Journal of Herbal Medicine xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Herbal Medicine

journal homepage: www.elsevier.com/locate/hermed

Research paper

In vitro enzyme inhibitory effects of *Rubus sanctus* Schreber and its active metabolite as a function of wound healing activity

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ARTICLE INFO

Article history:
Received 24 April 2015
Received in revised form 28 July 2015
Accepted 1 September 2015
Available online xxx

Keywords:
Collagenase
Elastase
Hyperoside
Hyaluronidase
Rubus sanctus
Wound

ABSTRACT

The young shoots of *Rubus* species have been used in folk medicine for the healing of wounds, infected insect bites and acnes for centuries. In order to investigate the potential wound healing activity, *n*-hexane, ethyl acetate (EtOAc) and methanol (MeOH) extracts of the aerial parts of *Rubus sanctus* Schreber, one of the widespread species growing wild in Turkey were successively prepared. The wound healing effect of the extracts was determined by assessing their potential inhibitory activity on hyaluronidase, collagenase and elastase enzymes, all of which have an essential role in the wound healing process. According to activity-guided fractionation assay, the methanol extract was fractionated by silica gel column chromatography. Preparative thin layer chromatography analysis afforded a pure compound which was elucidated by spectral techniques as "quercetin-3-*O*-β-galactoside", namely hyperoside. The *in vitro* assays demonstrated that hyperoside promoted the wound healing process by inhibiting the collagenase and elastase enzymes.

1. Introduction

Rubus species have been frequently used in traditional medicine due to their widespread distribution all over the world. According to the reports in Flora of Turkey and The East Aegean Islands, 9 *Rubus* species are growing wild in Turkey, namely, *Rubus saxatilis* L., *Rubus idaeus* L., *Rubus caesius* L., *Rubus sanctus* Schreber, *Rubus discolor* Weihe & Nees, *Rubus canescens* DC., *Rubus tereticaulis* P.J. Mueller, *Rubus hirtus* Waldst. & Kit. and *Rubus caucasicus* L. (Davis and Meikle, 1972). In ethnobotanical studies of Turkey, dried and crushed young shoots of *Rubus* species were reported to be applied onto wounds, infected insect bites and pimples (Uncini et al., 1999). Various other utilizations of *Rubus* species for the treatment of several ailments were also reported. For instance, the infusion prepared from the fresh leaves of *R. caesius* has been used for the treatment of diabetes mellitus, dried and powdered leaves for wound healing, the syrup made from the fruits for respiratory problems and the decoction of the fruits and leaves for the treatment of tonsillitis; the infusion prepared from the roots of *R. canescens* var. *canescens* for the treatment of gonorrhea and the decoction from the leaves as antipyretic. Infusion of fresh leaves of *R. hirtus* has been used to enhance the maturation process of oedema and the root decoction has been used to treat haemorrhoids (Akıllıgöz and Ezer, 2000). On the other hand, the fruits of *R. sanctus* have been used to stop diarrhoea and for the treatment of haemorrhoids, diabetes mellitus and rheumatism and as diuretic (Tombul and Altan, 1986). In Asia, Europe and North America, the roots, leaves and fruits of *Rubus* species have been used as diaphoretic, tonic, antispasmodic, cholagogue, to relieve menstrual pain and to help pass kidney stones, for wound healing and for the treatment of infertility, dysentery and malaria (Amico and Sorce, 1997; Ghalayini et al., 2011; Lewis and Elvin Lewis, 1976; Oh et al., 2007; Pal et al., 1991; Zhang et al., 2011a,b).

In the present study, the *in vitro* inhibitory effect of *R. sanctus* on the metalloproteinase enzymes was investigated to clarify the wound healing activity mechanism. Spectral analyses were also conducted to figure out the active compound/s of *R. sanctus* through activity-guided fractionation and isolation assays.

2. Materials and methods

2.1. Plant material

R. sanctus Schreber flowering aerial parts were collected from Kıbrısköy Village, Ankara, Turkey at the end of June in 2011. The plant was authenticated by Prof. Dr. Hayri DUMAN from Gazi University, Department of Biology, Faculty of Science and Art,

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http://dx.doi.org/10.1016/j.hermed.2015.09.002
2210-8033/

Ankara and a voucher specimen (GUE 2604) was deposited in the Herbarium of Faculty of Pharmacy, Gazi University, Ankara, Turkey.

2.2. Preparation of plant extracts

Shade dried and powdered plant material (400 g) was subjected to successive solvent extractions with *n*-hexane, EtOAc and MeOH at room temperature for 48 h (8 × 6 L). After filtration, the extracts were evaporated to dryness by using a rotary evaporator (Buchi, Switzerland) under reduced pressure at 40 °C to give "*n*-hexane" (44.36 g; yield: 11.09%) "EtOAc" (97.84 g; yield: 24.46%) and "MeOH" (121.56 g; yield: 30.39%) of extract, respectively.

2.3. Fractionation of MeOH extract by column chromatography

An amount of 7 g of the MeOH extract was weighed and fractionated by silica gel (70–230 mesh, 60 Å) column (4 × 40 cm) chromatography by eluting with a mobile phase consisting of $CHCl_3$:MeOH [(95:5); (90:10); (85:15); (80:20); (75:25); (70:30); (60:40)] with increasing polarity to give five subfractions; Fr. A (yield:4.56%); Fr. B (3.72%); Fr. C (10.99%); Fr.D (10.63%) and Fr. E (18.61%).

2.4. Fractionation of Fr.E by column chromatography

According to the biological activity-guided fractionation assays, the most active fraction, Fr.E (200 mg), was further fractionated by silica gel (70–230 mesh, 60 Å) column (1 × 20 cm) chromatography by eluting with a mobile phase consisting of $CHCl_3$:MeOH [(95:5); (90:10); (85:15); (80:20); (75:25); (70:30); (60:40)] with increasing polarity to give three fractions: $Fr.E_1$ (yield: 4.55%); $Fr.E_2$ (7.65%); $Fr.E_3$ (4.80%).

2.5. Purification and structure elucidation of the active compound from $Fr.E_2$ by preparative thin layer chromatography (TLC)

According to the biological activity-guided fractionation assays, the most active subfraction $Fr.E_2$ (15.3 mg) was further purified by using preparative TLC plates (Silica gel, 2 μm, Sigma–Aldrich, Z513059). Nuclear Magnetic Resonance (^1H and ^{13}C NMR) and time of flight-mass spectral (TOF-MS) techniques were used for the structure elucidation of the compound. NMR spectra were recorded on a Mercury 400 spectrometer (400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR) instrument, and using DMSO as solvent. TOF-MS analyses were performed using a Micromass LCT Premier XE spectrometer. The compound was identified as hyperoside (quercetin-3-*O*-β-galactoside) by comparison to their spectroscopic data with those of published in related references (Fig. 1) (Deng et al., 2009; Jung et al., 2011).

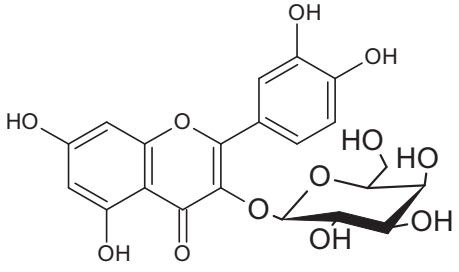

Fig. 1. Chemical structure of hyperoside.

2.6. Hyaluronidase inhibitory activity assessment

The inhibition of hyaluronidase enzyme was evaluated by measuring the amount of *N*-acetylglucosamine released from sodium hyaluronate. 50 μl of bovine hyaluronidase (7900 units/mL) was dissolved in 0.1 M acetate buffer (pH 3.6) and was mixed with 50 μl of different concentrations of the extracts dissolved in 5% DMSO. For the control group 50 μl of 5% DMSO was added instead of the extracts. After 20 min of incubation at 37°C, 50 μl of calcium chloride (12.5 mM) was added to the mixture and again incubated for another 20 min at 37 °C. 250 μl sodium hyaluronate (1.2 mg/mL) was added and incubated for 40 min at 37 °C. After incubation, the mixture was treated with 50 μl of 0.4 M NaOH and 100 μl of 0.2 M sodium borate and then incubated for 3 min inside the boiling water bath. 1.5 ml of *p*-dimethylaminobenzaldehyde solution was added to the reaction mixture after cooling to room temperature and was further incubated at 37 °C for 20 min to develop a color. The absorbance of this colored solution was measured at 585 nm (Beckmann Dual Spectrometer; Beckman, Fullerton, CA, USA) (Lee and Choi, 1999; Sahasrabudhe and Deodhar, 2010; Süntar et al., 2012).

2.7. Collagenase inhibitory activity assessment

The samples were dissolved in DMSO. *Clostridium histolyticum* (ChC) was dissolved in 50 mM Tricine buffer (with 0.4 M NaCl and 0.01 M $CaCl_2$, pH 7.5). Then, 2 mM *N*-[3-(2-Furyl)acryloyl]-Leu-Gly-Pro-Ala (FALGPA) solution was prepared in the same buffer. 25 μl buffer, 25 μl test sample and 25 μl enzyme were added to each well and incubated for 15 min. 50 μl substrate was added to the mixture to immediately measure the decrease of the optical density (OD) at 340 nm using a spectrometer.

The ChC inhibitory activity of each sample was calculated according to the following formula:

$$\text{ChC inibition activity}(\%) = \text{OD}_{\text{Control}} - \text{OD}_{\text{Sample}} \times 100/\text{OD}_{\text{Control}}$$

where $OD_{control}$ and OD_{sample} represent the optical densities in the absence and presence of sample, respectively (Barrantes and Guinea, 2003; Süntar et al., 2012).

2.8. Elastase inhibitory activity assessment

The sample solution and human neutrophil elastase enzyme (HNE) (17 mU/ml) were mixed in 0.1 M Tris–HCl buffer (pH 7.5), then incubated at 25 °C for 5 min. *N*-Methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide (MAAPVN) was added to the mixture and incubated at 37 °C for 1 h. The reaction was stopped by the addition of soybean trypsin inhibitor (1 mg/ml) and the optical density due to the formation of *p*-nitroaniline was immediately measured at 405 nm. The HNE inhibitory activities were calculated as described in the ChC inhibitory activity (Melzig et al., 2001a; Süntar et al., 2012).

2.9. Statistical analysis of the data

The data on percentage wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of $p \leq 0.05$ were considered statistically significant.

3. Results

In the present study, *in vitro* inhibitory activities of the extracts of *R. sanctus* on hyaluronidase, collagenase and elastase enzymes, all of which are involved in the wound healing process were investigated. *In vitro* hyaluronidase inhibitory activity assay results showed that neither the extracts nor the fractions obtained from

Please cite this article in press as: E. Küpeli Akkol, et al., *In vitro* enzyme inhibitory effects of *Rubus sanctus* Schreber and its active metabolite as a function of wound healing activity, J. Herbal Med. (2015), http://dx.doi.org/10.1016/j.hermed.2015.09.002