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Crystal structures and in vitro biological effects of two protocatechuic acid complexes

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ABSTRACT

Piperazine (**PRZ**) and ligustrazine (**TMP**) were respectively in combination with protocatechuic acid (**PA**) to form complexes **I** and **II**. The structures of complexes **I** and **II** were confirmed by ^{1}H NMR, ^{13}C NMR, and single crystal X-ray diffraction. The effects of **PA** and its complexes on chondrocytes growth were measured using the MTT assay. Furthermore, the anticoagulant activities of **PA** and two complexes were evaluated by four coagulation assays (PT, APTT, TT, and FIB). This study indicates that concurrently using **PA** and **TMP** may have novel therapeutic uses in age-related disorders such as osteoarthritis and cardiovascular disease.

KEYWORDS

Anticoagulant activities; Chondrocyte growth; Ligustrazine; Protocatechuic acid

Introduction

In modern society, aging is faster over the past decades. This is a major risk factor for developing age–related disorders such as osteoarthritis and cardiovascular disease [1]. These aging diseases have threatened the health of millions of patients and constituted a primary financial burden to the health care systems. Therefore, developing safer and more effective drugs as a treatment or prevention of elderly disorders is of great significance.

Nowadays, drug discovery from natural products has enjoyed a renaissance [2]. Protocatechuic acid (3,4-dihydroxy benzoic acid, **PA**), as a phenolic component present in many plants, attracts investigators to evaluate its activity as antioxidant, free radical scavenger, and antiphlogistic [3]. Ligustrazine (2,3,5,6-tetramethylpyrazine, **TMP**) is one of the alkaloids derived from the traditional Chinese medicine herb Chuanxiong (*Ligusticum wallichii Franchat*). **TMP**, containing a pyrazine ring, has been routinely used in clinic for the treatment of cardiovascular disease and ischemic stroke [4]. Furthermore, the effects of **TMP** on the osteoarthritis have been confirmed by Chinese scientists in recent years [5]. However, its rapid metabolism and quick elimination (in vivo its short half-life of $t_{1/2} = 2.89$ hours) limited its clinical application [6].

Considering to **PA** and **TMP**'s characters, we also chose piperazine (**PRZ**, another nitrogen-bearing six-member ring) to synthesized two complexes **I** (**PA** + **PRZ**) and **II**

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(**PA** + **TMP**). Subsequently the effects of **PA** and its complexes on chondrocytes growth and anticoagulation were preliminarily investigated. This combination therapy of traditional Chinese medicine, administering both antioxidant drug and anticoagulant drug, might prove more effective than using a single class of drug.

In this study, **PA** and its complexes all elevated chondrocytes growth and there were similar effects on the cell proliferation among them. The highest promotion appeared at day 2 under concentration 200 μg/mL. It revealed that the cell proliferation of two complexes maintained for a long time and were not eliminated so quickly owing to addition of antioxidant **PA**. On other hand, **PA** enhances the anticoagulant effects of **TMP** in thrombin time assay (TT) despite no anticoagulation effects of **PA** alone. The concurrent use of **PA** and **PRZ**/**TMP** could be considered to treat disabled people and elderly people who obsessed with osteoarthritis and cardiovascular disease.

Experimental

Materials and instruments

Protocatechuic acid was purchased from Shengshun Trading co., Ltd. of analytical grade (Zhuhai, China). Piperazine was purchased from Aladdin-reagent (Shanghai, China). Ligustrazine was purchased from J&K Scientific Ltd. of biological grade (Beijing, China).

^{1}H and ^{13}C NMR spectra were carried out in acetic acid-d_4 on a Bruker Ascend^{TM} 600 at 600 MHz for ^{1}H and 150 MHz for ^{13}C, respectively. The X-ray crystallographic study was carried out using a Nonius Kappa CCD diffractometer equipped with a graphite monochromator and an Oxford cryostream, using Mo-K_α radiation ($\lambda = 0.7107$ Å). Four blood coagulation tests were assessed by a blood coagulation analyzer (LG-PABER-I, GTM-STEELLEX Instrument Co., Ltd., Beijing, China). Absorbance (595 nm) of MTT was measured by a multi-detection microplate reader (EnSpire 2300, PerkinElmer Inc., USA). All other reagents were commercial materials of analytical purity without further purification.

Preparation of protocatechuic acid complexes [7]

Protocatechuic acid (**PA**, 1.54 g, 0.01 mol) was dissolved in absolute ethyl alcohol (7.5 mL) and piperazine (**PRZ**, 0.43 g, 0.005 mol) was dissolved in absolute ethyl alcohol (3.5 mL) at 40°C. The solution of piperazine was added into the solution of protocatechuic acid, and then the mixture was refluxed for 40 min at 50°C. After cooling, the crude product was precipitated. The crystal of the complex (**I**) was obtained after crystallization in 100 mL water–ethanol (1:1) solvent system at room temperature.

Protocatechuic acid (**PA**, 1.54 g, 0.01 mol) was dissolved in absolute ethyl alcohol (7.5 mL) and ligustrazine (**TMP**, 0.68 g, 0.005 mol) was dissolved in absolute ethyl alcohol (3.5 mL) at 40°C. The solution of ligustrazine was added into the solution of protocatechuic acid, and then the mixture was refluxed for 40 min at 50°C. After cooling, the crude product was precipitated. The crystal of the complex (**II**) was obtained after crystallization in 100 mL water–ethanol (1:1) solvent system at room temperature.

The complex (**I**) of protocatechuic acid and piperazine was obtained as brown block crystal. Yield: 62.3%. ^{1}H NMR (600 MHz, acetic acid-d_4): δ 7.55 (m, 2H), 7.54 (m, 2H), 6.92 (m, 2H), 3.69 (s, 8H) ppm. ^{13}C NMR (150 MHz, acetic acid-d_4): δ = 172.23, 151.13, 145.01, 124.65, 122.02, 117.72, 115.76, and 41.45 ppm.

The complex (**II**) of protocatechuic acid and ligustrazine was obtained as colorless block crystal. Yield: 37.4%. ^{1}H NMR (600 MHz, acetic acid-d_4): δ 7.53 (m, 1H), 7.52 (m, 1H), 6.88 (m, 1H), 2.50 (s, 12H) ppm. ^{13}C NMR (150 MHz, acetic acid-d_4): δ = 172.27, 150.96, 149.68, 144.87, 124.67, 121.90, 117.54, 115.82, and 19.87 ppm.

Biological effect on articular cartilage cells

Articular chondrocytes were dissociated from knee joint cartilage slices of a 1-week-old New Zealand rabbit (the Institute of Animal Science and Technology of Guangxi University, Nanning, China) by enzymatic digestion with 0.25% trypsin (Gibco, Carlsbad, USA) for 30 min at 37°C and followed with 2 mg/mL collagenase type II (Gibco) in alpha-modified Eagle's medium (*a*-MEM, Gibco) for 3 hours at 37°C. After 5 min centrifugation (1000 r/min), isolated chondrocytes were resuspended and were cultured with *a*-MEM containing 20% (v/v) fetal bovine serum (FBS, PAN) and 1% (v/v) antibiotics (penicillin 100 U/mLand streptomycin 100 U/mL, Gibco) at 37°C with 5% CO_2.

The effects on articular chondrocytes were carried out by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA) method [8]. Articular chondrocytes were seeded on 96-well microplates in 100 μL of medium (1×10^5 cells/mL). Cells were treated respectively with **PA** and two complexes (**I** and **II**) at a final concentration of 1.56–200 μg/mL for 1, 2, and 3 days. And a group without sample-treatment was served as a control. After culture, 10 μL of MTT solution (5 mg/mL) added into each well and incubated at 37°C for 4 hours. Cells were treated with 100 μL sodium dodecyl sulfate solution (10 % SDS dissolved in 0.01 M HCl) to dissolve the formazan solution. At the next day, the absorbances of the plates were read using a test wavelength of 595 nm. All measurements were performed in triplicate.

In vitro anticoagulant assay

Protocatechuic acid (**PA**) and two complexes (**I** and **II**) of four different concentrations were added to human plasma at a ratio of 1:9 (v/v), respectively. Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and fibrinogen (FIB) were detected using blood coagulation factor assay kits (Saiao Biotechnology Co., Ltd., Qingdao, China) according to the manufacturer's instructions. Ligustrazine (**TMP**) was used as a positive control, and sodium chloride solution (0.9%) was used as the negative control. All the determinations were performed in triplicate.

Statistical analysis

Statistical analysis was conducted to a one-way analysis of variance (ANOVA) and Dunnett's test with $P < 0.05$ versus blank control using IBM SPSS Statistics 20 software package. Results are expressed as means $\pm$ standard deviation (SD) of three parallel experimental measurements.

Results and discussion

Crystal structure description

A brown block crystal of complex **I** and a colorless block crystal of complex **II** were selected and mounted on a glass fiber. The structure was solved by the direct method and refined by

Table 1. Crystallographic data for complexes **I** and **II**.

Compound	Complex **I**	Complex **II**
Formula	$C_{18}H_{28}N_2O_{11}$	$C_{15}H_{20}N_2O_5$
Color/shape	Brown/block	Colorless/block
Formula weight	448.42	308.33
Temperature	293 K	104 K
Wavelength (Å)	0.71073	0.71070
Crystal system, space group	Monoclinic, C2/c	Monoclinic, P 21/n
Unit cell dimensions		
a (Å)	16.003 (3)	8.7252 (6)
b (Å)	10.997 (2)	13.8418 (7)
c (Å)	13.879 (3)	12.8449 (8)
α (°)	90.00	90.00
β (°)	119.84 (3)	98.006 (6)
γ (°)	90.00	90.00
Volume (Å^3)	2118.7 (10)	1536.18 (16)
Z	4	4
$D_{calc.}$ (Mg m^{-3})	1.406	1.333
Absorption coefficient (mm^{-1})	0.117	0.101
F(000)	952.0	656.0
Crystal size	0.50 × 0.50 × 0.50	0.65 × 0.60 × 0.55
θ range for data collection (°)	$3.38 < \theta < 25.99$	$3.35 < \theta < 26.00$
hkl limit	$-19 \le h \le 19$;	$-10 \le h \le 8$;
	$-13 \le k \le 8$;	$-16 \le k \le 17$;
	$-18 \le l \le 18$	$-15 \le l \le 15$
Reflections collected	4688	6982
Unique reflections	2072 [R(int) = 0.0271]	3006 [R(int) = 0.0217]
Completeness to $\theta = 25.000$	99.7%	99.7%
Data/restraints/parameters	2072/0/152	3006/0/206
Goodness-of-fit on F^2	1.084	1.068
R indices [$I > 2\sigma(I)$]	$R_1 = 0.0420$, $wR_2 = 0.1007$	$R_1 = 0.0396$, $wR_2 = 0.0948$
R indices (all data)	$R_1 = 0.0502$, $wR_2 = 0.1060$	$R_1 = 0.0493$, $wR_2 = 0.1008$
Largest diff. features (e Å^{-3})	0.576 and −0.253	0.255 and −0.205

The structure was solved by direct methods and refined by full-matrix least-squares fitting on F^2 by SHELXL-97.

full-matrix least squares procedure against F^2 using the SHELXS-97 and SHELXL-97 programs [9]. The crystal data and structure refinement details for complexes **I** and **II** are given in Table 1. Selected bond lengths (Å) and angles (°) are listed in Table 2. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication, CCDC No. 1421269 for complex **I** and CCDC No. 1421394 for complex **II**. These data can be obtained free of charge via www.ccdc.cam.ac.uk.

Coupled with the integrals in the NMR spectra, X-ray diffraction analysis intuitively provided the crystal structures of two complexes **I** and **II** to us. The crystal lattices of complexes **I** and **II** belong to the monoclinic system but have different space groups. As showed in Fig. 1(A), the complex **I** contains two protocatechuic acid molecules, one piperazine molecule, and three free water molecules (O5). Fig. 1(B) revealed the complex **II** contains one protocatechuic acid molecule, one ligustrazine molecule, and one free water molecule (O5). Free water molecules (O5) acted as the hydrogen bond donor or acceptor in both complexes **I** and **II**. Hydrogen bond distances and bond angles of complexes **I** and **II** are listed in Tables 3 and 4. The crystal structures contain extensive hydrogen bonding within the two complexes displaying an unusual hydrogen bonding motif.

Table 2. The selected bond lengths (Å) and angles (°) for complexes **I** and **II**.

Complex **I**			
C (1)-N (1)	1.496 (2)	C (1)-C (2) #1	1.504 (3)
C(3)-C(4)	1.490 (3)	C (4)-C (5)	1.395 (3)
C (7)-C (8)	1.403 (3)	C (5)-C (6)	1.383 (3)
C (8)-C (9)	1.378 (3)	C (6)-C (7)	1.392 (3)
N (1)-C (1)-C (2)#1	110.90 (14)	C (5)-C (4)-C (9)	119.17 (17)
N (1)-C (2)-C (1)#1	111.18 (15)	C (9)-C (4)-C (3)	119.75 (17)
C (5)-C (4)-C (3)	121.07 (17)	C (5)-C (6)-C (7)	120.60 (17)
C (6)-C (5)-C (4)	120.15 (17)	C (9)-C (8)-C (7)	119.75 (17)
C (6)-C (7)-C (8)	119.44 (17)	C (2)-N (1)-C (1)	111.94 (14)
C (8)-C (9)-C (4)	120.85 (17)		
Complex **II**			
C (6)-C (7)	1.378 (2)	C (1)-C (2)	1.477 (2)
C (2)-C (3)	1.391 (2)	C (6)-C (5)	1.403 (2)
C (4)-C (3)	1.385 (2)	C (2)-C (7)	1.397 (2)
N (2)-C (10)	1.344 (2)	C (4)-C (5)	1.390 (2)
N (1)-C (11)	1.3437 (19)	N (2)-C (9)	1.3412 (19)
C (10)-C (11)	1.397 (2)	N (1)-C (8)	1.3426 (19)
C (9)-C (8)	1.399 (2)		
C (3)-C (2)-C (7)	119.73 (14)	C (7)-C (6)-C (5)	120.10 (13)
C (4)-C (5)-C (6)	119.72 (14)	C (3)-C (4)-C (5)	119.97 (14)
C (6)-C (7)-C (2)	120.07 (14)	C (4)-C (3)-C (2)	120.32 (14)
C (8)-N (1)-C (11)	119.20 (13)	C (9)-N (2)-C (10)	118.86 (13)
N (2)-C (9)-C (8)	120.61 (14)	N (2)-C (10)-C (11)	120.72 (13)
N (1)-C (8)-C (9)	120.32 (13)	N (1)-C (11)-C (10)	120.24 (13)

Symmetry transformations used to generate equivalent atoms: #1: −x+1/2,−y+3/2,−z

Effects on the chondrocyte growth

Articular cartilage cells of rabbit were treated with **PA** and its complexes **I** (**PA** + **PRZ**) and **II** (**PA** + **TMP**) of various concentrations for 1, 2, and 3 days. The effects on the chondrocyte growth were examined by the MTT assay. As shown in Fig. 2, absorbance values for each sample were comparable to those of the blank control (no drugs) in the range of 1.56–200 μg/mL. For three tested samples, chondrocyte growth was promoted in a dose-dependent manner at days 1 and 2. Especially, at day 2 all the three samples increased cell growth evidently under the concentration 200 μg/mL. The medium consumption may be response to reduced cell viability at day 3, even if there was a little promotion at the highest concentration. Therefore,

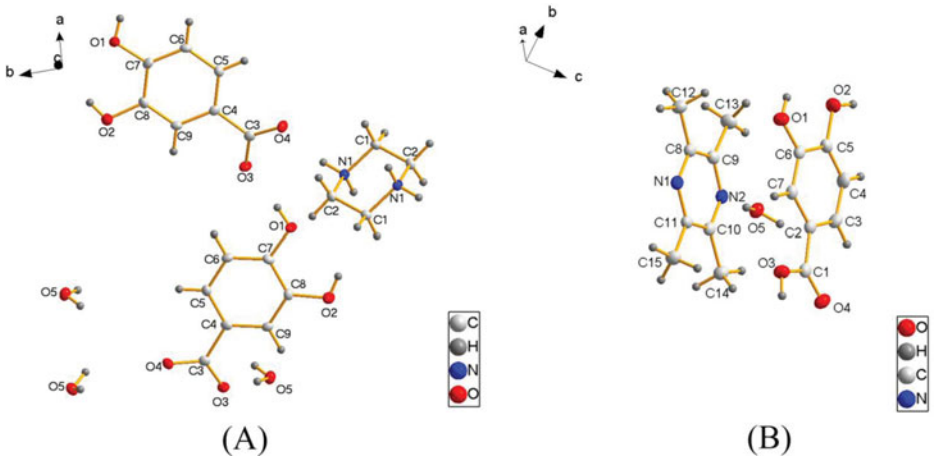

Figure 1. Molecular structures of protocatechuic acid complexes **I** (**PA** + **PRZ**, part A) and **II** (**PA** + **TMP**, part B).

Table 3. Hydrogen bond distances (Å) and bond angles (°) in complex **I**.

D-H···A	d(D-H)	d(H···A)	d(D···A)	<DHA
N1-H1A··· O2	0.90	1.85	2.654 (2)	147
N1-H1B··· O5	0.90	2.01	2.878 (2)	161
N1-H1B··· O4#1	0.90	2.57	2.927 (2)	104′/86′
O3-H3··· O4	0.82	2.31	2.739 (2)	114
O3-H3··· O5#2	0.82	2.04	2.801 (2)	154′/92′
O4-H4··· O1#2	0.82	1.76	2.583 (2)	177
O5-H5A··· O6#3	0.83 (3)	1.95 (3)	2.776 (2)	176 (2)
O5-H5B··· O1#4	0.82 (3)	1.92 (3)	2.733 (2)	172 (2)
O6-H6··· O2	0.84 (2)	1.89 (2)	2.6986 (16)	162 (2)

Symmetry transformations used to generate the equivalent atoms: #1: x, −y, 1/2+z; #2: x, −y, −1/2+z; #3: −x, 1−y, 1−z; #4: 1/2−x, 1/2+y, 3/2−z.

Table 4. Hydrogen bond distances (Å) and bond angles (°) in complex **II**.

D-H···A	d(D-H)	d(H···A)	d(D···A)	<DHA
O1-H1··· O2	0.82	2.26	2.7020 (15)	114
O1-H1··· O5#1	0.82	2.01	2.7646 (15)	152′/94′
O2-H2··· N1#2	0.82	1.90	2.7148 (16)	172
O3-H3··· O5#3	0.82	1.80	2.6140 (15)	170
O5-H5A··· N2	0.85	1.96	2.8123 (16)	176
O5-H5B··· O4#4	0.86	1.90	2.7509 (15)	177

Symmetry transformations used to generate the equivalent atoms: #1: 1/2-x, 1/2+y, 3/2-z; #2: 1/2+x, 3/2-y, 1/2+z; #3: −1+x, y, z; #4: −x, 1−y, 2−z.

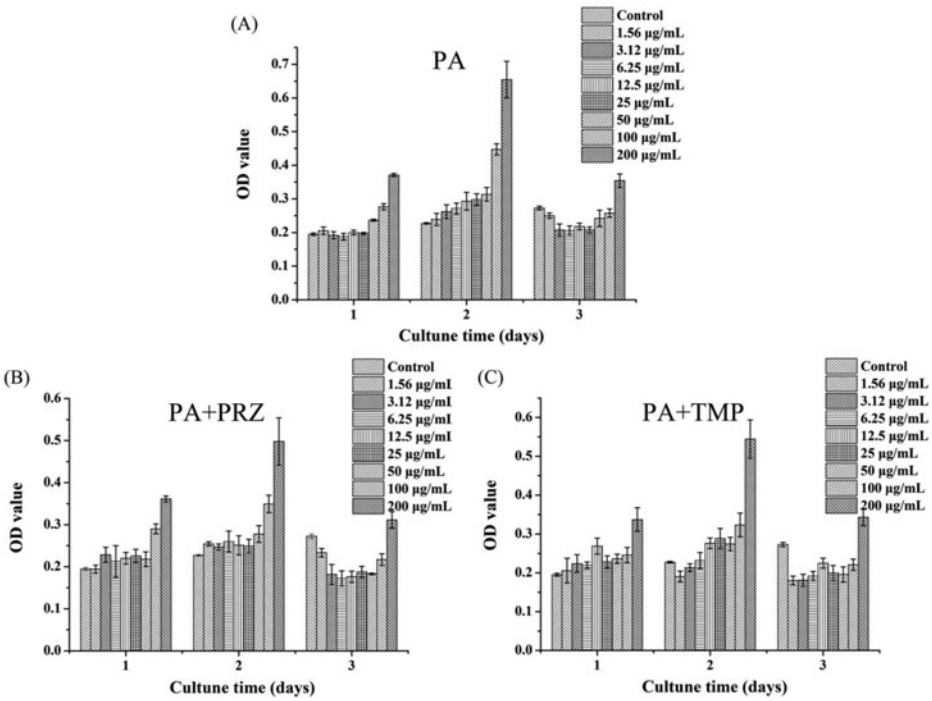

Figure 2. Effects of **PA** and its complexes **I** (**PA** + **PRZ**) and **II** (**PA** + **TMP**) on the chondrocyte growth.

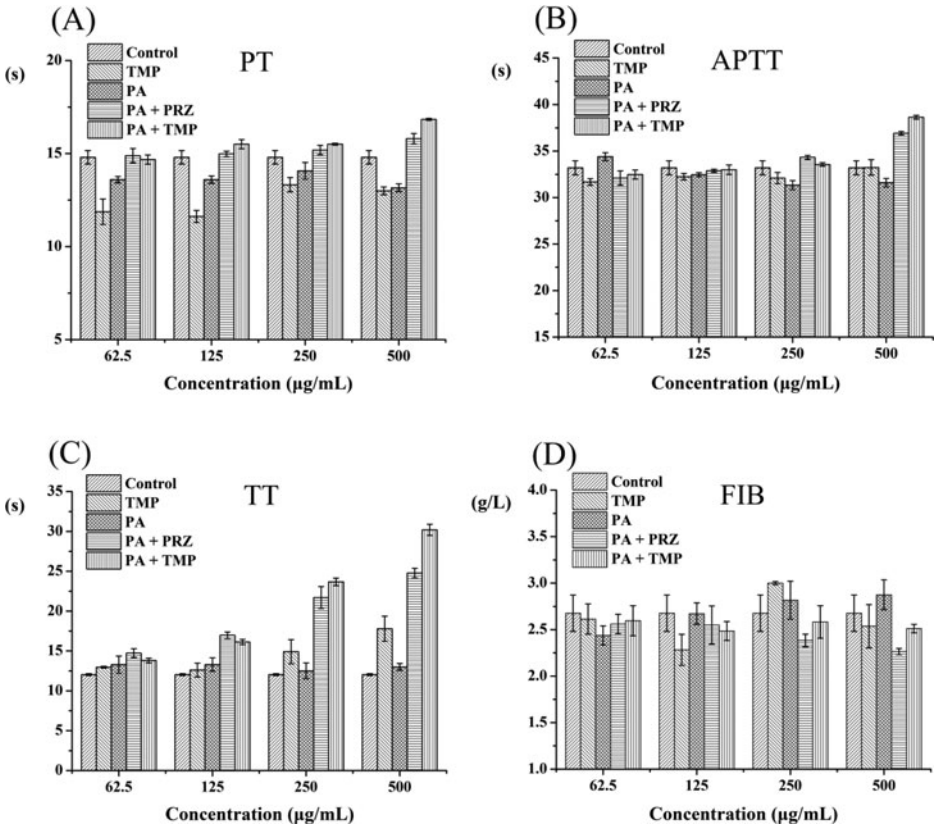

Figure 3. Anticoagulant activities of **PA** and its complexes **I** (**PA** + **PRZ**) and **II** (**PA** + **TMP**).

day 2 was the best time-point at when **PA** and its complexes significantly upregulated chondrocyte viability. These protective actions may be related to high antioxidant activity of **PA** [10]. **PA** can scavenge reactive oxygen species and nitric oxide to inhibit the osteoarthritis inflammatory cascade [11]. Additionally, the acceleration effects of two complexes were similar to that of **PA**. Nevertheless, it exhibited that the proliferation time lasted for a long time (2 days).

Anticoagulant activities

Anticoagulant activities of protocatechuic acid (**PA**) and its complexes **I** (**PA** + **PRZ**) and **II** (**PA** + **TMP**) were analyzed by the measurements of PT, APTT, TT, and FIB, with the standard **TMP** as a positive control. The results for the tested samples are summarized in Fig. 3.

The APTT assay was used as a screening test of the intrinsic coagulation pathway and the PT assay measured the activity of coagulation factors of the extrinsic coagulation pathway [12]. It can be found that **TMP** shortened clotting time in PT assay at lower concentrations (62.5–125 μg/mL). However, no significant difference in PT, APTT, and FIB analysis was observed between the two complexes and the blank control.

The TT assay detected the translation from fibrinogen to fibrin after the addition of known amounts of thrombin to the plasma sample [13]. In TT assay, the complexes **I** and **II** strongly prolonged the clotting times in a concentration-dependent manner, while **TMP** promoted an increase only at highest concentration (500 μg/mL). The TT analysis also showed that the

clotting time of complex **I** (at 500 μg/mL) and complex **II** (at 250–500 μg/mL) was about 2 times of the control sodium chloride. Comparatively, complex **II** showed better activities than complex **I**.

No anticoagulant effects were observed at any dose of **PA** alone. Interestingly, when **PA** and **PRZ**/**TMP** were administered together, the clotting times of TT were significantly prolonged in the higher-dose group (125–500 μg/mL), but not in the low-dose group (62.5 μg/mL).

From the obtained results, the strong prolongation of TT assays by complexes **I** and **II** suggest they inhibited thrombin activity or fibrin polymerization. There are no effects of the anticoagulant on the PT and APTT assays which suggest that the complexes **I** and **II** do not influence both intrinsic and extrinsic pathways of coagulation. Even if **PA** itself had no anticoagulant activity, the concurrent use of **PA** and **PRZ**/**TMP** potentiated bleeding events by increasing TT. Thus, **PA** played a vital role in promoting activities of anticoagulant drugs.

Conclusions

Protocatechuic acid (**PA**) possesses a wide spectrum of biological effects, especially antioxidative activity. Ligustrazine (**TMP**) is widely used as a vasodilator, and antithrombosis agent. They are both efficient components from Chinese traditional medicine herb. In this study, piperazine (**PRZ**, structurally related to **TMP**) and **TMP** were, respectively, combined with **PA** to form complexes **I** and **II**. The effects of **PA** and its complexes on chondrocytes growth were analyzed by MTT method. **PA** and its complexes **I** and **II** all exhibited high promotion on chondrocyte growth especially at day 2 with concentration 200 μg/mL. The chondrocyte proliferation attributed to the antioxidative activity of **PA**. Besides, different coagulation assays (PT, APTT, TT, and FIB) were used to determine anticoagulant activities of protocatechuic acid (**PA**) and its complexes **I** (**PA** + **PRZ**) and **II** (**PA** + **TMP**). The complexes **I** and **II** were devoid of PT, APTT, and FIB activities but presented great anticoagulant effects in TT assay. Their mechanism of action may be related to implication in the last step of the coagulation cascade, the conversion of fibrinogen to fibrin by thrombin. **PA** enhances the anticoagulant effects of **TMP** in TT assay despite no anticoagulation effect of **PA** alone. The findings of the present study may be useful for osteoarthritis and thrombosis among older people and these effects should also be considered in an herb-herb interaction.

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