Fitoterapia 97 (2014) 78–86

Contents lists available at ScienceDirect

Fitoterapia

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

The antibiofilm activity of lingonberry flavonoids against oral pathogens is a case connected to residual complexity

Kaisu R. Riihinen [a,b,*], Zhen M. Ou [c], Tanja Gödecke [d], David C. Lankin [d], Guido F. Pauli [a,d], Christine D. Wu [c]

[a] Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, IL 60612-7231, USA
[b] Institute of Public Health and Clinical Nutrition, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland
[c] Department of Pediatric Dentistry, College of Dentistry, University of Illinois at Chicago, Chicago, IL 60612-7231, USA
[d] Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, IL 60612-7231, USA

ARTICLE INFO

Article history:
Received 9 March 2014
Accepted in revised form 18 May 2014
Available online 29 May 2014

Keywords:
Flavonoids
Polyphenols
Dental caries
Vaccinium vitis-idaea
Streptococcus mutans
Fusobacterium nucleatum

Chemical compounds studied in this article:
Quercetin-3-rhamnoside (PubChem CID: 5280459)
Quercetin-3-glucopyranoside (PubChem CID: 5280804)
Quercetin-3-arabinofuranoside (PubChem CID: 5490064)
Proanthocyanidin A2 (PubChem CID: 124025)
Proanthocyanidin A1 (PubChem CID: 9872976)
Proanthocyanidin B2 (PubChem CID: 122738)
Cyanidin-3-galactoside (PubChem CID: 44256700)
Cyanidin-3-glucopyranoside (PubChem CID: 185664)
(+)-Catechin (PubChem CID: 9064)
(−)-Epicatechin (PubChem CID: 72276)

ABSTRACT

The antimicrobial activity of lingonberry (*Vaccinium vitis-idaea* L.) was evaluated against two oral pathogens, *Streptococcus mutans* and *Fusobacterium nucleatum*. Long-bed gel permeation chromatography (GPC; Sephadex LH-20) yielded purified flavonoids, with the most efficient minimum inhibitory concentrations (MICs) against planktonic cells in the anthocyanin and procyanidin primary fractions against *F. nucleatum* (63–125 μg/ml) and in the procyanidin rich fraction against *S. mutans* (16–31 μg/ml). The purified flavonol glycosides and procyanidins inhibited biofilm formation of *S. mutans* (MICs 16–31 μg/ml), while the corresponding reference compounds showed no activity. Secondary GPC purification yielded flavonol glycosides devoid of antibiofilm activity in the 50% MeOH fraction, while elution with 70% acetone recovered a brownish material with activity against *S. mutans* biofilm (MIC 8 μg/ml). Even after HPLC-PDA, NMR, and MALDI-TOF analyses, the structural identity of this material remained unknown, while its color and analytical characteristics appear to be consistent with flavonoid oxidation products.

* Corresponding author at: Institute of Public Health and Clinical Nutrition, University of Eastern Finland, P.O. Box 1627, 70211 Kuopio, Finland. Tel.: +358 40 5911425; fax: +358 17 162 792.
E-mail address: Kaisu.Riihinen@uef.fi (K.R. Riihinen).

http://dx.doi.org/10.1016/j.fitote.2014.05.012
0367-326X/

1. Introduction

The natural growing environment of lingonberry (*Vaccinium vitis-idaea* L.) is restricted to the Northern hemisphere. Nordic countries are known for the traditional consumption of food products prepared from these light red colored berry fruits. As a dietary supplement, lingonberry extract is available world-wide [1]. Flavonoids such as anthocyanins, flavonol glycosides, and proanthocyanidins are currently considered to be the health-promoting constituents of the berries [2]. Among Ericaceous berry fruits, lingonberries exhibit intermediate levels of anthocyanins (810 mg/100 g dry weight [DW]) and flavonol glycosides (82 mg/100 g DW), but high content of procyanidins (240–380 mg/100 g DW) compared to bilberries (*Vaccinium myrtillus* L.) [3,4]. Lingonberry extracts have been found to inhibit the promotional stage of chemically induced carcinogenesis [5], the intestinal tumorigenesis in multiple intestinal neoplasia [6], the growth of periodontal pathogens [7], the hemagglutination of *Escherichia coli* [8], the aggregation of oral bacterial cells [9], and the binding of *Neisseria meningitidis* pili to human epithelial cells [10]. Anticarcinogenic and antiadhesive activities were concentrated in the lingonberry fractions rich of flavonoids [5,9,10]. Antimicrobial and antiplaque activities of plant flavonoids, obtained from a variety of different plant sources, have been demonstrated in many in vitro studies [11–13]. Moreover, there is clinical evidence which demonstrates that the consumption of flavonoid-rich foods or beverages has a benefit to oral health by exhibiting anti-gingivitis and anti-caries properties [14–17].

The purification and structural characterization of berry flavonoids is a prerequisite for the determination of their in vitro biological activity [2]. Reported purification protocols are based on high speed countercurrent chromatography [18], gel permeation chromatography (GPC) [19–22], preparative HPLC [8,23], as well as combinations of these techniques. [5,24,25] However, the purity of the isolates has seldom been addressed and/or related to the purification method, even for the major flavonoids which are readily identified using contemporary NMR spectroscopy. In general, the loss of bioactivity during the final purification steps and the observation of inverse correlations between purity levels and bioactivity are indications of the potential presence of residual complexity, a phenomenon widely observed in natural products research [26]. For example, the abolishment of the anti-adhesion activity of cranberry against *P*-fimbriated *E. coli* to the human uroepithelial cell line T24 has been noted during the final reversed phase HPLC purification step [24]. A case of inverse purity-activity relationships (PARs) has been described for the antimycobacterial potential of ursolic acid and other triterpenes [27,28]. Therefore, residual complexity concerns pertain not only to flavonoids but to all associated bioactive compounds purified from plant materials [26,29,30]. Since ^{1}H NMR is a universal detector, recently established 100% quantitative ^{1}H NMR (qHNMR) methods allow effective determination of major and minor impurities in natural products [31]. The authors recently found noticeable discrepancies between the purities of isolated flavonol glycosides measured by HPLC (82–94 area-% at 250 nm) and the corresponding purities determined by qHNMR (52–70%) [21]. Similar forms of static residual complexity have been demonstrated to affect other natural product extracts and isolates such as ginkgo extracts, ginkgolide mixtures [32], and *Actaea* triterpenes [33].

The aim of the present study was to purify lingonberry flavonoids and to examine their antimicrobial activity against selected oral pathogens associated with dental caries and periodontal disease. For this purpose, repeated long-bed gel permeation chromatographic (GPC) separation on Sephadex LH-20 material was employed for the purification of flavonol glycosides [21], anthocyanins, procyanidins, and flavan-3-ols from lingonberry, as well as to remove the residual complex materials from the isolated flavonol glycosides. The effect of the enriched flavonoids on growth and biofilm formation of *S. mutans* and *F. nucleatum* was investigated. Identifications were performed by 1D and 2D NMR, HPLC–PDA, HPLC-IT–TOF-MS, UHPLC–PDA, and MALDI-TOF. Methods for quantification were based on the absorbance in HPLC–PDA and the total integration of ^{1}H NMR spectra.

2. Materials and methods

2.1. Standards

Quercetin-3-*O*-β-galactopyranoside (hyperoside or hyperin) was purchased from Indofine Chemical Co. Inc. and quercetin-3-*O*-α-rhamnopyranoside (quercitrin), quercetin-3-*O*-β-D-glucopyranoside, quercetin-3-*O*-α-arabinofuranoside (avicularin) were from ChromaDex Inc. (Santa Ana, CA). The following standards were purchased from Sigma Chemical Co.: cyanidin-3-galactoside (idaein chloride), vanillic acid, *p*-hydroxybenzoic acid, chlorogenic acid, *p*-coumaric acid, caffeic acid, ferulic acid, quercetin, (+)-catechin, (−)-epicatechin, and procyanidin B2, while cyanidin-3-glucoside was obtained from Extrasynthese (Genay, France). The structures of commercial materials were verified (1D/2D NMR experiments) and purities determined by 1D qHNMR.

2.2. Fractionation of flavonoids

A stepwise fractionation of lingonberry flavonoids was achieved on Diaion reversed-phase HP-20 resin and on Sephadex LH-20 based GPC as described previously [21]. Briefly, juice concentrate was pre-fractionated with a MeOH-water-gradient over HP-20 resin to enrich the flavonoids in fractions (Frs.) 3a–3d and 4 (Fig. 1). The composition of the Frs. was monitored by TLC [21]. Since Frs. 3a–3d displayed distinct spot profiles during TLC screening, they were not combined, even though they all eluted at 50% MeOH. Fr. 4 was released from the resin with 100% MeOH. The subsequent GPC fractionation was performed on a Sephadex LH-20 gel bed in a precolumn (85–95 ml/17–19 cm, I.D. 25 mm), or/and in a main column system (total 910 ml/960 cm, I.D. 11 mm). Two portions of Fr. 3a (0.8 and 1.0 g) and one portion of Fr. 3b (1.2 g) were re-chromatographed over the precolumn, eluting first with MeOH (1 ml/min) and finally recovering residual material by elution with 70% acetone. These fractions were combined according to TLC for further antimicrobial assays, compound identification, and quantification. The compounds eluting between 110 and 210 ml (1.3–2.5 bed volumes [BVs]) were visualized as red spots with vanillin sulfuric acid spray. These proposed flavan-3-ols and procyanidins were further eluted with MeOH (1 ml/min)