer, septic shock, gastroenteritis disease, inflammatory bowel disease, rheumatoid arthritis, and various inflammatory diseases (Aktan, 2004).

The polysaccharide hyaluronic acid (also called hyaluronan, HA) is one of the main components of the extracellular matrix of connective tissues. HA has essential structural and biological functions depending on their size. High-molecular-weight hyaluronic acid (HMW-HA) is responsible for tissue hydration, lubrication, structural integrity, free radical sequestration, and distribution of plasma proteins. Whereas low-molecular-weight hyaluronic acid (LMW-HA) acts as signaling molecules that intensify the inflammatory process. Therefore, maintaining the HMW-HA level is essential not only to inhibit inflammation but also to prevent or treat many diseases. HA polymers can be broken down by hyaluronidases (HYAL). In humans, HYAL plays essential roles in fertilization (Abdul-Aziz *et al*., 1995), embryonic development (Stern, 1984), and wound healing (Buhren *et al*., 2016). Therefore, HAYL inhibitors play an essential role in maintaining HMW-HA levels. Moreover, hyaluronidase inhibitors are considered as potent regulators that might serve as anti-inflammatory, anti-aging, anti-microbial, anti-cancer, anti-toxin and contraceptive agents (Girish *et al*., 2009).

Aglaonema simplex (Blume) Blume (family Araceae), generally known as 'Wan Khan Mak' in Thailand, distributed in Southeast Asia. This plant is classified as a biennial plant. The trunk is about 1.5 centimeters wide, and about 35-40 centimeters tall. It is propagated by seeds, and commonly found in shady and humid habitats in forests (Santisuk *et al*., 2012). *A. simplex* is one of the commercially valuable medicinal plants in Thailand because it is an herb that is used to longevity enhancer and is trusted to have anti-asthmatic activities (Perry and Metzger, 1980). However, there is no report of anti-hyaluronidase activities on this species.

In continuation of our studies of Thai medicinal plants, we report herein the isolation of known phytosterol and long-chain ester from the ethyl acetate extract of the rhizomes of *A. simplex*. Besides, *in vitro* and *in silico* anti-inflammatory activities against hyaluronidase are also discussed.

2. Experimental

2.1 General

All reagents were commercially available and were used without further purification. Infrared (IR) spectra were obtained by using a Bruker TENSOR II Fourier-transform infrared spectroscopy (FTIR) spectrometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 300 FT-NMR using $CDCl_3$ as the solvent. The internal standards were referenced from the residue of those solvents. Column chromatography (CC) was carried out on Merck silica gel 60 (0.040-0.063 mm, Merck KGaA, Darmstadt, Germany). Thin-layer chromatography (TLC) was carried

out with pre-coated Merck silica gel 60 PF_{254} (Aluminum sheet, Merck KGaA, Darmstadt, Germany). High resolution atmospheric pressure chemical ionization time of flight mass spectra (APCI-HR-TOF-MS) analyses were conducted using a Bruker maXis™ 4G mass spectrometer. Melting point (mp) measurements have been done on a Griffin melting point apparatus in degree Celsius of temperature. TLC spots were visualized by ultraviolet light (254 and 365 nm) and staining with *p*-anisaldeyde- sulphuric acid reagent.

2.2 Plant material

Fresh rhizomes of *A. simplex* were collected from at Nong Bua sub-district, Muang district, Kanchanaburi province, Thailand, in March 2018.

2.3 Extraction and isolation

Air-dried rhizomes of *A. simplex* (523.88 g) were ground to powder and then extracted at room temperature with ethyl acetate (EtOAc, 3 x 10 L). Removal of the solvent *in vacuo* gave brown viscous liquid crude EtOAc extract (20.60 g, 3.9 % yield). Fifty grams of the crude EtOAc extract were subjected to silica gel flash column chromatography (FCC), eluting with *n*-hexane/ acetone. The polarity was gradually increased with acetone to obtain six fractions (Fr. 1 - Fr. 6). Compound 1 (29.5 mg) was obtained from Fr. 1 after repeated purification by washing with methanol. Fr. 2 was re-chromatographed by column chromatography (CC) (9 : 1 hexane/ acetone) to give 3 subfractions (subfr. 2-1 - subfr. 2-3). Compound 2 was purified from subfraction 2-2 by recrystallization in methanol to obtain white amorphous solid of compound 2 (25.1 mg).

(1) butyl stearate

White waxy-solids; mp 38-40 ºC; R_f 0.76 (10 % hexane-acetone); IR (KBr) ν_{max} cm^{-1} : 2909, 2832, 1733, 1471, 1457, 1171, 719; ^{1}H-NMR ($CDCl_3$) δ : 0.87 (3H, t, H-3′) 0.89 (3H, t, H-18), 1.27-150 (28H, m, H-4-H-17), 1.63 (2H, m, H-3), 1.63 (2H, m, H-2′), 2.30 (2H, t, H-2), 4.07 (2H, t, H-1′); HR-APCI-TOFMS : (+ve) : m/z 341.3414 $[M+H]^+$ (calcd. for $C_{20}H_{44}O_2$: 340.3341)

(2) β-sitosterol

White amorphous solid; mp 130-138 ºC; Rf 0.50 (10 % hexane-acetone); ATR-FTIR ν_{max} cm^{-1} : 3371, 2916, 1732, 1462, 1068, 1021; ^{1}H-NMR ($CDCl_3$) δ : 0.77 (3H, s, H-18), 0.82 (3H, d, J = 6.4 Hz, H-27), 0.84 (3H, d, J = 6.4 Hz, H-26), 0.85 (3H, d, J = 3.3 Hz, H-29), 0.98 (3H, J = 6.6 Hz, H-21), 1.00 (3H, s, H-19), 3.49 (1H, m, H-3), 4.96 (2H, dd, J = 15.1, 8.4 Hz, H-22), 5.13 (2H, dd, J = 15.1, 8.4 Hz, H-23) 5.32 (1H, br d, J = 4.3 Hz, H-6); HR-APCI-TOFMS : (+ve): m/z 415.3934 $[M+H]^+$ (calcd. for $C_{29}H_{50}O$: 414.3862)

2.4 Evaluation of *in vitro* anti-hyaluronidase activities

The effects of the samples on hyaluronidase were determined by the previously reported procedure (Ndlovu *et al.*, 2013) with slight modifications. 50 µL hyaluronidase from bovine testes (Sigma-Aldrich, 400-1000 units/ mg dissolved in 0.1M acetate buffer, pH 3.5) was mixed with 50 µL of various concentrations of isolated compound 1 and 2 ranging from 25 µg/ mL to 1000 µg/ mL.

The mixture was incubated at 37 ºC for 20 minutes. The enzymatic reaction was originated by adding 50 µL of 12.5 mM calcium chloride in the mixture and incubated at 37 ºC for 20 min. Then 250 µL of hyaluronic acid sodium salt (Alfa Aesar) was added to the mixture and next incubated in the water bath at 100 ºC, for 3 min. The reaction mixture was allowed to cool to room temperature, then 1.5 mL of *p*-dimethyl amino benzaldehyde (PDMAB, Sigma-Aldrich) was added to the reaction mixture, and it was then incubated in water bath at 37 ºC for 20 min. The 200 µL of contents was transferred to respective a 96-well black clear flat bottom microplate. Fluorescence was detected using a VICTOR Nivo Multimode Microplate Reader (PerkinElmer Inc.) at 545 nm excitation and 612 nm emission. 6-O-palmitoylascorbic acid (Asc6Plm, Sigma-Aldrich), the known hyaluronidase inhibitor was used as the positive control (Botzki *et al.*, 2004). The blank, control, and all concentrations of inhibitors were analyzed in triplicate, and inhibition percentage was calculated by using the following equation:

$$\text{Inhibition (\%)} = \left(\frac{FI_{control} - FI_{sample}}{FI_{control}}\right) x100$$

whereas $FI_{control}$ refers to the fluorescence intensity at 612 nm of buffer, hyaluronidase + solvent; FI_{sample} means the fluorescence intensity at 612 nm of different concentrations of tested.

2.5 Statistical analysis

Data are represented as mean ± SD, and the half maximal inhibitory concentration (IC_{50}) values were calculated using Microsoft Excel 2016 package.

2.6 Molecular docking studies

The ligand setup was carried out using ACD/ ChemSketch and optimized using Gaussian 09 molecular simulation software (Gaussian, Inc., CT, USA) with the HF methods and the basis sets 3-21G. Auto Dock Tools 1.5.4 (ADT) and Auto Dock 4.2 programs were used for molecular docking for 100 runs and 2,500,000 maximum number of energy evaluation, 27000 maximum number of generations. The docking procedure was carried out with the setting size of grid box 100 x 100 x 100 Å points along x, y, z axes. The grid center was set at 55.813, -28.162, 6.854. The crystal of structures human hyaluronidase 1 [PDB entry code: 2PE4 (Chao *et al.*, 2007), resolution: 2 Å] was obtained from the Protein Data Bank. All water and noninteracting ions were removed. Then all missing hydrogen were added using the ADT program. Gasteiger charges were calculated for the system. Docking method validation was done by redocking natural ligand receptor on the active site. The docking validation method was evaluated depending on the root-mean-square deviation (RMSD) and declared accurate if the value of RMSD smaller than 3.5 Å (Kontoyianni *et al.*, 2004). In this study, the RMSD was 3.080, indicating this protocol and calculation parameter settings meet the criteria of validity docking methods.

3. Results and Discussion

3.1 Structure elucidation

Chromatographic separation of EtOAc fraction from the rhizomes of *A. simplex* led to

the isolation of compounds 1 and 2 (Figure 1). All the obtained compounds were identified by comparing the spectroscopic data with those published values.

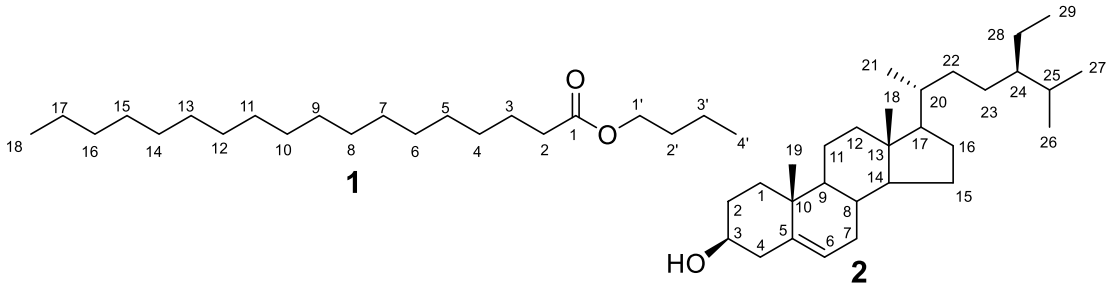

Figure 1 Structures of compounds 1 and 2

Compound 1 was a white waxy-solid. It was detected as purple color on TLC when visualized with anisaldehyde-sulphuric acid stain reagent. The R_f value is 0.93 (10 % hexane-acetone). The FT-IR (KBr) spectrum displayed C = O stretching at 1736 cm^{-1} and C-O stretching band at 1175 cm^{-1}. The APCI-TOFMS spectrum exhibits mass ion signals at m/z 341.3414 $[M+H]^+$, corresponding to the molecular formula $C_{20}H_{44}O_2$. The analysis of the ^{1}H-NMR spectrum indicates that characteristic peaks at δ_H 4.07 ppm (t) and 2.30 ppm (t) represent the -CH_2- groups adjacent to the oxygen of the ester and the carbonyl. The signals at δ_H 0.93 ppm (t) and 0.88 ppm (t) are characteristic of CH_3 groups, respectively, one that ends the methylene oxygen binder and the one ending those related to the carbonyl of the ester function. The multiplet signals at δ_H 1.27 ppm to 1.50 ppm corresponded to the -CH_2- groups between the extended hydrocarbon chain methylene group linked to the carbonyl. These values are similar to those found in the literature (Tshilanda *et al.*, 2014). According to this literature, compound 1 was identified as butyl stearate.

Compound 2 was isolated as a white amorphous solid (m.p. 130-138 ºC). It showed purple color on TLC when visualized with *p*-anisaldehyde-sulphuric acid spray reagent [R_f = 0.50 (10 % hexane-acetone)]. The ATR FT-IR spectrum showed hydroxyl absorption bands at 3371 cm^{-1} and methylene at 1462 cm^{-1}. The ^{1}H NMR spectrum of compound 2 varied between 0.77 to 5.32 ppm. This spectrum showed the presence of 6 high-intensity peaks indicating the presence of six methyl groups at δ 0.77 (3H, s, H-18), 0.82 (3H, d, J = 6.4 Hz, H-27), 0.84 (3H, d, J = 6.4 Hz, H-26), 0.85 (3H, d, J = 3.3 Hz, H-29), 0.98 (3H, d, J = 6.6 Hz, H-21) and 1.00 (3H, s, H-19) ppm. The proton corresponding to the H-3 of a sterol moiety has appeared as a multiplex at δ 3.49 ppm. The signals corresponding to three protons of ethylene group appeared at δ 5.13 ppm and δ 5.32 ppm. Its positive APCI-TOFMS showed a molecular ion peak at m/z 415.3934 $[M+H]^+$ corresponding to

DOI: 10.14456/tjst.2020.17

the molecular formula $C_{29}H_{50}O$. On this basis and in comparison with published data (Okoro *et al*., 2017), the compound 2 was identified as β-sitosterol.

3.2 *In vitro* anti-hyaluronidase activity evaluation

The biological activity profiles of the compounds 1 and 2 as hyaluronidase inhibitors were assayed in comparison with Asc6Plm as the reference compound. The IC_{50} value of each compound was determined by using the calibration curve, which gave a linear regression equation (R^2 > 0.9000), and the results are tabulated in Table 1. The study revealed that β-Sitosterol (2) was mild inhibitors of hyaluronidase with the IC_{50} value of 888.5±44.9 µg/mL, while 1 showed no inhibition (IC_{50} > 1000 µg/mL). In the case of the standard positive control, Asc6Plm showed an IC_{50} of 186.1±3.9 µg/mL. Following the anti-hyaluronidase activity evaluation, compound 2 was selected for further molecular docking simulations against crystal structures of human hyaluronidase.

3.3 Molecular docking analysis

Molecular docking study was carried out to determine the potential interactions and binding affinity between compound 2 and the target enzyme, hyaluronidase (2PE4). The docking study was carried out by using the Autodock software package. The docking results showed that compound 2 well accommodated in the binding pocket of hyaluronidase resulted in an energy conformation of -5.93 kcal/mol. In the case of butyl stearate (1) showed the lowest binding energy affinity of -3.42 kcal/mol, while the reference standard Asc6plm shows the binding free energy in the value of -8.70 kcal/mol. These results were consistent with the IC_{50} values. In the complex between β-sitosterol (2) and binding site of 2PE4, the residues of Glu304 and Asn350 were important for strong hydrogen bonding interaction with C-3 hydroxyl group of compound 2 with a bond length of 4.652 and 3.066 Å, respectively. The π···π interaction (distance 3.538 Å) between Glu304 and compound 2 was also observed. While the other residues which contributed toward the orientation of the substrate- binding site were Phe347, Leu379, Leu380, Leu381, Leu382, Asn383, Pro384, Asp408, Gln411, Met412, Glu415 and Phe416 as shown in Figure 2 and Table 2.

Table 1 Anti-hyaluronidase activity of isolated compounds

Sample	IC_{50} ± SD (µg/mL)
Butyl stearate (1)	inactive
β-sitosterol (2)	888.5±44.9
Asc6Plm*	186.1± 3.9

Values are mean ± SD (n = 3); SD = standard deviation; * = standard positive control; inactive at 1,000 µg/mL

4. Conclusions

Investigation on the chemical constituents from the rhizome of *A. simplex* resulted in the isolation of two- major compounds. Their structures were established by spectroscopic data as well as comparison of these data with the literature. These compounds were tested

against hyaluronidase inhibitory activity. β-sitosterol (2) showed the mild *in vitro* inhibitory activity against hyaluronidase (IC_{50} = 886.5±44.9 μg/mL). Furthermore, molecular docking studies indicated that compound 2 could bind to the binding site of hyaluronidase. This information is valuable for further development of new hyaluronidase inhibitors with higher potency.

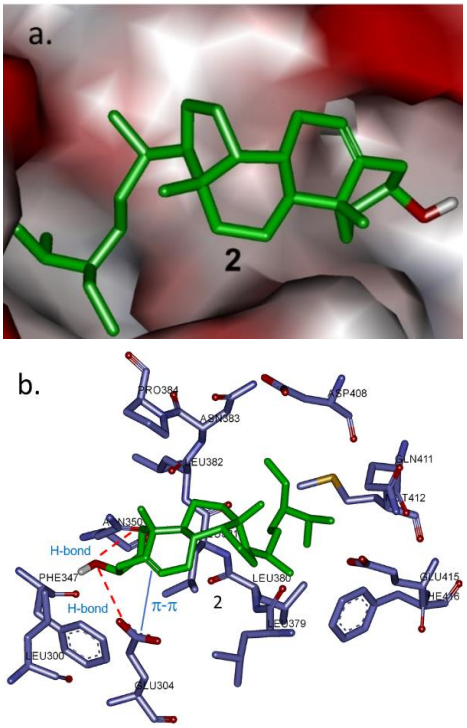

Figure 2 (a) Binding cavity of hyaluronidase with compound 2 (green, stick model). (b) The interactions of the compound 2 with the hyaluronidase residues (purple, stick model). Hydrogen-bond interactions with amino acid side chains are represented by a red dashed line. The π-π interactions are represented by a blue line with symbols indicating the interaction.

Table 2 The key binding interactions of β-sitosterol (2) with hyaluronidase (2PE4)

β-sitosterol (2) position	Residue	Interaction	Distance (Å)
3-OH	Glu304	H-bond	4.652
		π···π	3.538
3-OH	Phe347	O···π	3.647
3-OH	Asn350	H-bond	3.066
C-21	Leu379	C···O	3.116
C-26 or 27*	Leu380	C···C	3.238
C-29	Leu381	C···C	3.222
C-11	Leu382	C···O	2.996
C-21	Asn383	C···C	3.908
C-11	Pro384	C···C	3.885
C-29	Asp408	C···O	3.834
C-26 or 27*	Gln411	C···C	3.753
C-26 or 27*	Met412	C···N	3.500
C-21	Glu415	C···C	3.394
C-26 or 27*	Phe416	C···C	3.463

Position under * can be switch

5. Acknowledgments

This work was financially supported by Phranakhon Rajabhat University (grant No. 01. 38/2561). The authors are grateful to the Faculty of Science and Technology, Phranakhon Rajabhat University, and Department of Chemistry, Faculty of Science, Srinakharinwirot University, for providing necessary research facilities.

6. Conflict of interest

The authors declare that there is no

conflict of interest regarding the publication of this article.

7. References

Abdul-Aziz, M., MacLusky, N.J., Bhavnani, B.R. and Casper, R. F., 1995, Hyaluronidase activity in human semen: Correlation with fertilization *in vitro*, Fertil. Steril. 64: 1147-1153.

Aktan, F., 2004, iNOS- mediated nitric oxide production and its regulation, Life Sci. 75: 639-653.

Botzki, A., Rigden, D. J., Braun, S., Nukui, M., Salmen, S., Hoechstetter, J. and Buschauer, A., 2004, L- Ascorbic acid 6-hexadecanoate, a potent hyaluronidase inhibitor: X- ray structure and molecular modeling of enzyme-inhibitor complexes, J. Biol. Chem. 279: 45990-45997.

Buhren, B. A., Schrumpf, H., Hoff, N. P., Bölke, E., Hilton, S. and Gerber, P. A., 2016, Hyaluronidase: from clinical applications to molecular and cellular mechanisms, Eur. J. Med. Res. 21: 5-12.

Chao, K. L., Muthukumar, L. and Herzberg, O., 2007, Structure of human hyaluronidase-1, a hyaluronan hydrolyzing enzyme involved in tumor growth and angiogenesis, Biochemistry 46: 6911-6920.

Chen, L., Deng, H., Cui, H., Fang, J., Zuo, Z., Deng, J. and Zhao, L., 2017, Inflammatory responses and inflammation- associated diseases in organs, Oncotarget 9: 7204-7218.

Girish, K.S., Kemparaju, K., Nagaraju, S. and Vishwanath, B. S., 2009, Hyaluronidase inhibitors: A biological and therapeutic perspective, Curr. Med. Chem. 16: 2261-2288.

Kontoyianni, M., McClellan, L.M. and Sokol, G.S., 2004, Evaluation of docking performance: Comparative data on docking algorithms, J. Med. Chem. 47: 558-565.

Ndlovu, G., Fouche, G., Tselanyane, M., Cordier, W. and Steenkamp, V., 2013, *In vitro* determination of the anti- aging potential of four Southern African medicinal plants, BMC Complem. Altern. M. 13: 304-311.

Okoro, I.S., Tor-Anyiin, T.A., Igoli, J.O., Noundou, X. S. and Krause, R. W. M., 2017, Isolation and characterisation of stigmasterol and β-sitosterol from *Anthocleista djalonensis* A. Chev, Asian J. Chem. Sci. 3(4): 1-5.

Perry, L. M. and Metzger, J., 1980, Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses, Cambridge MIT Press, London.

Scotti, L., Singla, R.K., Ishiki, H.M., Mendonca, F.J.B., da Silva, M.S., Barbosa Filho, J.M. and Scotti, M. T., 2016, Recent advance ment in natural hyaluronidase inhibitors, Curr. Top. Med. Chem. 16: 2525-2531.

Stern, C. D., 1984, Mini-review: Hyaluronidases in early embryonic development, Cell Biol. Int. Rep. 8: 703-717.

Santisuk, T., Peter, K. L., Sookchaloem, D., Hetterscheid, W. L. A., Gusman, G., Jacobsen, N., Idei, T. and Van, D.N., 2012, Flora of Thailand, Volume 11, Part 2: Acoraceae & Araceae, Bangkok office of

the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Bangkok.

Tshilanda, D.D., Mpiana, P.T., Onyamboko, D.N., Mbala, B. M. , Ngbolua, K. T. , Tshibangu, D.S. and Kasonga, T.K., 2014, Antisickling activity of butyl stearate isolated from *Ocimum basilicum* (Lamiaceae), Asian Pac. J. Trop. Biomed. 4: 393-398.