SUPPLEMENTARY MATERIAL

Antioxidant and Hyaluronidase Inhibitory Activities of Divers Phenolics in

Phyllanthus emblica

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Fifty-eight phenolic compounds isolated from *P. emblica* were screened and compared for their *in vitro* and *in vivo* antioxidant properties, as well as hyaluronidase (HAase) inhibitory Activities. Among them, 20 compounds showed to be promising antioxidants due to the stronger scavenging activity in both DPPH radical and *Danio rerio* ROS assays, while nine compounds were potential HAase inhibitors with hundred fold stronger activities than that of the positive control, DSCG. The structure activity relationship was discussed.

Keywords: *Phyllanthus emblica*, phenolics, antioxidant, DPPH radical, *Danio rerio* ROS scavenging activity, HAase inhibition

3. Experimental

3.1. General

The solvents used for extracts (methanol and acetone) were glass-distilled prior to use. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), glutathione (GSH), *dl*- α -tocopherol, L-ascorbic acid, cetylpyridinium chloride, disodium cromoglycate (DSCG) and HAase (EC 3.2.1.35) used for *Danio rerio* ROS scavenging, DPPH radical scavenging and hyaluronidase (HAase) inhibition assays were purchased from Sigma/Aldrich (St. Louis, MO). CM-H₂DCFDA (C6827) was purchased from Invitrogen. Fifty-eight testing compounds were isolated from the fruits, roots, branches and leaves of *P. emblica*, by the groups at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS) and the Graduate School of Biomedical Sciences, Nagasaki University, Japan (Zhang et al, 2000a; Zhang et al, 2000b; Zhang et al, 2001a; Zhang et al, 2001b; Zhang et al, 2002; Liu et al, 2009; Lv et al, 2014), and confirmed by NMR and MS spectroscopy. These include phyllanemblinins A, C-F (**1**, **12**, **16-18**) (Zhang et al, 2001a), elaeocarpusin (**2**) (Zhang et al, 2001a), neochebulagic acid (**3**) (Zhang et al, 2001a), putranjivain A (**4**) (Zhang et al, 2001a), chebulagic acid (**5**) (Zhang et al, 2001a), punicafofin (**6**) (Zhang et al, 2001a), carpinusin (**7**) (Zhang et al, 2001a), mallonin (**8**) (Zhang et al, 2001a), geraniin (**9**) (Zhang et al, 2001a), tercatin (**10**) (Zhang et al, 2001a), corilagin (**11**) (Zhang et al, 2000b; Zhang et al, 2001a), furosin (**13**) (Zhang et al, 2001a), putranjivain B (**14**) (Zhang et al, 2001a), chebulanin (**15**) (Zhang et al, 2001a), 1,6-di-*O*-galloyl- (**19**) (Zhang et al, 2001a), 1,2,3,6-tetra-*O*-galloyl- (**20**) (Zhang et al, 2000b), 1,2,4,6-tetra-*O*-galloyl- (**21**) (Zhang et al, 2000b), 1,2,3,4,6-penta-*O*-galloyl- (**22**) (Zhang et al, 2000b), 1-*O*-digalloyl- (**23**) (Zhang et al, 2001a), and 1-*O*-galloyl- (**24**) (Zhang et al, 2001a) β -D-glucose, flavogallonic acid bislactone (**25**) (Zhang et al, 2001a), digallic acid (**26**), decarboxyellagic acid (**27**) (Zhang et al, 2000b), gallic acid 3-*O*-(6'-*O*-galloyl)-glucoside (**28**) (Zhang et al, 2001a), gallic acid (**29**) (Zhang et al, 2000b), L-malic acid 2-*O*-gallate (**30**) (Zhang et al, 2001b), mucic acid 2-*O*-gallate (**31**) (Zhang et al, 2001b), chebulic acid (**32**) (Zhang et al, 2001a), gallic acid 3-*O*-glucoside (**33**) (Zhang et al, 2001a), multifidol 1-*O*-(6''-*O*- β -D-apiofuranosyl)- β -D-glucoside (**34**) (Zhang et al, 2000b), multifidol glucoside (**35**) (Zhang et al, 2000b), epigallocatechin 3-*O*-gallate (**36**) (Zhang et al, 2000b), prodelphinidins B-2 (**37**) (Zhang et al, 2001b), B-1

(**38**) (Zhang et al, 2000b; Zhang et al, 2001a), and A-1 (**40**) (Zhang et al, 2000b), (-)-epicatechin 3-*O*-gallate (**39**) (Zhang et al, 2000b), (-)-epigallocatechin (**41**) (Zhang et al, 2000b), gallocatechin (**42**) (Zhang et al, 2000b), (-)-epicatechin (**43**) (Zhang et al, 2000b), (+)-catechin (**44**) (Zhang et al, 2000b), (*S*) eriodictyols 7-*O*-(6"-*O*-galloyl)-glucoside (**45**) (Zhang et al, 2002), 7-*O*-glucoside (**48**) (Zhang et al, 2002), and 7-*O*-(6"-*O*-*trans*-coumaroyl)-glucoside (**52**) (Zhang et al, 2002), quercetin (**46**) (Zhang et al, 2002), naringenins 7-*O*-(6"-*O*-galloyl)-glucoside (**47**) (Zhang et al, 2002), 7-*O*-(6"-*O*-*trans*-coumaroyl)-glucoside (**55**) (Zhang et al, 2002), and 7-*O*-glucoside (**58**) (Zhang et al, 2002), quercetins 3-*O*-glucoside (**49**) (Zhang et al, 2002), 3-*O*-(6"-*O*-glucosyl)-glucoside (**50**) (Zhang et al, 2002), and 3-*O*-rhamnoside (**51**) (Zhang et al, 2002), myricetin 3-*O*-rhamnoside (**53**) (Zhang et al, 2002), taxifolin 7-*O*-glucoside (**54**) (Zhang et al, 2002), naringenin (**56**) (Zhang et al, 2002), and kaempferol 3-*O*-rhamnoside (**57**) (Zhang et al, 2002). Among them, 17 compounds (**2**, **4-6**, **8**, **10**, **11**, **15**, **19**, **23**, **24**, **30**, **31**, **49**, **51**, **53**, and **57**) were yielded from the fruits, 25 compounds (**1**, **3**, **7**, **9**, **11**, **13-18**, **22**, **35**, **37-39**, **45-48**, **50**, **52**, **55**, **56** and **58**) were got from the branches and leaves, and 17 compounds (**20**, **21**, **25-29**, **32-34**, **36**, **39-44**) were isolated from roots of *P. emblica*. Compound **39** was obtained from both the roots and the branches and leaves of *P. emblica*.

3.2. Plant material

P. emblica were collected in Baoshan City, Yunnan Province, People's Republic of China, and identified by Prof. C. R. Yang (Kunming Institute of Botany, Chinese Academy of Sciences). A voucher specimen (KIB-ZL-0100020) has been deposited in State key laboratory of phytochemistry and plant resource in west China of Kunming Institute of Botany, Chinese Academy of Sciences.

3.3. HAase Inhibition Assay

The inhibitory activity of HAase was measured by the turbidimetric assay with slight modifications (Ferrante, 1956). Samples dissolved in 0.1 M acetate buffer (0.2 mL) and HAase (EC 3.2.1.35) from the bovine testes type I-S in buffer (final concentration: 4000 unit/mL, 0.1 mL) were mixed and the mixture was incubated at 37 °C for 20 min. Then, tested compounds was added and incubated at 37 °C for 20 min. After HAase potassium salt (in buffer (final concentration: 0.4 mg/mL, 0.5 mL) had been added, the mixture was incubated at 37 °C for 20 min. Then, the enzymatic reaction was terminated by the addition of 100 μ L of 1% CTAB (hexadecyltrimethyl-

ammonium bromide) in 2% aqueous NaOH. The turbidity at 595 nm was measured after 30 min. DSCG (disodium cromoglycate) was used as positive control. The IC₅₀ (the concentration required to inhibit 50% HAase) values of tested compounds were calculated using the software of SPSS 16.0 and expressed as means ± standard errors (SE) in triplicate.

In addition, the data suggested that The function of hyaluronidase should be related to antioxidant activity due to some antioxidants reported herein with the potential HAase inhibitory activities (Figure S3-S5).

3.4. DPPH radical scavenging assay

The DPPH assay was performed according to Shan et al (Shan et al, 2005). 50 µL of different concentrations (ranging from 0.15 to 15 µM) of the methanolic samples was added to 1.95 ml of DPPH solution (60 µM in methanol). The reaction was carried out at 23 °C in the dark for 40 min and then the absorbance of the reactive mixture was recorded using a spectrophotometer at 517 nm. Pure ethanol was used as a control sample. The radical scavenging activities of samples, expressed as a percentage inhibition of DPPH, were calculated according to the formula: inhibition% = $[(A_0 - A_1)/A_0] \times 100$, where A₀ and A₁ are the absorbance values of the blank sample and of the tested samples, respectively. *dl*-Tocopherol and L-ascorbic acid were used as positive control. The SC₅₀ (the concentration required to scavenge 50% radicals) values of tested compounds toward DPPH were calculated using the software of SPSS 16.0 and expressed as means ± standard errors (SE) in triplicate.

3.5. ROS scavenging assay

Adult AB strain zebrafish were housed in a light and temperature controlled aquaculture facility with a standard 14 : 10 hours light/dark photoperiod and fed with live brine shrimp twice daily and dry flake once a day. Four to five pairs of zebrafish were set up for nature mating every time. On average, 200 – 300 embryos were generated. Embryos were maintained at 28 °C in fish water (0.2% Instant Ocean Salt in deionized water, pH 6.9 – 7.2, an conductivity 480 – 510 ms/cm and hardness of 53.7 - 71.6 mg/L CaCO₃). The embryos were washed and staged at 6 and 24 hours post-fertilization (hpf) (Kimmel et al, 1995). The 3 dp larval zebrafish were used to the antithrombotic test. The zebrafish facility at Hunter Biotechnology, Inc. is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

The experiment was carried out on 96-well cell culture plates, of which it included a negative control group, a solvent control group, a positive control group and a ROS model group and eight samples groups. Each group processed 6 wells, and the larval zebrafish were seeded 1 fish per well. No fish was in the negative control group. The CM-H₂DCFDA (C6827, Invitrogen) was used to test the ROS in larval zebrafish (Akshata et al, 2007). The negative control group was used to prove that the solvent did not have negative effects on the testing results. The solvent group not only represented the background value of the test group, but also made sure that the ROS of larval zebrafish were normal. GSH was used as the positive control. The tested compounds were added separately as the test group with a final concentration of 30 μ M. The quantitative analysis of emulsion power (F) of ROS was carried on a multifunction microplate reader (Mithras LB940, Berthold Technologies). The inhibition ratio was calculated by the following equation, and before calculations, the F (test group) and F (model group) should minus the background values. All incubations were performed in triplicate. The CM-H₂DCFDA was absorbed by larval zebrafish and were hydrolyzed to DCFH. The ROS *in vivo* of larval zebrafish oxidized DCFH with no fluorescence to DCF with strong fluorescence. The level of ROS *in vivo* of larval zebrafish was suggested by testing the fluorescence of DCF. The ROS scavenging rate of tested compounds were calculated using the software of SPSS 16.0 and expressed as means $\pm$ standard errors (SE) in triplicate. The results were shown as $\bar{X} \pm \text{SE}$.

$$\text{ROS clearance rate (\%)} = \left[1 - \frac{\text{F (test group)}}{\text{F (model group)}}\right] \times 100$$

3.6. Statistical analysis

The results expressed as the means $\pm$ standard error (SE) of four independent experiments. Test was used to compare means of the positive control to each tested compounds. For data analysis, softwares Graph Pad Prism 5.0 and Origin Pro. 8.0 for Windows were used.

3.7. Zebrafish

A breeding stock of healthy mature zebrafish (4 to 5 pairs) was used for naturally embryos production. Each pair can yield up to 200 to 300 embryos and the dead embryos were removed 6 and 24 hours post-fertilization (dpf). The rest suitable embryos, according to the embryonic stage of development (Kimmel et al., 1995),

R_6O
 OR_1
 OR_3
 OR_4
 OR_2

R_6O
 R_4O
 R_3O
 OR_1
 OR_2

1 $R_1 = G, R_2, R_4 = \text{Ela}, R_3 + R_6 = \text{HHDP}$
3 $R_1 = G, R_2 = H, R_4 = \text{Neoche}, R_3, R_6 = \text{HHDP}$
4 $R_1 = G, R_2, R_4 = \text{Put}, R_3, R_6 = \text{HHDP}$
5 $R_1 = G, R_2, R_4 = \text{Che}, R_3, R_6 = \text{HHDP}$
6 $R_1 = R_2 = R_4 = G, R_3, R_6 = \text{HHDP}$
7 $R_1 = G, R_2, R_4 = \text{Che}, R_3 = \text{Neoche}, R_6 = H$
8 $R_1 = G, R_2, R_4 = \text{Ela}, R_3 = R_6 = H$
9 $R_1 = G, R_2, R_4 = \text{DHHDP}, R_3, R_6 = \text{HHDP}$
10 $R_1 = R_4 = G, R_2 = H, R_3, R_6 = \text{HHDP}$
11 $R_1 = G, R_2 = R_4 = H, R_3, R_6 = \text{HHDP}$
13 $R_1 = G, R_2, R_4 = \text{DHHDP}, R_3 = R_6 = H$
14 $R_1 = G, R_2 = R_4 = \text{Put}, R_3 = R_6 = H$
15 $R_1 = G, R_2, R_4 = \text{Che}, R_3 = R_6 = H$

16 $R_1 = G, R_4 = \text{Neoche}, R_2 = R_3 = R_6 = H$
17 $R_1 = G, R_3 = \text{Neoche}, R_2 = R_4 = R_6 = H$
18 $R_1 = G, R_6 = \text{Neoche}, R_2 = R_3 = R_4 = H$
19 $R_1 = R_6 = G, R_2 = R_3 = R_4 = H$
20 $R_1 = R_2 = R_3 = R_6 = G, R_4 = H$
21 $R_1 = R_2 = R_4 = R_6 = G, R_3 = H$
22 $R_1 = R_2 = R_3 = R_4 = R_6 = G$
23 $R_1 = G_2, R_2 = R_3 = R_4 = R_6 = H$
24 $R_1 = G, R_2 = R_3 = R_4 = R_6 = H$

1 $R_1, R_2 = \text{HHDP}$
12
16 $R = H$
G2 $R = G$
Neoche
HHDP
Put
DHHDP

Figure S1. Structures of ellagitannins and gallotannins **1-24**.

Figure S2. Structures of aromatic compounds and organic acid gallates, condensed tannins and their monomers, and flavonoids **25-58**.

Figure S3. The inhibitory activity on HAase of compounds **1-58**. (a-e) compounds **1-58** inhibited on HAase. (f) Relationship between structural type and inhibitory activities on HAase. (g) Relationship between the number of phenol and inhibitory activities on HAase. (h) Relationship between the number of 1,2 dihydroxy phenol and inhibitory activities on HAase. The IC₅₀ of inhibitory activity on HAase expressed as mM; DSCG is the positive control; Error bars are means $\pm$ SE

Figure S4. The DPPH radical scavenging activity of compounds 1-58. (a-e) Activities according to structural types. (f) Relationship between structural types and activities. (g) Relationship between phenol number and activities. (h) Relationship between *ortho*-dihydroxy phenol numbers and activities. The SC₅₀ of DPPH radical scavenging activity expressed as μM; *dl*-α-tocopherol and L-ascorbic acid are the positive control; Error bars are means ± SE.

Figure S5. The ROS scavenging activity of compounds 1-58. (a-e) Activities according to structural types. (f) Relationship between structural types and activity. The ROS scavenging rate expressed as %; GSH is the positive control; Error bars are means $\pm$ SE

Table S1. HAase inhibitory, DPPH radical and *Danio rerio* ROS scavenging activities of compounds **1-58** from *P. emblica*

Compounds	HAase ^a IC ₅₀ (mM)	DPPH ^b SC ₅₀ (μM)	ROS ^c SR (%)
Ellagitannins			
Phyllanemblinin C (1)	8.71 ± 0.12	0.25 ± 0.04	67.0 ± 2.94
Elaeocarpusin (2)	0.03 ± 0.007	0.37 ± 0.02	105.2 ± 2.96
Neochebulagic acid (3)	0.14 ± 0.014	0.43 ± 0.12	92.5 ± 2.16
Putranjivain A (4)	0.06 ± 0.006	0.45 ± 0.10	82.6 ± 2.73
Chebulagic acid (5)	0.05 ± 0.004	0.51 ± 0.14	71.0 ± 2.67
Punicafolin (6)	0.03 ± 0.002	0.55 ± 0.02	62.0 ± 2.33
Carpinusnin (7)	0.15 ± 0.04	0.57 ± 0.12	94.1 ± 2.76
Mallonnin (8)	0.27 ± 0.02	0.58 ± 0.15	110.6 ± 2.82
Geraniin (9)	0.17 ± 0.012	0.58 ± 0.17	83.1 ± 1.98
Tercatain (10)	0.05 ± 0.007	0.67 ± 0.11	67.9 ± 3.74
Corilagin (11)	0.36 ± 0.048	0.70 ± 0.16	72.4 ± 4.19
Phyllanemblinin A (12)	0.20 ± 0.018	0.82 ± 0.19	-15.0 ± 7.17
Furosin (13)	1.17 ± 0.35	0.90 ± 0.20	95.0 ± 2.17
Putranjivain B (14)	0.06 ± 0.004	0.98 ± 0.21	85.3 ± 2.41
Chebulanin (15)	0.05 ± 0.002	1.03 ± 0.46	80.9 ± 3.17
Phyllanemblinin E (16)	0.48 ± 0.12	1.17 ± 0.50	82.8 ± 3.07
Phyllanemblinin D (17)	1.21 ± 0.46	1.24 ± 0.09	92.1 ± 2.11
Phyllanemblinin F (18)	0.21 ± 0.04	1.27 ± 0.14	85.1 ± 2.57
Gallotannins			
1,6-Di- <i>O</i> -galloyl-β-D-glucose (19)	3.64 ± 0.14	0.50 ± 0.15	92.3 ± 2.23
1,2,3,6-Tetra- <i>O</i> -galloyl-β-D-glucose (20)	0.11 ± 0.04	0.52 ± 0.09	77.4 ± 2.60
1,2,4,6-Tetra- <i>O</i> -galloyl-β-D-glucose (21)	0.18 ± 0.009	0.55 ± 0.16	79.7 ± 2.85
1,2,3,4,6-Penta- <i>O</i> -galloyl-β-D-glucose (22)	0.01 ± 0.002	0.82 ± 0.17	88.4 ± 2.44
1- <i>O</i> -Digalloyl-β-D-glucose (23)	0.40 ± 0.03	0.96 ± 0.18	89.2 ± 2.11
1- <i>O</i> -Galloyl-β-D-glucose (24)	10.8 ± 0.15	1.85 ± 0.23	73.4 ± 3.13
Aromatic compounds and organic acid gallates			
Flavogallonic acid bislactone (25)	N	0.56 ± 0.12	93.5 ± 1.55
Digallic acid (26)	N	0.59 ± 0.18	93.4 ± 2.19
Decarboxyellagic acid (27)	N	0.67 ± 0.22	94.2 ± 1.66
Gallic acid 3- <i>O</i> -(6'- <i>O</i> -galloyl)-glucoside (28)	0.61 ± 0.02	1.05 ± 0.46	77.1 ± 2.64
Gallic acid (29)	11.4 ± 2.89	1.24 ± 0.35	82.6 ± 2.86
L-malic acid 2- <i>O</i> -gallate (30)	6.40 ± 0.84	1.98 ± 0.23	92.3 ± 2.26
Mucic acid 2- <i>O</i> -gallate (31)	7.12 ± 0.12	2.17 ± 0.12	95.2 ± 2.15
Chebulic acid (32)	2.97 ± 0.14	2.64 ± 0.09	90.5 ± 1.70
Gallic acid 3- <i>O</i> -glucoside (33)	13.55 ± 1.12	4.83 ± 0.54	98.1 ± 2.50
Multifidol 1- <i>O</i> -(6"- <i>O</i> -β-D-apiofuranosyl)-β-D-glucoside (34)	N	27.94 ± 0.91	95.1 ± 1.60
Multifidol glucoside (35)	6.33 ± 0.78	33.79 ± 3.89	101.5 ± 1.98
Condensed tannins			
Epigallocatechin 3- <i>O</i> -gallate (36)	0.12 ± 0.042	0.81 ± 0.12	96.5 ± 1.57
Prodelphinidin B-2 (37)	0.35 ± 0.014	0.86 ± 0.10	76.9 ± 2.23
Prodelphinidin B-1 (38)	0.19 ± 0.019	0.87 ± 0.17	80.9 ± 2.19
Epicatechin 3- <i>O</i> -gallate (39)	0.43 ± 0.035	0.94 ± 0.035	73.7 ± 3.13
Prodelphinidin A-1 (40)	0.14 ± 0.018	1.02 ± 0.23	65.3 ± 2.97
Epigallocatechin (41)	3.17 ± 0.14	1.29 ± 0.35	108.4 ± 3.57
Galocatechin (42)	1.35 ± 0.35	1.71 ± 0.23	107.6 ± 3.70
Epicatechin (43)	1.07 ± 0.35	1.73 ± 0.26	73.4 ± 4.08
(+)-Catechin (44)	1.85 ± 0.23	1.97 ± 0.35	108.1 ± 4.00
Flavonoids			
Eriodictyol 7- <i>O</i> -(6"- <i>O</i> -galloyl)-glucoside (45)	0.09 ± 0.12	1.01 ± 0.10	102.1 ± 4.27
Quercetin (46)	N	1.04 ± 0.11	111.6 ± 3.45
Naringenin 7- <i>O</i> -(6"- <i>O</i> -galloyl)-glucoside (47)	0.35 ± 0.02	1.23 ± 0.05	69.6 ± 3.74
Eriodictyol 7- <i>O</i> -glucoside (48)	0.58 ± 0.035	1.54 ± 0.09	83.6 ± 4.26

Quercetin 3-O-glucoside (49)	0.19 ± 0.009	1.54 ± 0.10	105.0 ± 2.49
Quercetin 3-O-(6"-O-glucosyl)-glucoside (50)	2.95 ± 0.14	1.55 ± 0.11	77.3 ± 2.63
Quercetin 3-O-rhamnoside (51)	1.90 ± 0.23	1.70 ± 0.23	107.3 ± 2.36
Eriodictyol 7-O-(6"-O-trans-coumaroyl)-glucoside (52)	0.28 ± 0.02	1.87 ± 0.24	96.0 ± 1.83
Myricetin 3-O-rhamnoside (53)	0.54 ± 0.12	1.91 ± 0.26	105.8 ± 1.94
Taxifolin 7-O-glucoside (54)	1.57 ± 0.46	1.92 ± 0.28	85.7 ± 2.15
Naringenin 7-O-(6"-O-trans-coumaroyl)-glucoside (55)	N	2.04 ± 0.14	104.2 ± 2.38
Naringenin (56)	N	10.41 ± 0.91	108.4 ± 3.00
Kaempferol 3-O-rhamnoside (57)	3.51 ± 0.76	13.76 ± 0.48	101.8 ± 1.93
Naringenin 7-O-glucoside (58)	N	19.63 ± 0.58	102.2 ± 2.34
Positive control			
DSCG	6.99 ± 0.78		
L-ascorbic acid		4.46 ± 0.22	
dl- α -tocopherol		3.86 ± 0.69	
GSH			82.9 ± 3.25

^a: HAase inhibition activity; ^b: DPPH radical scavenging activity; ^c: *Danio rerio* ROS scavenging activity; SR: scavenging rate; N: None testing.

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