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Spirostanol saponins and esculin from *Rusci rhizoma* reduce the thrombin-induced hyperpermeability of endothelial cells

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ABSTRACT

Rusci rhizoma extracts are traditionally used against chronic venous disorders (CVD). To determine the effect of its secondary plant metabolites on the endothelium, phenolic compounds and saponins from Butcher's broom were isolated from a methanolic extract, and their activity on the thrombin-induced hyperpermeability of human microvascular endothelial cells (HMEC-1) was investigated *in vitro*.

In addition to the six known spirostanol saponins deglucoruscin (**5**), 22-O-methyl-deglucoruscoside (**6**), deglucoruscoside (**7**), ruscin (**8**), ruscogenin-1-O-(α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside (**9**) and 1-O-sulpho-ruscogenin (**10**), three new spirostanol derivatives were isolated and identified: 3′-O-acetyl-4′-O-sulphodeglucoruscin (**1**), 4′-O-(2-hydroxy-3-methylpentanoyl)-deglucoruscin (**2**) and 4′-O-acetyl-deglucoruscin (**3**). Furthermore, the coumarin esculin (**4**), which is also prominently present in other medicinal plants used in the treatment of CVD, was isolated for the first time from *Rusci rhizoma*. Five of the isolated steroid derivatives (**2**, **5**, **8**, **9** and **10**) and esculin (**4**) were tested for their ability to reduce the thrombin-induced hyperpermeability of endothelial cells *in vitro*, and the results were compared to those of the aglycone neoruscogenin (**11**). The latter compound showed a slight but concentration-dependent reduction in hyperpermeability to 71.8% at 100 μM. The highest activities were observed for the spirostanol saponins **5** and **8** and for esculin (**4**) at 10 μM, and these compounds resulted in a reduction of the thrombin-induced hyperpermeability to 41.9%, 42.6% and 53.3%, respectively. For **2, 5** and **8,** the highest concentration tested (100 μM) resulted in a drastic increase of the thrombin effect. The effect of esculin observed at a concentration of 10 μM was diminished at 100 μM. These *in vitro* data provide insight into the pharmacological mechanism by which the genuine spirostanol saponins and esculin can contribute to the efficacy of Butcher's broom against chronic venous disorders.

1. Introduction

Extracts of Butcher's broom (*Rusci rhizoma*, *Ruscus aculeatus*, Ruscaceae) are traditionally used against chronic venous disorders (CVD). As its efficacy has been demonstrated in several clinical studies, the ESCOP (European Scientific Cooperative on Phytotherapy) recommends a daily intake of *Rusci rhizoma* that contains a total of 7–11 mg of ruscogenins (ESCOP, 2003). Recent studies have also demonstrated the efficacy of a combination of *Rusci rhizoma* with hesperidin methylchalcone and ascorbic acid against CVD (Allaert et al., 2011; Boyle et al., 2003). Furo- and spirostanol glycosides with different sugars and various esterifications represent the most abundant group of secondary plant metabolites (Mimaki et al., 1998a,b,c). As these metabolites seem to be important for the efficacy of the drug (ESCOP, 2003), the European Pharmacopoeia (7. Ed., 2011) demands their quantification; however, due to various analytical difficulties, the furo- and spirostanol glycosides have to be hydrolysed and quantified as the corresponding aglycones (1.0% total sapogenins quantified as neoruscogenin and ruscogenin). Most *in vitro* and *in vivo* experiments with *R. aculeatus* and CVD were performed with extracts of Butcher's broom or the steroidal aglycones (ESCOP, 2003 for an overview) and not with the genuine saponines. For example, Huang et al. (2008) showed that ruscogenin inhibits NF-κB and ICAM-1 expression. In addition, the inhibition of elastase by the steroidal aglycones has been reported (Facino et al., 1995). Nevertheless, the steroidal glycosides, not the aglycones, are the relevant secondary metabolites in Butcher's broom extracts. In a key study, Rauwald and Grünwidl (1991) demonstrated that steroid glycosides from *Rusci rhizoma* are absorbed and can be detected in their unmodified form in human plasma after oral administration. Therefore, it seems necessary to

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work with isolated genuine steroid glycosides *in vitro* and not with their aglycones to mimic what occurs *in vivo*. Surprisingly, pharmacological data on the activity of these compounds are scarce; thus, a rational explanation of the clinical effects of extracts of Butcher's broom at the cellular and molecular level is still lacking. As increased permeability of the venous endothelium plays a pivotal role in chronic venous disorders, the aim of this work was to find *in vitro*-based explanations for the efficacy of Butcher's broom's extracts in CVD by the isolation of single compounds and the determination of the ability of these compounds to alter the thrombin-induced hyperpermeability of endothelial cells.

2. Results

From a butanol fraction of a methanolic extract of *Rusci rhizoma*, 10 compounds were isolated using a combination of different chromatographic techniques (see Fig. 1 for the new and tested substances). The spirostanol derivatives deglucoruscin (**5**), 22-O-methyl-deglucoruscoside (**6**), deglucoruscoside (**7**; all three are described by Mimaki et al., 1998b), ruscin (**8**; Mimaki et al., 1998b), ruscogenin-1-O-(α-L-rhamnopyranosyl-(1 → 2)-β-D-galactopyranoside (**9**; Mimaki et al., 1998a) and 1-O-sulpho-ruscogenin (**10**; Oulad-Ali et al., 1996) had been previously isolated from *R. aculeatus*. Their structures were elucidated with modern spectroscopic (1D- and 2D-NMR) and spectrometric (ESIMS) techniques, and the data were identical to those published in the literature.

Compound **1** exhibited a ^{1}H NMR spectrum that was similar to that of deglucoruscin (**5**), with the majority of signals for the aglycone core between ~0.80 and ~3.00 ppm and the majority of the sugar protons between ~3.80 and ~5.80 ppm (Table 1). In addition, the ^{13}C NMR spectrum of **1** shows characteristic signals for C-1 (δ_C = 83.9 ppm), C-3 (δ_C = 68.0 ppm), C-5 (δ_C = 139.4 ppm), C-6 (δ_C = 125.0 ppm), C-22 (δ_C = 109.4 ppm), C-25 (δ_C = 144.6 ppm) and C-27 (δ_C = 108.6 ppm) as well as for the methyl groups H-18 (δ_H = 0.82 ppm, s), H-19 (δ_H = 1.38 ppm, s) and H-21 (δ_H = 0.99 - ppm, d, J = 7.0 Hz). COSY, HSQC, HMBC and ROESY experiments confirmed the presence of neoruscogenin as the aglycon portion of the molecule, with arabinose and rhamnose as the sugars. The ^{3}J couplings of H-1′ (arabinose) to C-1 (aglycone) and H-1″ (rhamnose) to C-2′ (arabinose), which were detectable in the HMBC spectrum, determined the linkage of both sugars and the connection to the aglycone. The identity and absolute configuration of L-arabinose and L-rhamnose were confirmed by cleavage of the glycosidic bonds, derivatisation with S-(−)-1-phenylethylamine and CE-analysis with reference sugars (Noe and Freissmuth, 1995). The relatively large $^{3}J_{H\text{-}1'/H\text{-}2'}$ (6.4 Hz) and the very small $^{3}J_{H\text{-}1''/H\text{-}2''}$ (<1 Hz) values indicated the presence of α-L-arabinose and α-L-rhamnose. In contrast to **5**, a carbon signal (δ_C = 171.0 ppm) correlating to a methyl group (δ_H = 1.98 ppm, *s*) indicated the presence of an additional acetyl group. HMBC experiments and the downfield shift of H-3′ (δ_H = 5.48 ppm) revealed the acetylation of this position of the arabinose. Another difference was observed for the molecular masses; **1** had a pseudomolecular ion peak at m/z 827 for $[M-H]^-$ in negative ion ESIMS, whereas **5** displayed a pseudomolecular ion at m/z 705 for $[M-H]^-$. The fragment at m/z 746 ($[M-H-81]^-$) indicated the presence of a sulphate group. This group was connected to C-4′, which was determined because H-4′ was shifted downfield to δ_H = 5.48 ppm. All other NMR signals were comparable to those of **5**. Consequently, the structure of **1** was unambiguously determined to be 3′-O-acetyl-4′-O-sulphodeglucoruscin.

Compound **2** is also a derivative of deglucoruscin (**5**). In contrast to **5**, **2** showed a pseudomolecular ion peak at m/z for 819 $[M-H]^-$ in negative ion ESIMS and 6 additional carbon signals in the ^{13}C NMR experiment. A cross peak in the HMBC spectrum between a carbonyl signal (δ_C = 175.1 ppm) and H-4′ (δ_H = 5.53 ppm, *s*) indicated an esterification of deglucoruscin at position H-4′. The side chain was determined using 2D-NMR experiments to be 2-hydroxy-3-methylpentanoate (HMP), which is a typical modification of steroid glycosides from *R. aculeatus* (Mimaki et al., 1998b). Thus, compound **2** was identified from NMR (Table 1) and MS data to be 4′-O-(2-hydroxy-3-methylpentanoyl)-deglucoruscin.

Compound **3** exhibited ^{1}H- and ^{13}C NMR spectra that were very similar to those of **1**. In addition to the signals of deglucoruscin, typical signals for an acetyl group could be observed. A downfield shift of the H-4′ signal (δ_H = 5.38 ppm) and an HMBC cross-peak between H-4′ and the acetyl carbonyl indicated acetylation at C-4′. The pseudomolecular ion peak at m/z 747, corresponding to the $[M-H]^-$ ion, in negative ion ESIMS, together with the NMR data (Table 1), confirmed the structure of **3** to be 4′-O-acetyl-deglucoruscin.

Additionally, the coumarin esculin (**4**) was isolated for the first time from *Rusci rhizoma*. The structure was elucidated by 1D- and 2D-NMR and ESIMS, and these data were compared with the literature values (Dubois et al., 1990).

To investigate the effect of the compounds on endothelial hyperpermeability *in vitro*, confluent human microvascular endothelial cells (HMEC-1) were pre-incubated in a Transwell® insert with **2**, **4**, **5**, **8**, **9**, **10** and the aglycone neoruscogenin (**11**, 0.1 to 100 μM) for 30 min before the addition of thrombin (3 U/ml). As shown in Fig. 2, the isolated compounds **4** (53.3%), **5** (41.9%), **8** (42.6%), **9** (63.7%) and **10** (62.6%) reduced the thrombin induced permeability at 10 μM. In contrast, **2** (257.6%), **5** (368.3%) and **8** (390.2%) augmented the permeability at a tenfold higher concentration. Neoruscogenin slightly decreased the permeability at concentrations up to 100 μM (71.8%).

3. Discussion

From a methanolic extract of Butcher's broom, nine spirostanol saponins and one coumarin were isolated. Three of the isolated saponins, 3′-O-acetyl-4′-O-sulphodeglucoruscin (**1**), 4′-O-(2-hydroxy-3-methylpentanoyl)-deglucoruscin (**2**) and 4′-O-acetyl-deglucoruscin (**3**), are previously undescribed natural compounds. The esterification of furo- and spirostanol saponins with acetic acid, 2-hydroxy-3-methylpentanoic acid and sulphuric acid seems to be a characteristic modification for *R. aculeatus*. Some typical structural variations that are observed include different positions and combinations of esters and variations of the sugar side chain, e.g., the presence of a galactose moiety instead of arabinose at position 1 (Oulad-Ali et al., 1996; Mimaki et al., 1998a-c).

Only sparse information exists for phenolic compounds in Butcher's broom. In addition to benzofurans and dibenzofurans (Elsohly et al., 1974; Elsohly et al., 1977), a new class of phenolic compounds with a phenyl-benzoxepin carbon skeleton was recently isolated in very low quantities (Barbič et al., 2012). With the identification and isolation of the coumarin esculin (**4**), another class of phenolic compounds was found in *Rusci rhizoma* that is also prominently present in other medicinal plants used in the treatment of CVD.

A key feature of CVD is the pathologically increased permeability of the venous endothelium (hyperpermeability), i.e., a strong extravasation of fluid, macromolecules (proteins) and even erythrocytes from the vessel into the interstitial space, which is manifested in the formation of oedema (Jünger et al., 2000). The Transwell® assay is an *in vitro* test system that values the influence of compounds on the macromolecular endothelial permeability when thrombin has been used to induce hyperpermeability. The isolated compounds and the aglycone neoruscogenin showed different effects on the thrombin-induced hyperpermeability of endothelial cells after 60 min (Fig. 2). The neoruscogenin-diglycoside **5**