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The position of prenylation of isoflavonoids and stilbenoids from legumes (Fabaceae) modulates the antimicrobial activity against Gram positive pathogens

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ABSTRACT

The legume plant family (Fabaceae) is a potential source of antimicrobial phytochemicals. Molecular diversity in phytochemicals of legume extracts was enhanced by germination and fungal elicitation of seven legume species, as established by RP-UHPLC–UV–MS. The relationship between phytochemical composition, including different types of skeletons and substitutions, and antibacterial properties of extracts was investigated. Extracts rich in prenylated isoflavonoids and stilbenoids showed potent antibacterial activity against *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* at concentrations between 0.05 and 0.1% (w/v). Prenylated phenolic compounds were significantly ($p < 0.01$) correlated with the antibacterial properties of the extracts. Furthermore, the position of the prenyl group within the phenolic skeleton also influenced the antibacterial activity. Overall, prenylated phenolics from legume seedlings can serve multiple purposes, e.g. as phytoestrogens they can provide health benefits and as natural antimicrobials they offer preservation of foods.

1. Introduction

Natural plant extracts have been shown to exert a wide range of biological activities, such as anti-oncogenic, anti-inflammatory and antimicrobial activity (Ververidis et al., 2007). These properties of plant extracts have been associated with phytochemicals, secondary metabolites involved in the adaptation of plants to their environment (Cowan, 1999).

The Fabaceae or legume family is an important source of bioactive phytochemicals, including saponins and phenolic compounds (Ahuja, Kissen, & Bones, 2012; Güçlü-Üstündağ & Mazza, 2007). Saponins are triterpenoid glycosides (Vincken, Heng, de Groot, & Gruppen, 2007). Their amphiphilic structure enables them to complex with triterpenes or sterols in biological membranes (such as cholesterol in animal cells, ergosterols in fungi and hopanoids in bacteria), and thereby alter membrane permeability (Osbourn, Goss, & Field, 2011). The membranolytic activity of saponins depends on both the structure of the saponin itself (e.g. number of saccharide chains, aglycone skeleton), and on the type of sterol-like components present in the membrane to complex with (Oleszek, 2000). In nature, saponins act as pre-formed barriers against pathogens (Osbourn et al., 2011). Legumes mainly contain oleanane-class soyasaponins (Vincken et al., 2007), with members of the A, B, E and 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP)-conjugated groups (Güçlü-Üstündağ & Mazza, 2007).

Phenolic compounds from the isoflavonoid and stilbenoid classes have been described as the main phytoalexins in legumes, i.e. secondary metabolites synthesized *de novo* in response to (microbial) stress (Ahuja et al., 2012). Phenolic compounds have high structural diversity derived from the number of (aromatic) rings (defining their skeleton), modifications of the skeleton by oxygenation of the (aromatic) carbons, C- or O-glycosylation, C- or O-alk(en)ylation (e.g. methylation or prenylation), and modification of the substituents (e.g. oxygenation of prenyl groups, cyclization with a phenolic hydroxyl group) (Tahara & Ibrahim, 1995). The presence of a prenyl group (5-carbon isoprene substituent) attached to a phenolic skeleton is a special chemical feature of many legume phytoalexins.

Prenylated compounds have been reported to have interesting biological and pharmacological properties. For example, the

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pterocarpans *glyceollins* from soybean (*Glycine max*), have shown strong antioxidant, anti-inflammatory and anti-estrogenic activities (Kim, Lim, Kim, & Kim, 2012; van de Schans et al., 2016). It has been hypothesized that prenylated molecules have better partitioning to membranes and better interaction with target proteins than non-prenylated molecules, due to the increase in the compound's hydrophobicity by the addition of the prenyl group (Botta et al., 2009).

The content of saponins and phenolic compounds, isoflavonoids in particular, increases during germination of legume seeds (Ayet et al., 1997; Wang et al., 2015). The combination of germination with fungal elicitation can further increase the structural diversity of these bioactive compounds, including induction of new subclasses and induction of prenylation (Aisyah, Gruppen, Madzora, & Vincken, 2013). The effect of fungal elicitation of legume seedlings on the antibacterial properties of extracts against human pathogens has not been systematically studied. Furthermore, the contribution of different types of phenolics (e.g. (iso)flavonoids, stilbenoids and phenolic acids), different types of substitutions (e.g. sugar units, prenyl groups) or other phytochemicals present (e.g. saponins) to the antibacterial activity of seedling extracts needs to be investigated.

In this study, the occurrence of phenolic compounds and saponins in legume seedling extracts was correlated with their antibacterial properties. In order to obtain large structural diversity in phytochemicals, seeds from seven different legume species (mung bean, kidney bean, soybean, peanut, white, yellow and blue lupine) were used, and each species was subjected to germination alone or in combination with fungal elicitation. The antibacterial properties of the extracts were tested using *Listeria monocytogenes* and methicillin-resistant *Staphylococcus aureus* (MRSA) as target bacteria. *L. monocytogenes* is a foodborne pathogen known to be resistant to acid, disinfection, high salt concentrations and, in recent years, to clinically relevant antibiotics (Obaidat, Bani Salman, Lafi, & Al-Abboodi, 2015). MRSA is a major cause of community and healthcare-associated infections worldwide and has been the most important antibiotic resistant bacterium in humans and livestock for the last 50 years (Moellering, 2012).

We hypothesize that prenylated phenolics can increase the antibacterial activity of extracts from legume seedlings against human pathogenic bacteria, considering that prenylation of phenolic compounds is a response to microbial attack in legume seeds (Aisyah et al., 2013). We also hypothesize that saponins, having membranolytic activity (Oleszek, 2000), contribute to the activity of the extracts.

2. Material and methods

2.1. Materials

Seeds of mung bean (*Vigna radiata*), kidney bean (*Phaseolus vulgaris*), soybean (*Glycine* max), white lupine (*Lupinus albus*), blue lupine (*L. angustifolius*), yellow lupine (*L. luteus*) and peanut (*Arachis hypogaea*) were purchased from Vreeken's Zaden (Dordrecht, The Netherlands). Daidzein, genistein, *trans*-resveratrol and soyasaponin Bb were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile (ACN; ULC/MS grade), water acidified with 0.1% (v/v) acetic acid (HOAc) or 0.1% (v/v) formic acid (FA) (ULC/MS grade), and methanol (MeOH) (ULC/MS grade) were purchased from Biosolve (Valkenswaard, The Netherlands). Water for purposes other than UHPLC was prepared using a Milli-Q water purification system (Millipore, Molsheim, France). Growth media, Bacto brain heart infusion (BHI) broth was purchased from BD (Franklin Lakes, NJ, USA); tryptone soya broth (TSB) and agar bacteriological from Oxoid Ltd (Basingstoke, UK) and peptone physiological salt solution (PPS) from Tritium Microbiologie (Eindhoven, The Netherlands) All other chemicals were purchased from Merck (Darmstadt, Germany) and Sigma-Aldrich.

2.2. Microorganisms

Food grade fungi *Rhizopus oryzae* (LU 581) and *Rhizopus oligosporus* (LU 575) (Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands), were used to elicit the legume seeds during germination. *L. monocytogenes* EGD-e and methicillin-resistant *Staphylococcus aureus* 18HN (MRSA, kindly provided by RIVM, Bilthoven, The Netherlands) were used to test the antimicrobial properties of the extracts from legume seedlings. Glycerol stocks (50% (v/v)) of these microorganisms were kept at −80 °C.

2.3. Rhizopus elicitation and extraction of legume seedlings

Legume seeds were germinated in the presence of *Rhizopus* sp. as previously described (Aisyah et al., 2013). Briefly, legume seeds were soaked in water for 1 day, germinated for 2 days at 25 °C (100% RH), and subsequently elicited with the fungus (approx. 1.5×10^7 CFU/g seed) for 5 days at 30 °C (55–85% RH). After the elicitation period, seedlings were freeze-dried, milled, defatted with hexane and extracted with 80% (v/v) aqueous MeOH, as described elsewhere (Aisyah et al., 2013). To obtain a fraction enriched in isoflavonoid or stilbenoid compounds, the MeOH extract obtained from the seedling meal was cleaned from the presence of sugars and other water-soluble components by solid-phase extraction (SPE) with Sep-Pak Vac C18 cartridges (Waters, Milford, MA, USA), following the protocol of the manufacturer. The final SPE-cleaned seedling extract was dried with a flow of nitrogen gas, solubilized in *tert*-butanol and freeze-dried to obtain a powder. All extracts were kept at −20 °C until further analysis.

2.4. Compositional analysis by RP-UHPLC–PDA–ESI-MS

Compositional analysis was performed on an Accela ultra high performance liquid chromatography (RP-UHPLC) system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler, photodiode array (PDA) detector and ESI-ion trap mass spectrometer (MS).

For flavonoid, isoflavonoid and saponin analysis, seedling extracts (2 μL; 5 mg/mL in MeOH) were injected onto an Acquity UPLC BEH Shield RP18 column (2.1 mm i.d. × 150 mm, 1.7 μm particle size) with an Acquity UPLC Shield RP18 Vanguard guard-column (2.1 mm i.d. × 5 mm, 1.7 μm particle size; Waters, Milford, MA, USA). Water containing 0.1% (v/v) HOAc and 1% (v/v) ACN, eluent A, and ACN containing 0.1% (v/v) HOAc, eluent B, were used as solvents at a flow rate of 300 μL/min. The following elution gradient was used: 0–1 min isocratic on 9% (v/v) B; 1–2.5 min, linear gradient from 9 to 25% B; 2.5–9.5 min, linear gradient from 25 to 50% B; 9.5–12.5 min isocratic at 50% B; 12.5–22.5 min, linear gradient from 50 to 100% B; 22.5–24.5 min isocratic at 100% B; 24.5–25 min, linear gradient from 100 to 9% B; 25–30 min, isocratic at 9% B. Column temperature was set at 35 °C and the PDA detector was set to measure from 200 to 600 nm.

For stilbenoid analysis, seedling extracts (2 μL, 5 mg/mL in MeOH) were injected onto a Hypersil Gold C18 column (2.1 mm i.d. × 150 mm, 1.9 μm particle size, Thermo Scientific, San Jose, CA, USA). Water containing 0.1% (v/v) FA + 1% (v/v) ACN, eluent A, and MeOH containing 0.1% (v/v) FA, eluent B, were used as solvents at a flow rate of 300 μL/min. The following elution gradient was used: 0–1 min isocratic on 0% B; 1–2 min linear gradient from 0 to 30% B; 2–18 min linear gradient from 30 to 80% B; 18–23 min linear gradient from 80 to 95% B; 23–24 min linear gradient 95 to