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Inhibitory activity of *Podospermum canum* and its active components on collagenase, elastase and hyaluronidase enzymes

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ABSTRACT

Present study is aimed to investigate *in vitro* inhibitory effects of the extract prepared from the aerial parts of *Podospermum canum* (syn: *Scorzonera cana* var. *jacquiniana*) (Asteraceae) on hyaluronidase, collagenase, and elastase enzymes using a bioassay-guided fractionation. Inhibitory effects of the extract, sub-extracts, fractions obtained by column chromatography, and isolated compounds on collagenase, elastase, and hyaluronidase were performed by using *in vitro* enzyme inhibitory assays based on spectrophotometric evaluation. The methanolic extract obtained from *P. canum* exhibited strong inhibitory activities on elastase and collagenase while the insignificant activity was observed on hyaluronidase. Through bioactivity-guided fractionation, the ethyl acetate and remaining water sub-extracts obtained from the methanolic extract displayed significant inhibitory activities on collagenase and elastase, while petroleum ether and chloroform extracts did not show any inhibitory activity. Eleven known compounds: arbutin, 6́-*O*-caffeoylarbutin, cichoriin, 3,5-dicaffeoylquinic acid methyl ester, apigenin 7-*O*-*β*-glucoside, luteolin 7-*O*-*β*-glucoside, apigenin 7-*O*-*β*-rutinoside, isoorientin, orientin, vitexin, procatechuic acid, and new compound 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-*β*-glucopyranosyl) benzyl ester have been obtained from ethyl acetate sub-extract. Results of the present study have revealed that apigenin 7-*O*-*β*-glucoside, luteolin 7-*O*-*β*-glucoside, apigenin 7-*O*-*β*-rutinoside, and isoorientin showed potent enzyme inhibitory activities. However, methanolic extract of *P. canum* displayed a greater inhibitory activity than fractions and isolated compounds both on collagenase and elastase.

1. Introduction

Podospermum canum C. A. Meyer (syn.: *Scorzonera cana* (C.A. Meyer) Hoffm. var. *jacquiniana* (W. Koch) Chamb.), a plant from Asteraceae family, is a perennial herb with cylindrical root-stock, entire to pinnatipartite basal leaves and yellow flowers [1]. This plant which was formerly known as *S. cana* var. *jacquiniana*, is now systematically classified into *Podospermum* genus based on studies of Mavrodiev et al. [2], which reclassified *Scorzonera*, *Podospermum*, and *Lasiospora* into separate genera.

P. canum grows naturally in central and south parts of the Europe, west part of the Syria, Iraq, Caucasia, Iran, and Anatolia [1]. *P. canum* is known as "*karakök*" or "*tekesakalı*" in Turkey. Its leaves are used as a vegetable [3] and are used for their galactagogue and appetizing activities in Turkish folk medicine [4]. Furthermore, *Scorzonera* plants are used as a food in Anatolia and as medicinal plants to combat arteriosclerosis, hypertension, rheumatism, kidney diseases, diabetes mellitus [5], and for wound healing [6,7]. Previous reports have revealed the significant anti-inflammatory effects of extracts of *P. canum* in carrageenan-and PGE_2-induced hind paw edema model at doses of 100 mg/kg, while no activity in serotonin induced hind paw edema and TPA induced ear edema models. *P. canum* displayed also antinociceptive activity *in vivo* [8]. Wound healing effects of the *P. canum*, evaluated *in vivo* by linear incision and circular excision experimental wound models with subsequent histopathological analysis, and an anti-hyaluronidase activity were established as remarkable [9].

Hyaluronidase, collagenase, and elastase are matrix metalloproteinases (MMPs), and comprise a family of ECM-degrading enzymes. They play essential role in remodeling and repair of tissues. They have important role in regulation of extracellular matrix degradation and deposition which is essential for wound re-epithelialization. Hyaluronidase, collagenase and elastase are involved in the

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pathogenesis of different diseases, such as cancer, cardiovascular diseases, inflammation, bone destruction, fibrosis, as well as in development and healing of wounds [10–13]. The aim of the current study was to confirm medicinal usage of *P. canum* based on wound healing and to investigate, using bioactivity-guided fractionation and isolation, which compound(s) is/are responsible for significant wound healing activity that was established previously *in vivo*.

2. Materials and methods

2.1. Plant material

P. canum (syn: *S. cana* var. *jacquiniana*) was collected in Çamlıdere (Ankara, Turkey, N 40°29′15.6″; E 32°28′09.8″, located in the northwest of the Anatolia), 100 km from the Ankara city center. Plants were growing naturally at roadsides and in the fields. The age of the harvested plants was 5–8 months and the samples were collected during flowering period (19th of June 2016). The identification of the plant was performed by Prof. Dr. Hayri Duman (Department of Biology, Faculty of Science, Gazi University) according to the description of the plant material in the Flora of Turkey and the East Aegean Islands [1]. A voucher specimen was deposited in the herbarium of the Faculty of Pharmacy at Ankara University (AEF Number: 23834).

2.2. Preparation of the plant extracts

Dried and powdered aerial parts (flowers, leaves and stems; 600 g) were macerated in methanol at room temperature, five times, each following, in 2.5 L of methanol for 24 h. The extraction was finished by 1 h of a sonication. The combined extracts were filtered, and the solvent was evaporated at 40–50 °C under reduced pressure to obtain the crude extract (87.98 g). The crude extract was dissolved in water and then subjected to partitioning by petroleum ether, chloroform, and ethyl acetate, respectively, to obtain four sub-extract with different polarity. According to the results of bio-assays, the ethyl acetate fraction (4.34 g) was selected for further separation.

2.3. General experimental procedures

Mass spectra were measured using a Waters 2695 Alliance Micromass ZQ, LC/MS. Varian Mercury 400, 400 MHz High Performance Digital FT-NMR Spectrometer was used for ^{1}H NMR, ^{13}C NMR and 2D NMR (HMBC, HSQC, COSY, TOCSY, NOESY, DEPT) (in CD_3OD).

2.4. Isolation procedure

The ethyl acetate portion, selected as the active, was separated using column chromatography on silica gel (40–63 µm, Merck) by eluting with isocratic solvent system ethyl acetate:methanol:water (100:13.5:10, v/v/v). Fractions obtained from column chromatography were combined according to their TLC behavior to obtain six main fractions (Fr. A-F). Fraction A and B (as the most active fractions) were used for further isolation procedures. Both Fraction A and B were subjected to Sephadex column and eluted with methanol. Fraction A gave 60 sub-fractions; sub-fraction 2 gave **1** (8 mg) and **4** (6 mg) by preparative thin layer chromatography on reverse phase TLC plates (Merck 5559) by eluting with methanol:water (1:1, v/v). Sub-fraction 3 gave compound **2** (6.3 mg). Compounds **3** (4 mg), **7** (15 mg) and **12** (5.1 mg) were obtained from sub-fraction 14 and 15–17, respectively, on silicagel TLC plates (Merck 5554) using $CHCl_3$:MeOH:water (65:25:4, v/v/v). From sub-fraction 18–21 and 28–30, **5** (11 mg), **10** (4.28 mg), and **6** (7.53 mg) were isolated using reversed phase preparative TLC with methanol:water (1:1, v/v). **11** (12.26 mg) (Fig. 1) was isolated from subfraction 9–13 by running preparative TLC on reversed phase with methanol:water (1:1, v/v).

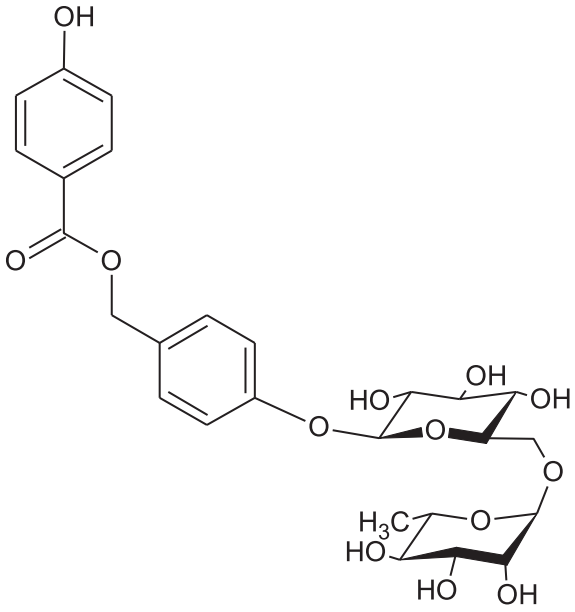

Fig. 1. Structure of 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-β-glucopyranosyl) benzyl ester (**11**).

Fraction B, that contains the compounds **8**, **9** and **6,** was chromatographed on Sephadex column to obtain 40 sub-fractions by eluting with methanol for obtaining **9** (12 mg) and **8** (13 mg) from sub-fractions 24–29 and 34–40, respectively which were selected due to their content **8** and **9** in high amount than the other subfractions. On the other hands **6** has already been isolated from the Fraction A, was not applied to further separation procedure.

The structures of the compounds were elucidated by using MS and ^{1}H- and ^{13}C NMR, 2D-NMR techniques and by comparing the results with the literature.

2.5. In vitro enzyme inhibitory assays

2.5.1. Assessment of collagenase inhibitory activity

The assay of collagenase inhibitory activity was based on the spectrophotometric methods reported by Vanwart and Steinbrink [14], with some modifications in order to use a microplate reader. Tricine buffer was used for this assay. Collagenase from *Clostridium histolyticum* (ChC) was added to Tricine buffer (50 mM, pH 7.5, 400 mM NaCl and 10 mM $CaCl_2$) to get a concentration of 0.8 units/mL. The substrate *N*-[3-(2-furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) was added to Tricine buffer to obtain a concentration of 2 mM. In each case, the total final volume of the mixture (150 µL) included Tricine buffer (25 µL), 0.8 mM FALGPA (75 µL), 0.1 units of ChC (25 µL) and 25 µL of the solution of the tested materials in 5% DMSO (100 µg/mL). Each test material was incubated with the enzyme in buffer for 15 min before the substrate was added to trigger the reaction. After adding the substrate, the absorbance at 335 nm was measured in 2 min intervals for 20 min using a Beckmann Dual Spectrometer (Beckman, Fullerton, CA, USA). Water was used as a negative and epigallocatechin gallate (EGCG) (250 µM, 0.114 mg/mL) as a positive control [15]. Measurements were done in three replications. The formula % inhibition = $(OD_{Control} - OD_{Sample}) \times 100 / OD_{Control}$ was used for the calculating the inhibition value.

2.5.2. Assessment of elastase inhibitory activity

The elastase assay was based on the method of Kim et al. [16] and was conducted in 0.2 mM Tris-HCL buffer (pH 8.0). Porcine pancreatic elastase (PE) was added to sterile water to prepare a stock solution at a concentration of 3.33 mg/mL. The substrate *N*-Succinyl-Ala-Ala-Ala-*p*-

nitroanilide (AAAPVN) was dissolved in buffer to get a concentration of 1.6 mM. Each test material was mixed with the enzyme, and the mixture was incubated for 15 min before the substrate was added. The final mixture (250 µL of total volume) included buffer, 0.8 mM AAAPVN, 1 µg/mL of PE, and 25 µL of the test materials (100 µg/mL in 5% DMSO). EGCG (250 µM, 0.114 mg/mL) was used as a positive control [15], with water as a negative control. The substrate was added and the mixture was incubated for 20 min, whereupon the absorbance between λ 381 and 402 nm (according to the pre-screening scans) was measured using a Beckmann Dual Spectrometer. Measurements were done in three replications. The formula % inhibition = $(OD_{Control} - OD_{Sample}) \times 100 / OD_{Control}$ was used for the calculating the inhibition value.

2.5.3. Assessment of hyaluronidase inhibitory activity

Prior to the hyaluronidase assays, the total UV/VIS absorbance spectra of all extracts were scanned (Cary 300 UV–visible spectrophotometer) to check any potential interference with the colorimetric methods used for the assay. The inhibition of hyaluronidase was determined by measuring the amount of *N*-acetylglucosamine released from sodium hyaluronate, using a slightly modified version of methods described in literature [17,18]. Bovine hyaluronidase (7900 units/mL) was added to 0.1 M acetate buffer (pH 3.6, 50 µL) and this solution was then mixed with 50 µL of each of the test materials dissolved in 5% DMSO (100 µg/mL). The mixture was incubated for 20 min at 37 °C, 50 µL of calcium chloride (12.5 mM) was then added, and the mixture was again incubated for another 20 min at 37 °C. 250 µL of sodium hyaluronate (1.2 mg/mL) was then added and the mixture was incubated for next 40 min at 37 °C. After this incubation, 50 µL of 0.4 M NaOH and 100 µL of 0.2 M sodium borate were added and the mixture incubated for 3 min in a water bath at 100 °C. When the reaction mixture had cooled to room temperature, *p*-dimethylaminobenzaldehyde solution (1.5 mL) (stock *p*-dimethylaminobenzaldehyde reagent − 10% w/v in 12.5% v/v concentrated hydrochloric acid in glacial acetic acid; stock reagent diluted 1 to 10 with glacial acetic acid before use) was added and the mixture was incubated for another 20 min at 37 °C. The absorbance of the colored solution was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA). Tannic acid was used at the concentration of 100 µg/mL as a positive control. Fifty microliters of 5% DMSO was used as a negative control. Measurements were done in three replications. The formula % inhibition = $(OD_{Control} - OD_{Sample}) \times 100 / OD_{Control}$ was used for the calculating the inhibition value.

2.6. Statistical analysis of the data

All experiments were done in triplicates. The data for the percentage of enzyme inhibitory activity were analyzed statistically using one-way analysis of variance (ANOVA-Dunnett's). Values of $p \leq 0.05$ were considered to be statistically significant. Statistical analyses were done using GraphPad Prism 6.0.

3. Results and discussion

We aimed to investigate the *in vitro* inhibitory effects of the extracts prepared from the aerial parts of *P. canum* against collagenase, elastase, and hyaluronidase. Results of the experiments have revealed that neither the extracts and fractions nor the isolated compounds obtained from the *P. canum* exerted a significant inhibitory activity on hyaluronidase at a concentration of 100 µg/mL compared to tannic acid as a positive control, which showed the strongest inhibitory activity on hyaluronidase (Fig. 2). On the other hand, total methanolic extract displayed effect on both collagenase and elastase with the value of inhibition 35.7% and 51.7%, respectively. In the same experiment, EGCG - a positive control in both elastase and collagenase activity assays, exhibited stronger inhibitory activities of 50.58% and 74.93%, respectively (Figs. 3 and 4). According to activity-guided fractionation, the active total MeOH extract was subjected to first step fractionation process through subsequent liquid-liquid extraction to obtain petroleum ether, chloroform, ethyl acetate and remaining water sub-extracts. When compared to the control group (V), ethyl acetate and remaining water sub-extracts were found to have significant collagenase and elastase inhibitory activities with the values of 32.6% and 25.7% for collagenase, and 47.9% and 37.3% for elastase at a concentration of 100 µg/mL, respectively.

Fractionation of the active sub-extract on column chromatography combined 6 main sub-fractions (assigned as Fr. A-F). The activity was traced to Fr A (29.37% inhibition of collagenase and 41.78% inhibition of elastase), and Fr B (25.15% inhibition of collagenase and 32.16% inhibition of elastase). These were re-chromatographed to obtain 11 known compounds and one new substance possibly responsible for the effect. The structures of the isolated compounds were elucidated by using MS, one-dimensional and two-dimensional ^{1}H and ^{13}CNMR techniques, and by comparison of data with published literature; and these compounds were identified as arbutin (**1**) [19], 6́-*O*-caffeoylarbutin (**2**) [20], cichoriin (**3**) [21], 3,5-dicaffeoylquinic acid methyl ester (**4**) [22], apigenin 7-*O*-*β*-glucoside (**5**) [23], luteolin 7-*O*-*β*-glucoside (**6**) [24] apigenin 7-*O*-*β*-rutinoside (**7**) [25], isoorientin (**8**) [26], orientin (**9**), vitexin (**10**) [27], and procatechuic acid (**12**) [28].

Compound **11** revealed a molecular peak *m/z* $[M+H]^+$ 575.8 in ESI-MS analysis. ^{13}C NMR spectrum displayed signals that belong to 26 carbon atoms (see Table 1). The proton NMR gave signals observed at *δ* 7.86 (d, *J* = 8.8 Hz, 2H), *δ* 6.80 (d, *J* = 8.8 Hz, 2H), *δ* 7.39 (d, *J* = 8.8 Hz, 2H) and *δ* 7.09 (d, *J* = 8.4 Hz, 2H) ppm. This revealed presence of two AÁ XX́ aromatic systems. Proton signals at *δ* 4.88 (d, *J* = 7.6 Hz, 1H) ppm and *δ* 4.69 (d, *J* = 1.6 Hz, 1H) ppm, which correlate with the *δ* 102.3 and *δ* 102.2 carbon signals in HSQC spectrum, indicated presence of two different sugar units, assignable for H-1́ and H-1̃ positions of sugars. An anomeric proton signal H-1́ of compound **11** appeared at *δ* 4.88 (d, *J* = 7.6 Hz, 1H) and the resonances in the region of *δ* 4.02–3.45 (protons H-2́, H-3́, H-4́, H-5́, H-6́, m, 6H) together with the corresponding carbon resonances in the HSQC spectrum suggested the presence of *β*-glucopyranose unit. The presence of a doublet at *δ* 1.18 integrated for three protons in ^{1}H NMR and a signal at *δ* 17.9 ppm in ^{13}C NMR were typical for the rhamnose methyl group. The interglycosidic linkage was verified as glucose (6́→1̃) rhamnose by the HMBC spectrum and the downfield shift of C-6́ of glucose (*δ* 67.8) characteristic for rutinoside – *β*-glucose and *α*-rhamnose moiety, respectively. HMBC spectrum proved that signal at *δ* 168.1 ppm in ^{13}C NMR spectrum correlating with a proton signal at *δ* 5.23 (brs, 2H) ppm and a carbon signal at *δ* 67.15 ppm showed a presence of ester bond. All these data suggested that compound **11** is a glucorhamnoside of 4-hydroxy-benzoic acid benzyl ester. In the HMBC spectrum, a crosspeak between C-4 and H-1́ established the linkage point of 4-hydroxy-benzoic acid benzyl ester and sugar moieties. The compound was therefore identified as 4-hydroxybenzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-*β*-glucopyranosyl) benzyl ester. All data were confirmed by the published data by Zidorn, Ellmerer-Muller and Stuppner [29] for glucoside of 4-hydroxybenzoic acid benzyl ester.

The isolated compounds were evaluated to elucidate their anti-collagenase and anti-elastase activities. Among the tested compounds at a concentration of 100 µg/mL, apigenin 7-*O*-*β*-glucoside (**5**), apigenin 7-*O*-*β*-rutinoside (**7**) and isoorientin (**8**) showed 43.04%, 40.36% and 30.66% inhibition of elastase; apigenin 7-*O*-*β*-glucoside (**5**), luteolin 7-*O*-*β*-glucoside (**6**), and apigenin 7-*O*-*β*-rutinoside (**7**) displayed inhibitory activities on collagenase with values of 26.48%, 29.96% and 24.28% (Figs. 3 and 4).

Wound healing is a very complex process, associating cellular, molecular, biochemical and physiological events, which result in a regeneration and repair of the damaged tissue. Wound repair can be described as a three-stage process: inflammation, cell proliferation and resynthesis of extracellular matrix [30,31]. Inflammatory phase starts

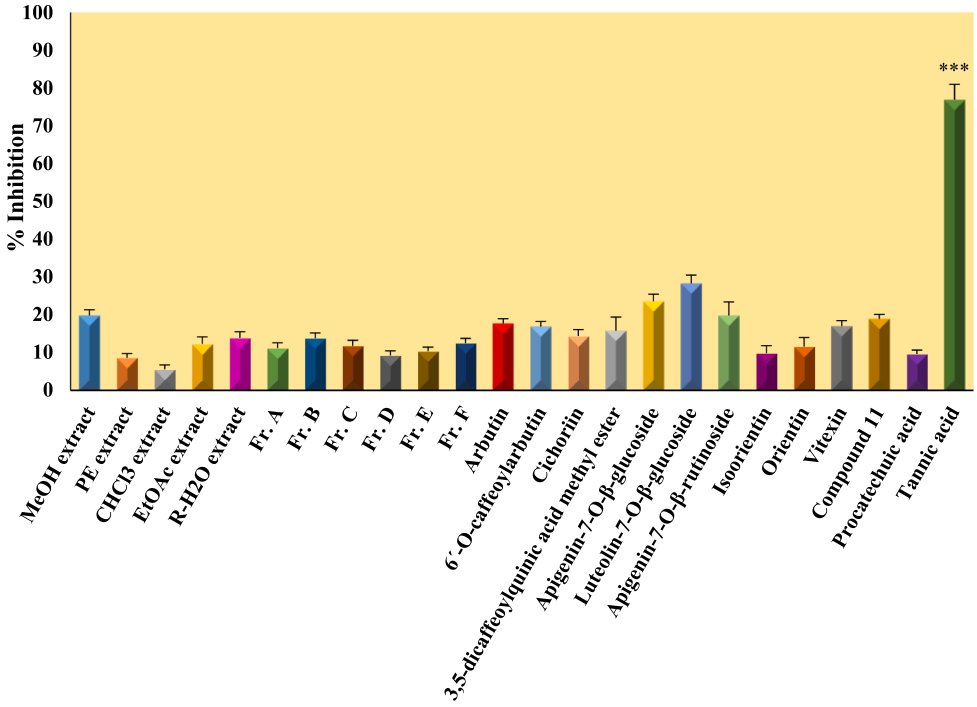

Fig. 2. Effects of the extracts, fractions and isolated compounds from *P. canum* on hyaluronidase enzyme inhibitory activity. The results are expressed as the mean ± S.D. Asterisks indicate a significant difference in comparison with the vehicle-treated cells (V), *** $p < 0.001$. Compound **11**: 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-β-glucopyranosyl) benzyl ester.

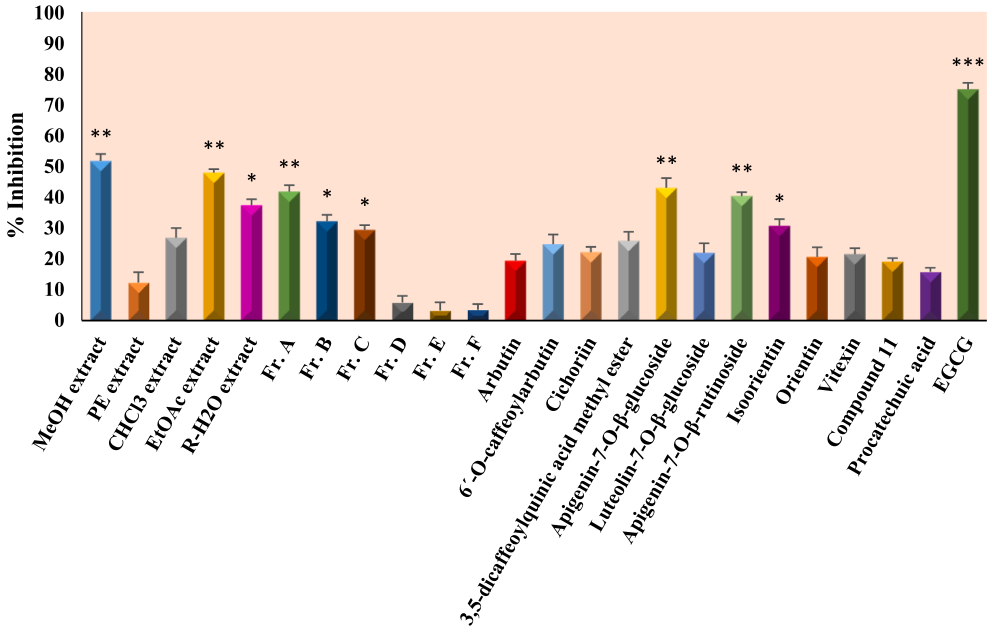

Fig. 3. Effects of the extracts, fractions and isolated compounds from *P. canum* on elastase enzyme inhibitory activity. The results are expressed as the mean ± S.D. Asterisks indicate a significant difference in comparison with the vehicle-treated cells (V), *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$. Compound **11**: 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-β-glucopyranosyl) benzyl ester.

immediately after injury with vasoconstriction which induces hemostasis and triggers a release of inflammatory mediators [32]. Controlled inflammation is important in the wound healing process [33,34]. The proliferative stage is aimed to reduce the area of lesioned tissue by contraction and fibroplasia, establishing a viable epithelial barrier to activate keratinocytes [30]. The third stage, resynthesis of the ECM, plays a major role in control of the wound healing process in regulation of cellular processes. It has been reported that nearly all the ECM components are able to regulate cell behaviour. Modifications of ECM occuring during wound healing are therefore very important players in this process [31]. Hyaluronidase, collagenase and elastase are MMPs, and comprise a family of ECM degrading enzymes. They are playing essential role in tissue remodeling and repair. They have important role in regulating extracellular matrix degradation and deposition essential for wound re-epithelialization [10–13]. Hyaluronic acid (HA) is the predominant glycosaminoglycan in the skin and keeps the moisture. HA in tissues is degraded by hyaluronidases. Degradation of HA in the ECM results in a breakdown of structural integrity and an increase in tissue permeability [35,36]. Hyaluronidases in the ECM are present in an inactive or suppressed form, bound to their natural inhibitors. The inhibition of hyaluronidase activity prevents otherwise rapid degradation of HA in the ECM and functions to maintain structural integrity. Previous studies reported that hyaluronidase inhibitors may serve as anti-aging, anti-inflammatory, and anti-microbial agents [36]. *P. canum* methanolic extract did not exert inhibitory activity on hyaluronidase. Collagen and elastin are the other major components of connective tissue and also collagen and elastin are the extracellular matrix components which provide specific functions to the cells by forming a supportive framework and maintaining the skin's elasticity. Collagen is responsible for the tensile strength of the skin. Elastin provides the

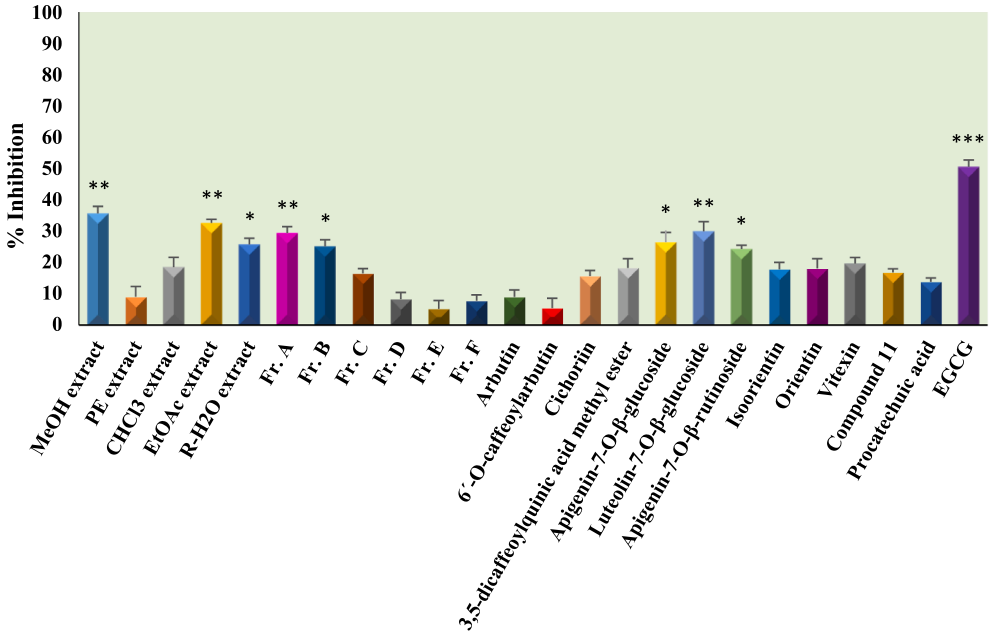

Fig. 4. Effects of the extracts, fractions and isolated compounds from *P. canum* on collagenase enzyme inhibitory activity. The results are expressed as the mean ± S.D. Asterisks indicate a significant difference in comparison with the vehicle-treated cells (V), *p < 0.05, **p < 0.01, *** p < 0.001. Compound **11**: 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-β-glucopyranosyl) benzyl ester.

Table 1
^{13}C and ^{1}H NMR data of the 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosil-*β*-glucopyranosyl) benzyl ester (**11**).

Position	δ_C	δ_H, *J* in Hz	HMBC correlations
p-OH-benzyl alcohol moiety			
A	67.2	5.24 (brs, 2H)	
C-1	131.8	–	H-α
C-2	130.9	7.49 (d, *J* = 8.8, 2H)	H-α
C-3	117.9	7.09 (d, *J* = 8.4, 2H)	
C-4	159	–	H-2́/6, H-3/5 H-1́
C-5	117.9	7.09 (d, *J* = 8.4, 2H)	
C-6	130.9	7.49 (d, *J* = 8.8, 2H)	H-α
Acyl moiety			
α	168.1	–	H-2́, H-α
C-1́	122.2	–	H-3́
C-2́	132.9	7.86 (d, *J* = 8.8, 2H)	
C-3́	116.3	6.81 (d, *J* = 8.8, 2H)	
C-4́	163.9	–	H-2́
C-5́	116.3	6.81 (d, *J* = 8.8, 2H)	
C-6́	132.9	7.86 (d, *J* = 8.8, 2H)	
Sugar link	*β*-Glc		
C-1́	102.3	4.88 (d, *J* = 7.6, 1H)	
C-2́	74.9	3.45 (m)	
C-3́	78.1	3.46 (m)	
C-4́	71.6	3.34 (m)	
C-5́	77	3.58 (m)	
C-6́	67.9	4.02 (d, *J* = 7.6, 1H) 3.61 (m)	H-1́́
	α-Rha		
C-1́́	102.2	4.69 (d, *J* = 1.6, 1H)	
C-2́́	72.2	3.84 (dd, *J* = 2.0, 3.2, 1H)	H-1́́
C-3́́	72.4	3.69 (dd, *J* = 2.7, 9.6, 1H)	
C-4́́	74.1	3.34 (m)	
C-5́́	69.9	3.65 (m)	H-1́́
C-6́́	18	1.18 (d, *J* = 6.4, 3H)	

elasticity of the skin [15]. Thus, inhibition of collagenase and elastase enzymes which break down these components could be beneficial for the wound healing process [15,37–39]. As described above, methanolic extract of *P. canum* showed inhibition of collagenase and elastase and thuse can effect elasticity of skin and tensile strength of skin.

In current study, phenolic compounds, mainly flavonoids, are suggested to be substances responsible for here described activity of *P. canum* extracts. Flavonoids have been isolated as a result of bio-activity guided fractionation. They display wide variety of activities, including anti-inflammatory, antibacterial, and antioxidant, which are reported as important in wound healing process [33,34]. Reactive oxygen species (ROS) that arise after cutaneous injury may worsen the healing process and contribute to an induction of apoptosis. Normal levels of ROS play important roles in wound repair and transduction of signals for proliferation of cells and re-epithelialization, such as the collagenase activity and the epidermal growth factor signaling. Higher levels of ROS can cause oxidative stress and may damage macromolecules such as DNA, lipids, and proteins. Therefore, regulating oxidative stress and the inflammatory response is important during the cutaneous wound healing process.

A study conducted by Tatemoto et al. reported that tannic acid inhibited hyaluronidase enzyme even at low concentrations (2–10 μg/mL) [40]. However, previous studies exhibited that tannic acid has various side effects, including nausea, stomach irritation, and liver damage. Additionally, it can lead to excessive astringency on the mucous membrane, and, thus, it can cause irritating effects on the skin [41]. Thring et al. found that EGCG displayed strong anti-elastase and -collagenase activities [15]. However, according to previous studies, EGCG could cause some undesired effects such as anemia, liver and kidney failures [42]. In this study tannic acid and EGCG were used as positive controls and they showed strong inhibitory activities on hyaluronidase, collagenase and elastase enzymes. Despite of their strong inhibitory activities, they have some severe side effects as mentioned before. Thus, authors considered that *in vivo* biological activity studies should be performed on the extracts, subextracts, fractions, isolated compounds, EGCG and tannic acid to observe their activity and toxicity profiles.

In current study flavonoids have been isolated as a result of bioassay guided fractionation. Apigenin 7-*O*-*β*-glucoside (**5**), apigenin 7-*O*-*β*-rutinoside (**7**) exhibited the significant inhibitory activity against both collagenase and elastase enzymes. However, activity of all isolated compounds were determined to be lower than activity of ethyl acetate sub-extract and total methanolic extract.

Plants of Scorzonerinae subtribe, including *Scorzonera* and *Podospermum* genera, are chemically characterized by the accumulation of different compounds, such as terpenoids (especially sesquiterpenes and triterpenes), dihydrostilbenes, dihydroisocoumarines, bibenzyl derivatives, lignans, quinic and caffeic acid derivatives, as well as

flavonoids. However, flavonoid profile of this subtribe has not been fully investigated; it is known that *C*-glycosides of both apigenin and luteolin are important. Different types of caffeic acid derivatives also occur and new structures have been isolated from these genera [43–46]. In the view of such information, it could be suggested that activity of *P. canum* extracts is probably based on a synergic interaction of its terpenic, phenolic and especially flavonoid content.

4. Conclusion

This study deepened the knowledge about the phytochemical profile of *P. canum*. A new phenolic compound, 4-hydroxy-benzoic acid 4-(6-*O*-*α*-rhamnopyranosyl-*β*-glucopyranosyl) benzyl ester, was isolated and identified. According to the literature, all other compounds described here have been isolated for first time from this plant. In the present study, *P. canum* extract significantly inhibited collagenase and elastase, suggesting that this plant could be used for the treatment of wounds. Flavonoids isolated from the aerial parts of the plant apigenin 7-*O*-*β*-glucoside (**5**), luteolin 7-*O*-*β*-glucoside (**6**), apigenin 7-*O*-*β*-rutinoside (**7**) and isoorientin (**8**), showed inhibitory activities on elastase and collagenase. Apigenin 7-*O*-*β*-glucoside (**5**), and apigenin 7-*O*-*β*-rutinoside (**7**) were established to be statistically significant inhibitors for both enzymes. Results may indicate that flavonoids could be major compounds responsible for wound healing activity of *P. canum*. However the higher observed activity of total methanolic extract could show the synergic interaction of more compounds present in the complex extract.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103330.

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