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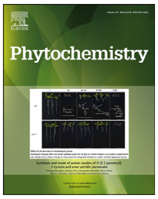

Phytochemical analysis of salal berry (*Gaultheria shallon* Pursh.), a traditionally-consumed fruit from western North America with exceptionally high proanthocyanidin content

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ABSTRACT

Salal (*Gaultheria shallon* Pursh.) is a wild perennial shrub of the Ericaceae and common in coastal forests of western North America, and its berries were an important traditional food for First Nations in British Columbia. Salal berries were investigated for phytochemical content and antioxidant capacity over the course of fruit development. The proanthocyanidin content was extremely high in young berries (280.7 mg/g dry wt) but dropped during development to 52.8 mg/g dry wt. By contrast, anthocyanins accumulated only at the late berry stages. Total antioxidant capacity, as measured by the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) method, reflected both proanthocyanidin and anthocyanin content, and in mature berries reached 36 mmol Trolox equivalents/100 g dry wt. More detailed phytochemical analysis determined that delphinidin 3-*O*-galactoside is the dominant anthocyanin, and that the berries are also rich in procyanidins, including procyanidin A2 which has been implicated in anti-adhesion activity for uropathogenic *E. coli*. Proanthocyanidins were 60% prodelphinidin, and overall concentrations were higher than reported for many *Vaccinium* species including blueberry, lingonberry, and cranberry. Overall, the phenolic profile of salal berries indicates that these fruit contain a diversity of health-promoting phenolics.

1. Introduction

Plants produce an impressive number of phenolic secondary plant metabolites with diverse ecological roles that allow plants adapt to their environment. A prominent group within the phenolics are the flavonoids, which include the anthocyanins, flavonols, flavones, isoflavonoids, flavan-3-ols, and proanthocyanidins (PAs; *syn.* condensed tannins). While most flavonoids share a common three-ring structure and are synthesized via the general flavonoid pathway, they have diverse bioactivities and functions. For example, anthocyanins are common red and blue plant pigments, which function to attract pollinators and seed dispersers to flowers and fruit (Davies et al., 2012). By contrast, the biosynthetically related PAs are known to bind proteins and act as defenses against mammalian herbivores and fungal pathogens (Barbehenn and Constabel, 2011). They also have general antimicrobial activity (Scalbert, 1991); when deposited in soils as leaf litter, PAs can inhibit microbial activity and nutrient cycling (Schweitzer et al., 2004). In most species, PAs consist of a mixture of oligomeric and polymeric flavan-3-ols with varying mean degrees of polymerization (mDP), ranging from 2 to 30 or higher (Porter, 1988). They may be linked via several types of C—C linkages; most common are C4 → C8 linkages, but others structures, such as double-linked A2 type oligomers, are also found. PAs are particularly abundant in trees and woody plants, and occur at high concentrations in roots, bark, as well as leaves (Porter, 1988). They are considered the most widespread secondary plant metabolite.

The PAs are also common phytochemical constituents of berry fruits (Prior and Gu, 2005; Rasmussen et al., 2005). Fruits are thought to be the biggest source of PAs in western diets, although cereals, beans and nuts are also important. Comprehensive surveys indicate a wide range in both PA content and structure in

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commercially grown fruits (Prior and Gu, 2005; Hosseinian et al., 2007). Cranberry, blueberry, and strawberry show among the highest PA contents, accumulating 150–420 mg/100 g fr. wt. Wild berries also demonstrate a broad range of PA concentrations. In a detailed analysis of twelve native berries from northern Canada, Dudonné et al. (2015) found the greatest PA concentrations (700 mg/100 g fr. wt) in highbush cranberry (*Viburnum trilobum* Marsh.). Anthocyanins are also prevalent in many dark berries, with concentrations as high as 500 mg/100 g fr. wt in black crowberry (*Empetrum nigrum* L.). Other phenolic compounds such as flavonols or hydroxycinnamic acids including chlorogenic acid, also occur in many berries, but generally in lower concentrations (Dudonné et al., 2015).

Phenolic phytochemicals, in particular the PAs and anthocyanins, have been studied intensively as antioxidants and for their benefits to human health (Quideau et al., 2011; Prior and Gu, 2005). Many phenolics have strong *in vitro* free radical scavenging capacity (Quideau et al., 2011; Hagerman et al., 1998), since a phenolic ring with at least two hydroxyls in the *ortho* position is typically an effective antioxidant (Quideau et al., 2011). Commonly occurring examples are the flavonol quercetin, anthocyanins such as cyanidin, and flavan-3-ols, for example catechin and PA. The radical scavenging and antioxidant ability of PAs is further increased in polymers due to the large number of aromatics and hydroxyls in close proximity to each other (Hagerman et al., 1998). Antioxidant capacity of phenolics is readily measured using the common ABTS (2,2′- azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay, which has facilitated surveys demonstrating the substantial antioxidant capacity of many fruits and vegetables (Re et al., 1999; Määttä-Riihinen et al., 2005; Prior et al., 2005).

In parallel, epidemiological studies suggest that consumption of foods high in PAs has long-term benefits to human health, including a reduced risk of cardiovascular and neurodegenerative diseases (Prior and Gu, 2005; Santos-Buelga and Scalbert, 2000), as well as hypoglycaemic and anti-inflammatory effects (Grace et al., 2014; Esposito et al., 2014). Other potential health benefits include increasing serum antioxidant capacity (Serafini et al., 1998) and antihypertensive benefits (Furuuchi et al., 2012). Whether the well-established *in vitro* antioxidant capacity of PAs and other phenolics is mechanistically linked to their health-promoting function is still an open question, since this is likely not their only effect. It is important to note that the PA content of a food does not reflect its bioavailability *in vivo;* this varies with the specific structures involved, and depends on their propensity to be absorbed by the intestine as well as metabolic conversions, and interactions with gut flora (Santos-Buelga and Scalbert, 2000). However, despite incomplete knowledge of their interactions with human metabolic processes, the potential beneficial effects of dietary PAs continue to drive investigations of the phytochemistry of fruit.

Salal (*Gaultheria shallon* Pursh.) is an ericaceous shrub common in temperate rainforests of the Pacific Northwest (Tappeiner et al., 2001). Salal berries are similar in shape and size to blueberries, huckleberries and other *Vaccinium* species; they are a traditional food of coastal First Nations who consumed the berries fresh, or mashed and dried into cakes for consumption throughout the winter (Turner and Bell, 1971, 1973). Salal forms a dense understory, and was once thought compete with reforestation by outcompeting seedlings (Tappeiner et al., 2001). An earlier survey found that salal berries exhibit high antioxidant capacity (Acuña et al., 2002; Einbond et al., 2004), and contain a range of simple phenolics including notably high levels of caffeic acid (Towers et al., 1966). Recently, two reports identified additional phenolic constituents, including small oligomeric PAs, anthocyanins, and flavonols in salal fruit (McDougall et al., 2016), and measured total antioxidant activity, anthocyanins, and phenolic content (Martin et al., 2015). These authors did not include polymeric PAs in their analysis, and did not provide quantitative data on individual compounds.

The suggestion of strong antioxidant activity, reports of high PA concentrations in salal leaves (Preston, 1999), and traditional First Nations use of salal berries prompted us to investigate these fruit in detail. We hypothesized that the reported antioxidant capacity of salal berry is due to PA concentrations, and that the berries could thus be an important source of dietary PAs. Furthermore, we took a developmental approach and profiled PAs and other phytochemicals throughout fruit development. Our aim was to understand patterns of antioxidant capacity and phytochemicals over time, which ultimately should reflect their biological function.

2. Results

2.1. Developmental profile of salal anthocyanins, proanthocyanidins, and antioxidant capacity

Salal berries from the eight stages of berry development and ripening showed a predictable increase in weight and size (Fig. 1a and b). Anthocyanins were detected in all stages of flower and berry, but were lowest in the white open flower stage (Fig. 1c). A low concentration of total anthocyanins was maintained until stage B7, when their concentration increased dramatically. Mature salal berries (B8) contained very high levels of anthocyanins, more than 1500 mg/100 g dry wt of total anthocyanins.

To determine how berry antioxidant capacity changed over the profile, we carried out antioxidant assays of MeOH extracts using the colourimetric ABTS method (Re et al., 1999). Antioxidant capacity of salal extracts was highest in the flower and young berry stages, with maximal levels reaching 106 mmol TE/100 g dry weight at stage B2 (Fig. 2**).** As berries matured, antioxidant activity diminished several-fold, to approximately 36 μmol TE/100 g dry weight. The reduction in antioxidant capacity from B4 to B5 coincided with a rapid increase in mean berry mass, presumably diluting out the antioxidant levels. For comparison, we tested antioxidant capacity of mature highbush blueberries (cv. Rubel), and measured 13 μmol TE/100 g dry weight, approximately one-third the capacity of mature salal berries.

Since the antioxidant levels of berries did not follow the anthocyanin concentrations, we investigated the PA concentrations using the butanol-HCl assay (Fig. 3). Using a purified salal leaf PA standard, we determined that salal berries contained very high concentrations of PAs in younger stages, which declined several fold thereafter to a mean of 5280 mg/100 g dry wt in mature berries (stage B8). High concentrations of PAs were also found in the flowers. In general, the PA concentration profile paralleled the antioxidant activity, suggesting this activity is mostly due to salal berry PAs. Indeed, we determined a strong correlation between PA concentration and antioxidant capacity of samples ($R^2 = 0.969$) (Supplemental Fig. S1). When PA content was replotted on a per berry basis, it became apparent that total PAs continued to accumulate during ripening (Fig. 3). The PA concentration profile is thus the balance of biosynthesis and dilution by berry expansion. Overall, the PA concentration of mature berries is almost ten-fold higher than those we measured in blueberries (Zifkin et al., 2012), suggesting that salal could be an excellent source of dietary PAs.

2.2. UHPLC-MS/MS analysis of individual salal phenolics

The high antioxidant and flavonoid content of salal prompted us to investigate the phytochemistry of these berries in more detail. We subjected a selection of berry stages to a more detailed analysis